Title: Intravenous sulforhodamine B reduces alveolar surface tension, improves oxygenation and reduces ventilation-induced lung injury in a respiratory distress model

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Summary: In a model of respiratory distress, intravenously administered sulforhodamine B (SRB) crosses the injured alveolar-capillary barrier to reduce alveolar surface tension specifically in injured lung regions. This novel therapy improves oxygenation and reduces ventilation-induced lung injury. Intravenous SRB administration might help not-yet-ventilated respiratory distress patients avoid mechanical ventilation and ventilated respiratory distress patients survive.

Abstract

BACKGROUND: In the neonatal (NRDS) and acute (ARDS) respiratory distress syndromes, mechanical ventilation supports gas exchange but can cause ventilation-induced lung injury (VILI). Also in NRDS and ARDS, surface tension, *T*, is elevated and VILI may be proportional to *T*. Surfactant therapy is effective in NRDS but not ARDS. Intravenous sulforhodamine B (SRB) administration is a potential alternative *T*-lowering therapy.

METHODS: In anesthetized rats, we injure the lungs with 15 min of 42 ml/kg tidal volume, V_T, and zero end-expiratory pressure ventilation. Then, we lower V_T to 6 ml/kg; apply positive end-expiratory pressure; and administer intravenous non-*T*-altering fluorescein (27 μM in plasma) without or with therapeutic SRB (10 nM in plasma). We increase inspired oxygen fraction, as necessary, to maintain >90% arterial oxygen saturation. After 4 hrs, we sacrifice the rat; sample systemic venous blood for injury marker ELISAs; excise the lungs; combine confocal microscopy and servo-nulling pressure measurement to determine *T*; image fluorescein in flooded alveoli to assess local permeability; and lavage the right middle lobe and quantify total protein content to assess global permeability.

RESULTS: Lungs exhibit focal injury. Surface tension is elevated 72% throughout control lungs and in uninjured regions of SRB-treated lungs, due to lack of recruitment maneuvers, but is normal in injured regions of treated lungs. Intravenous SRB marginally reduces global permeability at the expense of marginally increased local permeability, improves oxygenation and reduces injury markers.

CONCLUSION: Intravenous SRB reduces alveolar surface tension specifically in injured regions and holds promise as treatment for VILI.

Introduction

In the neonatal (NRDS) and acute (ARDS) respiratory distress syndromes, regions of the lungs are injured – edematous, hemorrhagic or collapsed – and mechanical ventilation often causes additional ventilation induced lung injury (VILI). Several VILI mechanisms have been proposed. First, lung inflation that fails to expand injured regions may injuriously overexpand adjacent regions (7). Second, if inflation expands injured regions but there is insufficient positive end-expiratory pressure (PEEP) to prevent marked alveolar diminishment or collapse on deflation, then there may be atelectrauma (22, 28). Third, in regions with heterogeneous alveolar flooding but without alveolar collapse, we find that inflation injuriously exacerbates stress concentrations present in regional septa (26, 36). These injury mechanisms likely co-exist.

Lowering surface tension, T, should reduce VILI. Lowering T should reduce opening pressure, P_O, of injured regions and alveolar flooding within injured regions (8, 19, 37), thus reduce overexpansion injury of adjacent regions. Lowering T should reduce normal stresses that may cause injury during re-opening of flooded or collapsed regions, thus reduce atelectrauma (2). And lowering T reduces over-distension injury of septa in heterogeneously flooded regions (36).

Surfactant therapy is highly effective in NRDS, but not ARDS. In NRDS, T is elevated due to a low surfactant level in the immature lungs and exogenous surfactant administration reduces VILI. Even in surfactant-treated NRDS, however, mechanical ventilation can cause barrier permeability that allows plasma protein entrance into the airspace liquid (16). Further lowering T should further reduce VILI (36). In ARDS, likewise, T should be elevated (10, 23, 24). Unexpectedly, however, surfactant therapy has failed to reduce mortality (3). We previously demonstrated that surfactant delivery via the trachea washes T-raising mucins to the alveolus, thus *raises* alveolar T (23); this problem is likely specific to the mature lung, in which mucin production is greater than in the neonatal lung (4). Additionally, under ARDS conditions, in which T is elevated not due to a paucity of surfactant but due to inactivation or contamination of surfactant, it is questionable whether even surfactant delivered mucin-free to the alveolus could reduce T (23, 24). An alternative means of lowering T in ARDS is required.

We previously found that the dye sulforhodamine B (SRB), working in concert with albumin, increases the efficacy of native lung surfactant (19). *In situ* in surface alveoli of isolated rat lungs, we determine T according to the Laplace relation by combining servo-nulling measurement of alveolar liquid pressure with confocal microscopic determination of interfacial curvature (18, 23). Applying this method in healthy lungs, we found that the combination of SRB and albumin lowers T 27% below normal and, as assessed by barrier permeability to a small solute, reduces VILI in heterogeneously flooded regions (19). With albumin already present in the airspace liquid in NRDS or ARDS, SRB administration may therapeutically reduce VILI.

Here, in an *in vivo* rat model, we injure the lungs with high tidal volume, V_T, and zero end-expiratory pressure (ZEEP) ventilation. Then, we lower V_T, apply PEEP and test intravenous (IV) SRB for the ability to reduce T and VILI (Fig. 1). With alveolar-capillary barrier injury present, solutes pass from blood plasma to alveolar liquid. Thus, as in NRDS and ARDS, the albumin required to facilitate SRB is present in the airspace liquid. And SRB, too, crosses the barrier. Sulforhodamine B is non-toxic, including in pregnant rats and fetuses, and approved as a food dye in Japan (19, 29, 35). Intravenous SRB administration constitutes a potential new therapeutic agent, which may be delivered via a new route, for VILI.

Results

After 15 min of injuriously high V_T ventilation, at the 'injury baseline' time point, SRB or control saline is administered and ventilation is continued for 4 hrs with a protective V_T and a PEEP of 2 or 10 cmH_2O. At 4 hrs, there is focal injury on the lung surface. Sulforhodamine B lowers *T* specifically in injured regions, improves oxygenation and reduces plasma injury markers.

In vivo physiologic parameters. Over the course of the experiment, there are no significant differences over time or between groups in heart rate (HR), peripheral arterial oxygen saturation, S_PO_2, or body temperature, which average 368 ± 33 beats/min, 94.1 ± 1.2% and 37.0 ± 0.3 °C, respectively. End-tidal CO_2 does not differ between groups but differs between the injury and post-injury periods, in which it averages 21.5 ± 4.7 and 38.1 ± 5.4 mmHg, respectively.

To maintain S_PO_2 above 90% as lung condition degrades, we progressively increase the fraction of inspired oxygen, F_IO_2. Applied PEEP level does not alter required F_IO_2 (Fig. 2). At either PEEP level, however, SRB administration reduces required F_IO_2 thus improves oxygenation. Sulforhodamine B also eliminates variability between replicate experiments – at 3-4 hrs, with SRB, required F_IO_2 is always 0.4.

In vivo lung mechanics. As we do not administer a paralytic, the rats sometimes exhibit significant spontaneous breathing (>4 cmH_2O decrease in minimum airway pressure below set PEEP). Significant spontaneous breathing during the acclimation period, when the lungs are healthy, does not affect reported results. Significant spontaneous breathing later on affects barrier permeability, as detailed below, but not other analyzed metrics.

During the 15 min injury period, peak inspiratory pressure (PIP) averages 37 ± 2 cmH_2O and exhibits no characteristic trend; PIP increases, decrease or remains constant in different animals. From injury baseline to the 4-hr time point in the 2 cmH_2O PEEP/0 nM SRB group, the PIP-PEEP difference increases thus elastance increases (Fig. 3). Administration of SRB delays the increase. At injury baseline, PIP-PEEP is greater with 10 than with 2 cmH_2O PEEP. In the 10 cmH_2O PEEP group, regardless of SRB administration, 4 hrs of subsequent ventilation do not alter PIP-PEEP. At the 4-hr time point, PIP-PEEP is the same regardless of PEEP level or SRB administration.

Lung appearance. After isolating lungs at the PEEP volume, all 4-hr time point lungs exhibit focal regions of surface injury (Fig. 4). The same pattern is observed for 83% of baseline injury lungs and even 50% of healthy control lungs. The pattern is consistent with that observed previously for high PIP-induced lung injury (6). Bright-field microscopy, which illuminates the alveolar air-liquid interface, shows interfaces in injured regions to have disappeared; fluorescent confocal microscopy shows alveoli in injured regions are flooded and diminished in size. The presence of flooded/diminished alveoli on lung isolation at 10 cmH_2O PEEP indicates this PEEP was insufficient for maintaining end-expiratory alveolar patency.

By histology, despite inflation to 30 cmH_2O before left lung fixation, all left lungs including healthy controls exhibit multi-layers of nuclei in the parenchyma that indicate septal apposition and may constitute early atelectasis (Fig. 5A). Noting that euthanasia is performed by isoflurane overdose in 100% oxygen, which requires a particularly long time in healthy controls (Fig. 1), this result is consistent with oxygen- and anesthesia-induced atelectasis (32, 33). The initiation of atelectasis, in turn, might explain the presence of focal surface injury in 50% of ventilated healthy control lungs.

Edema. Also by histology of left lungs, PEEP worsens but SRB does not alter lung edema. Perivascular edema is more prevalent in the 4-hr time point/10 cmH_2O PEEP groups than in other groups (Fig. 5B). For alveolar edema, there tend to be one to three foci present in sections from injury baseline and 4-hr time point/2 cmH_2O PEEP groups. One of those regions is always located adjacent to the main branch

point of the airways (Fig. 5C). More numerous foci are generally present in sections from 4-hr time point/10 cmH_2O PEEP groups.

Wet-to-dry ratio (W/D) of lower right lobes is elevated at 4 hrs (Fig. 6). Positive end-expiratory pressure does not alter W/D. Administration of SRB marginally reduces W/D.

Surface tension. Surface tension at transpulmonary pressure, P_L, of 15 cmH_2O is normal (18) in healthy control lungs and marginally elevated at injury baseline. At 4 hrs, regardless of PEEP or SRB treatment, T is elevated in the liquid lining layer of aerated alveoli in uninjured lung regions (Fig. 7). Also at the 4 hrs, and regardless of PEEP, T is comparably elevated in flooded alveoli in injured regions of untreated control lungs yet normal in flooded alveoli of SRB-treated lungs. Thus, intravenous SRB lowers T specifically in injured regions. The reduction of T shows that both SRB and albumin, which must be present to facilitate SRB (19), are present in the liquid phase. The specific reduction in injured areas may be due in part to greater extravasation of 559 Da SRB (36) but is likely largely attributable to greater extravasation of 67 kDa albumin.

Surface tension in flooded alveoli in injured regions of non-SRB-treated lungs averages 18.9 ± 2.2 mN/m, with a minimum value of 15.2 mN/m, across both PEEP groups (Fig. 7). To determine whether the elevated T is attributable to omission of recruitment maneuvers from our protocol, we performed the following experiment: injure the lungs with 15 min of 42 ml/kg V_T/ZEEP ventilation, do not administer SRB and then ventilate for 4 hrs with 6 ml/kg V_T ventilation, a PEEP of 2 cmH_2O and, every 30 min, a recruitment maneuver (RM) that raises PIP, over the course of 3-5 breaths, to 32-36 cmH_2O. In this experiment, after lung isolation at 4 hrs, the focal injury pattern is the same as in other lungs and T in a flooded alveolus in an injured region is a normal 10.7 mN/m. While we cannot rule out the possibility that periodic RMs prevent a form of regional injury that causes surfactant inactivation, these results suggests that T in our experiments without RMs may be elevated – in injured and aerated regions – not for the reasons that T is elevated in ARDS but due to a low surfactant level as in NRDS. Further, that recruitment maneuvers can normalize T in a non-SRB-treated injured region where plasma proteins are present in the alveolar liquid (i) supports our prior finding that plasma proteins do not raise T in the lungs (18) and (ii) extends that the finding to a condition with pathophysiologic cyclic surface area compression.

Liquid phase. As expected from the Laplace relation, the trends in liquid pressure, P_{LIQ}, and meniscus radius, R, are generally inverse and proportional, respectively, to that in T (Fig. 7). The one exception is that, at 4 hrs, T elevation in flooded alveoli of untreated lungs is principally due to increased R while P_{LIQ} is close to normal, and SRB normalizes T by reversing the increase in R. The SRB-induced decrease in R suggests the possibility of greater meniscus penetration into flooded alveoli, thus a thinner liquid layer.

Permeability. Having administered IV fluorescein to all animals at the injury baseline time point, we characterize permeability from (i) fluorescein intensity in alveolar edema liquid (4-hr time point only) and (ii) protein content of lavage liquid (all time points). Not surprisingly, one of our permeability metrics, fluorescein intensity, is influenced by spontaneous breathing. Thus we exclude fluorescein intensity data and, for consistency between permeability metrics, lavage liquid protein content data from experiments with significant spontaneous breathing after the acclimation period.

In alveolar edema liquid, PEEP does not affect fluorescein intensity but SRB tends to increase intensity (Fig. 8). In lavage liquid, protein concentration is normal at injury baseline and tends to be elevated 4 hrs later. At 4 hrs, PEEP tends to increase and SRB tends to decrease protein concentration. Noting that fluorescein intensity indicates local permeability severity and lavage liquid protein indicates a combined effect of global permeability severity and spatial extent, the opposite trends of fluorescein intensity and lavage protein concentrations, while not significant, suggest that SRB may, by increasing local

permeability severity, limit spatial injury propagation over time. Such spatial propagation has been observed previously in model and clinical ARDS/VILI (5, 6).

Injury markers. By enzyme-linked immunosorbent assay (ELISA) analysis of plasma, we show that 15 min of high V_T ventilation begin to initiate injury and 4 subsequent hrs of protective ventilation increase injury. Increasing PEEP from 2 to 10 cmH_2O tends to increase injury. Treatment with SRB tends to reduce injury.

In particular, 4 hrs of ventilation without, but not with, SRB injures type I cells (Fig. 9). High PEEP causes type II cell injury; at low PEEP, even while surfactant protein D (SP-D) is not significantly elevated, SRB tends to be protective of type II cells. Regardless of PEEP, SRB reduces tumor necrosis factor α (TNF-α) level. High PEEP increases interleukin 6 (IL-6) level and, at high PEEP, SRB tends to be protective. Finally, after 4 hrs of ventilation, one measure of systemic inflammation, plasminogen activator inhibitor 1 (PAI-1), is elevated only without, not with, SRB administration but another measure, von Willebrand Factor (vWF), is elevated without and tends to be exacerbated by SRB administration.

Discussion

In this study, we injure the lungs with high V_T mechanical ventilation. The protocol causes focal injury on the lung surface. Due to a lack of recruitment maneuvers, T is elevated. In treated lungs, however, sufficient levels of SRB and albumin diffuse from blood to alveolar liquid to normalize T in injured regions. The SRB-plus-albumin-induced normalization of T in the presence of reduced surfactant, like SRB-plus-albumin-induced reduction of T below normal in healthy lungs (19), demonstrates that the combination of SRB and albumin improves the efficacy of native lung surfactant.

With spatially heterogeneous injury, the effect of lowering T specifically in injured regions could elicit opposing effects (25). Intravenous SRB administration causes a T differential – lower T in injured than surrounding uninjured regions – that should subject injured regions to greater stress and tend to exacerbate local permeability. In contrast, lowering T should both reduce T-dependent stress concentrations in septa separating aerated/flooded alveoli within heterogeneously flooded regions and, in the presence of cyclic re-opening, reduce atelectrauma. As lowering T marginally increases local permeability (Fig. 8A), the former tendency appears to dominate. Why lowering T also marginally reduces global permeability (Fig. 8B) remains to be determined. Possible explanations include: (i) preferential inflation-induced expansion of injured regions should, albeit to an unknown extent, reduce inflation-induced over-expansion of adjacent tissue at peak lung pressures and (ii) if there is cyclic re-opening, reduced P_O of injured alveoli should reduce overexpansion of surrounding tissue at pressures between the former, higher and the new, lower P_O values. Regardless of mechanical mechanism, SRB administration reduces multiple downstream plasma markers of lung injury. Although it is not known whether SRB has direct anti-inflammatory effects, SRB most likely acts by reducing T and, in turn, improving lung mechanics.

Sulforhodamine B administration improves oxygenation via an unknown mechanism. Possible explanations include reducing P_O of injured alveoli, thus recruiting additional gas exchange surface area at lower volumes; thinning the alveolar liquid layer in injured regions, once open (Fig. 7, bottom panel); expanding capillaries in injured regions thus increasing local gas exchange area or reducing shunting (15, 34); and limiting the spatial extent of lung injury.

In contrast to the effect of lower T, a higher PEEP of 10 cmH_2O is generally detrimental. As this PEEP level is insufficient for maintaining end-expiratory alveolar patency in this model (Fig. 4B), it increases the prevalence of edema throughout the lungs (Fig. 5), tends to increase global permeability (Fig. 8B),

increases multiple plasma injury markers (Fig. 9) and fails to improve oxygenation (Fig. 2). In general, though PEEP often improves oxygenation, it can tend to do so at the expense of increased lung injury. Reducing *T*, in contrast, should improve oxygenation while also tending to reduce lung injury.

Comparing the effects of *T* and PEEP also elucidates the relative contributions of *T* and tissue elastance to lung elastance. At injury baseline, with almost-normal *T*, PIP-PEEP is greater with 10 than 2 cmH_2O PEEP due to increased tissue elastance at increased lung volume. Over the next 4 hrs, *T* increases throughout all control (0 nM SRB) lungs but PIP-PEEP increases only with 2, not 10, cmH_2O PEEP. This result suggests that *T* may only affect lung mechanics at relatively low lung volumes.

The animal model of this study is an ARDS-NRDS hybrid. The architecture of the adult lung is that of ARDS. The spatially heterogeneous ventilation injury and proteinaceous edema liquid are common to ARDS and NRDS. The cause of elevated *T* is likely that of NRDS – a low surfactant level. As SRB improves the efficacy of low surfactant levels, SRB might be considered as an complement or alternative to surfactant therapy in NRDS.

Elevation of *T* in ARDS has been attributed to various causes including plasma proteins or cholesterol in the edema liquid; debris from damaged cells (membrane lipids or cytosolic contents, including hemoglobin) in the edema liquid; elevated phospholipase activity; oxidation of surfactant lipids or proteins; and aspirated gastric contents (9–11, 13, 21, 23, 27, 30). We and others have demonstrated (12, 14, 18), and we confirm here, that plasma proteins do not raise *T* in the presence of an intact surfactant monolayer such as exists in the lungs. However other factors, individually or in combination, likely contribute to elevated *T* in ARDS. In gastric aspiration, neither exogenous surfactant nor SRB is likely to be beneficial (23, 24). In non-aspiration ARDS, SRB is likely to reduce *T* (24).

Our study has limitations, including the following. With a compliance chamber between anesthesia vaporizer and ventilator, pressure at the ventilator inlet may have been positive such that actual V_T may have exceeded set V_T. Nonetheless, consistent injury-period PIP levels indicate consistent injury across animals. We are not able to determine *T in vivo* but only in lungs isolated following animal sacrifice. With just two histologic sections from each left lung, we are unable to correlate surface injury with interior injury. Inflating lungs to a P_L of 30 cmH_2O before fixation at 5 cmH_2O may alter alveolar collapse or flooding patterns. This protocol step could have obscured SRB effects on collapse or flooding pattern that might contribute to the SRB-induced improvement in oxygenation. Additionally, our permeability results do not differ significantly between 4-hr time point groups so our discussion of implications, including for oxygenation, is speculative. Further studies will be necessary to clarify why SRB administration improves oxygenation.

In summary, we demonstrate that intravenous SRB administration can lower alveolar liquid *T* in regions with barrier injury. In a model of heterogeneous lung injury, intravenous SRB administration constitutes a novel approach to simultaneously improving oxygenation and reducing ventilation injury. As ventilation injury can occur with spontaneous breathing in the presence of elevated *T* (1), intravenous SRB has the potential to help not-yet ventilated respiratory distress patients avoid mechanical ventilation as well as the potential to help ventilated respiratory distress patients survive.

Methods

In anesthetized, mechanically ventilated rats, we generate high V_T lung injury. We then reduce V_T to a protective level, administer *T*-lowering SRB intravenously, monitor the rats for four hours and euthanize the rats. We isolate the lungs and determine *T*. We also determine W/D, assess barrier permeability, quantify plasma levels of injury and inflammation markers and assess histologic pathology.

In vivo protocol

We handle all animals in accord with a protocol approved by the Stevens Institute of Technology Institutional Animal Care and Use Committee. We anesthetize male Wistar-Han rats (n=53, 270–375 g, Charles River, Wilmington, MA) with 3% isoflurane in 100% oxygen and perform a tracheotomy. We connect the tracheotomy tube to the circuit of a mechanical ventilator (model 683 with 30 ml cylinder, Harvard Apparatus, Holliston, MA; compliance chamber between anesthesia vaporizer and ventilator inlet) using a stopcock that also connects a transducer for recording airway entrance pressure. We begin ventilation with a F_IO_2 of 1.0, a V_T of 6 ml/kg, a respiratory rate (RR) of 75 breaths/min and 2 cmH_2O PEEP. Throughout the experiment, we maintain body temperature at 37 °C and, every five minutes, record: end-tidal CO_2 with a capnograph and S_PO_2 and HR by pulse oximetry (PhysioSuite, Kent Scientific, Torrington, CT).

High V_T injury. Following a 15 min acclimation period, we injure the lungs by ventilating for 15 min with a F_IO_2 of 1.0, a nominal V_T of 42 ml/kg, a RR of 11 breaths/min and ZEEP (31). The ZEEP is achieved by the combination of 2 cmH_2O set PEEP and capnograph suction during the prolonged end-expiratory period.

Treatment and continued ventilation. At the injury baseline, we reduce F_IO_2 to 0.21, reduce V_T to 6 ml/kg, increase RR to 75 breaths/min and apply a PEEP of 2 or 10 cmH_2O. We administer a tail vein injection (normal saline base, total volume 100 μL) of non-*T*-altering (18) fluorescein (target 27 μM in plasma based on reported 45% hematocrit (Charles River, Wilmington, MA) and calculated blood volume (20); Cole Parmer, Vernon Hills, IL) for visualization and barrier permeability quantification after lungs are excised at the end of the experiment. In experimental groups only, we additionally include *T*-lowering SRB (target 10 nM in plasma; 341738, Sigma Aldrich, St. Louis, MO) as a therapy. Injection time averages 7.5 ± 3.5 min. From the injury baseline, we ventilate for four hours. Whenever S_PO_2 drops to 90%, we increase F_IO_2 by 0.20. If increasing F_IO_2, does not increase S_PO_2 above 90% in 5 min, we again increase F_IO_2 by 0.20. Finally, we euthanize the rat with 5% isoflurane in 100% oxygen.

Additional control groups. We additionally employ two earlier-time-point control groups. In a healthy control group, we anesthetize and ventilate the rats, administer a tail vein injection of fluorescein and euthanize at the end of the 15-min acclimation period. In an injury baseline group, we follow the same protocol but continue ventilating through the 15 min injury period before returning to low V_T ventilation and euthanizing.

Post-mortem protocol

Post-mortem, we collect a blood sample and isolate the lungs for the procedures below.

Thoracotomy and blood collection. After euthanizing the rat, we close the tracheal stopcock to maintain lung volume at the PEEP level and open the chest. We ligate the inferior vena cava and, proximal to the ligature, collect ~4 ml blood, which we heparinize (20 U/ml; Penn Veterinary Supply Inc, Lancaster, PA), centrifuge at 1000 x g and 4 °C for 10 min, aliquot and store at -80 °C.

Lung isolation and imaging. We isolate the lungs and heart at PEEP, position the lungs costal surface upward, photograph the lungs and place the lungs on the stage of an upright confocal microscope (SP5, Leica Microsystems, Buffalo Grove, IL). In select experiments, we document morphology of an injured surface region by bright-field microscopy and fluorescent confocal imaging of fluorescein (x20, 0.5 N.A. water immersion objective; cover slip just in contact with lung surface (38); and, for fluorescent imaging, 488 nm/≥493 nm excitation/emission). We then inflate the lungs to a P_L of 30-33 cmH_2O and deflate to 15 cmH_2O. In 4-hr time point lungs, to quantify alveolar liquid fluorescein intensity, we obtain confocal images (x40, 0.8 N.A. water immersion objective; cover slip just in contact with lung surface; 488

nm/≥493 nm excitation/emission and 798 gain) at a 20 μm sub-pleural depth in three flooded costal-surface regions, or fewer regions in the 40% of lungs in which we cannot locate three.

T determination. We determine *T* in surface alveoli at P_L of 15 cmH_2O as described above and detailed previously (23, 18). In healthy control and baseline injury lungs, we determine *T* in aerated alveoli. At the 4-hr time point, we determine *T* in both aerated and flooded alveoli.

Wet-to-dry ratio. We reduce P_L to 5 cmH_2O, apply a vascular clamp (S&T B1-V, Fine Science Tools, Foster City, CA) to the connective tissues between the right middle and lower lobes and separate the lower lobe distal to the clamp. We weight the lower lobe before and after drying it at 76 °C for 24 hrs, and determine W/D.

Lavage. We ligate the bronchus to the right middle lobe, cut the bronchus distal to the ligation, cannulate the bronchus and lavage by instilling 1.54 μL/g body weight room temperature normal saline. We collect 127 ± 33 μL lavage liquid, which we centrifuge at 1000 x g and 4 °C for 10 min, aliquot and store at -80 °C.

Lung fixation and histology. With the remaining lung lobes still at P_L of 5 cmH_2O, we ligate the trachea, submerge the remaining lung in normal saline and microwave for 4 min to above 60 °C (17, 37). We transfer the lung to 4% paraformaldehyde for 48 hrs and then to 70% ethanol. We obtain two hematoxylin- and eosin-stained, 3 μm-thick sagittal sections through the left lung that are located ~33 and ~66% of the distance along the transverse axis of the lungs (Histowiz, Brooklyn, NY). The inner section contains the main airway tree.

Post-experimental analysis

Lung mechanics. We assess mechanics by analyzing the PIP-PEEP difference which, for fixed V_T, varies proportionately with respiratory system elastance. We determine injury baseline PIP-PEEP by averaging the value over 10 ventilation cycles starting 2 min after V_T reduction to 6 ml/kg and 4-hr time point PIP-PEEP by averaging the value over the last 10 cycles before commencing euthanasia. Starting at injury baseline, we fit an exponential function to PIP to determine the time constant of elastance increase.

Image analysis. For a given lung, in each 4-hr time point fluorescent image of a flooded region, obtained at P_L of 15 cmH_2O, we quantify fluorescein intensity in the liquid of three flooded alveoli. Then we average all fluorescein levels measured for that lung.

Histologic analysis. Histologic analysis of perivascular and alveolar edema is performed by Dr. Jerrold Ward (Global VetPathology, Montgomery Village, MD).

Protein extravasation and plasma biomarker quantification. We thaw lavage liquid or blood plasma aliquots. In the lavage liquid, we determine protein concentration by Bradford assay (Thermo Fisher Scientific, Waltham, MA). For plasma, we use ELISAs to determine levels of the receptor for advanced glycation end-products (sRAGE; Mybiosource, San Diego, CA), SP-D (Lifespan Biosciences, Seattle, WA), TNF-α (R&D Systems, Minneapolis, MN), IL-6 (Invitrogen, Carlsbad, CA), PAI-1 (Lifespan Biosciences) and vWF (Lifespan Biosciences). We follow manufacturer instructions, including duplicate sample testing, for each assay.

Statistical analysis. We report data as means ± SD. We perform individual comparisons by Student's t-test and multiple comparisons by ANOVA and post-hoc Tukey's analysis or, when data are not parametric (F_IO_2 at 3- and 4-hr time points, permeability indices and ELISA data), by Kruskal-Wallis ANOVA on ranks and post-hoc Dunn's analysis, accepting significance at $p < 0.05$. When comparing multiple independent parameters, e.g., PEEP and absence/presence of SRB, we report differences between groups for which only one independent parameter differs.

Author contributions

WY designed the study, performed the experiments, analyzed data and wrote and edited the manuscript. TLN analyzed data and reviewed and edited the manuscript. CEP designed the study, analyzed data and wrote and edited the manuscript.

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Figure Legends

Figure 1: Protocol. Schematic of experimental protocol. F_IO_2: fraction of inspired oxygen; S_PO_2: peripheral arterial oxygen saturation; V_T: tidal volume; PEEP: positive end-expiratory pressure; RR: respiratory rate. Euthanasia requires 20-40, 15-25 and 10-20 min in healthy control, injury baseline and at 4-hr time point groups, respectively.

Figure 2: Oxygenation. F_IO_2 vs. time, from injury baseline onward, where F_IO_2 is increased as necessary to maintain arterial oxygen saturation above 90%. The two sulforhodamine B (SRB) administration groups lack error bars at final 4-hr time point because F_IO_2 was identical across all replicate experiments. Statistics: n = 9-11/group; pooling same-PEEP data, *$p < 0.05$ between 0 and 10 nM SRB groups at 3- and 4-hr time points.

Figure 3: Lung compliance. A. Airway pressure traces during acclimation period, injury period and subsequent 4 hrs of ventilation, with indicated PEEP during 4-hr period, in absence of SRB administration. **B.** Time constant for increase in peak inspiratory pressure (PIP), starting at injury baseline, in 2 cmH_2O PEEP groups. Despite increase, SRB does not alter appearance of time traces at either PEEP. **C.** Difference between PIP and PEEP at the injury baseline time point just prior to tail vein injection and at the 4-hr time point. Statistics: *$p < 0.01$ between indicated groups; $^{o}p < 0.001$ vs. injury baseline/2 cmH_2O PEEP group.

Figure 4: Lung appearance. A. Photograph of lung in 10 cmH_2O PEEP/10 nM SRB group excised at PEEP volume and at 4-hr time point and exhibiting a focal injury pattern. Lungs excised from other groups look the same. **B.** Bright-field image (left) and fluorescent confocal image of fluorescein (right) for an injured region on the surface of the lung shown in (A), still held at PEEP volume. Higher-magnification fluorescent image located principally within central injured region of bright-field image.

Fig. 5: Histology. A. Multilayers of nuclei (arrows), indicating septal apposition, are present in lungs from all groups. **B.** Perivascular edema is more frequent at high than low PEEP. **C.** Focal alveolar injury always present adjacent to main airway branch point (arrow in left image of 2 cmH_2O PEEP/0 nM SRB group lung). Injury is sometimes hemorrhagic (upper right image, from same lung as on left), sometimes edematous (lower right image, from 10 cmH_2O PEEP/10 nM SRB group lung). Treatment with SRB does not alter histologic findings.

Fig. 6: Wet-to-dry ratio. Wet-to-dry ratio of lower right lobe in all experimental groups. Statistics: *$p < 0.001$ vs. healthy control and injury baseline groups.

Fig. 7: Surface tension and underlying measurements. We calculate alveolar interfacial surface tension, T, from the Laplace relation after experimentally setting alveolar air pressure, P_{ALV}, to 15 cmH_2O; determining alveolar liquid phase pressure, P_{LIQ}, in the liquid lining layer in the corner of an aerated (A) alveolus or below the meniscus of a flooded (F) alveolus by servo-nulling pressure measurement; and determining three-dimensional interfacial radius of curvature, R, from a z-stack of confocal microscopic images. Statistics for T: *$p < 0.001$ vs. healthy control, 2 cmH_2O PEEP/flooded/10 nM SRB and 10 cmH_2O PEEP/flooded/10 nM SRB, and $p < 0.01$ vs. injury baseline. Statistics for P_{LIQ}: *$p < 0.001$ vs. healthy control, injury baseline and all flooded alveoli at 4 hrs; $^{O}p < 0.05$ between indicated groups. Statistics for R: *Same meaning as for T data; $^{O}p < 0.001$ vs. healthy control and $p < 0.01$ vs. 2 cmH_2O PEEP/flooded/10 nM SRB and 10 cmH_2O PEEP/flooded/10 nM SRB.

Fig. 8: Permeability. **A.** Alveolar liquid fluorescein intensity indicates local permeability severity. Following inflation to 30 cmH_2O and deflation to 15 cmH_2O, confocal images obtained at 20-μm subpleural depth in flooded regions (4-hr time point only, as no flooded regions identified in healthy control or injury baseline groups). Representative images from 10 cmH_2O PEEP group exhibit average

intensities of 44 and 68 gray levels with 0 and 10 nM SRB, respectively. For display purposes, contrast of both images increased to same degree. Note typical pattern of diminished-sized flooded alveoli and over-expanded aerated alveoli. Graph presents group data. **B.** Total protein content of middle right lobe lavage liquid indicates global permeability – a metric combining injury severity and extent. Statistics: $^{o}p < 0.05$ vs. healthy control.

Fig. 9: Injury markers. Injury markers quantified by enzyme-linked immunosorbent assays of blood plasma: soluble receptor for advanced glycation end products (sRAGE) and surfactant protein D (SP-D) are markers of alveolar epithelial type I and II cell injury, respectively; tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6) are cytokines; and plasminogen activator inhibitor 1 (PAI-1) and von Willbrand factor (vWF) are markers of systemic inflammation. Data normalized by average value of 2 cmH_2O/0 nM SRB group. Statistics: $*p < 0.05$ vs. healthy control and injury baseline groups or between indicated groups; $^{o}p < 0.05$ vs. healthy control group; $^{\#}p < 0.05$ vs. group with same SRB concentration at 2 cmH_2O PEEP.

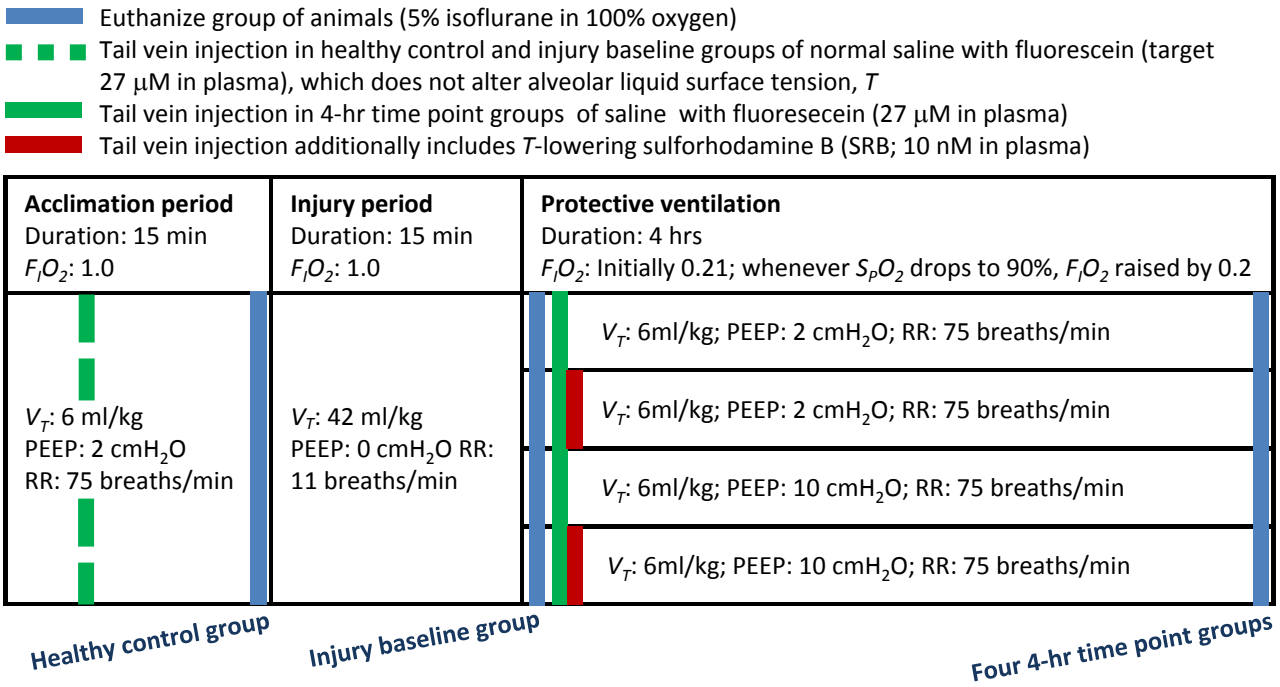

Euthanize group of animals (5% isoflurane in 100% oxygen)
Tail vein injection in healthy control and injury baseline groups of normal saline with fluorescein (target 27 μM in plasma), which does not alter alveolar liquid surface tension, T
Tail vein injection in 4-hr time point groups of saline with fluoresecein (27 μM in plasma)
Tail vein injection additionally includes T-lowering sulforhodamine B (SRB; 10 nM in plasma)
Acclimation period
Duration: 15 min
FIO2: 1.0
Injury period
Duration: 15 min
FIO2: 1.0
Protective ventilation
Duration: 4 hrs
FIO2: Initially 0.21; whenever SpO2 drops to 90%, FIO2 raised by 0.2
VT: 6 ml/kg
PEEP: 2 cmH2O
RR: 75 breaths/min
VT: 42 ml/kg
PEEP: 0 cmH2O RR: 11 breaths/min
VT: 6ml/kg; PEEP: 2 cmH2O; RR: 75 breaths/min
VT: 6ml/kg; PEEP: 2 cmH2O; RR: 75 breaths/min
VT: 6ml/kg; PEEP: 10 cmH2O; RR: 75 breaths/min
VT: 6ml/kg; PEEP: 10 cmH2O; RR: 75 breaths/min
Healthy control group
Injury baseline group
Four 4-hr time point groups

Figure 1

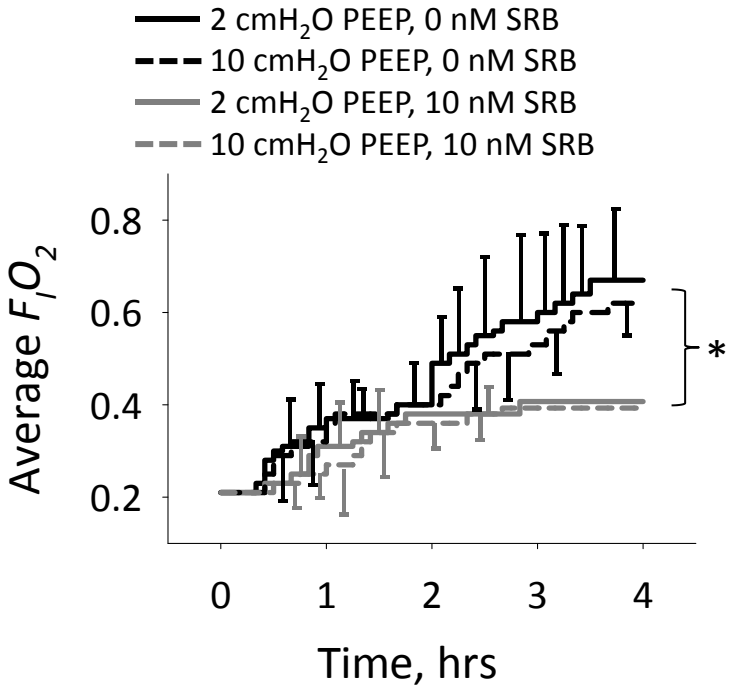

2 cmH2O PEEP, 0 nM SRB
10 cmH2O PEEP, 0 nM SRB
2 cmH2O PEEP, 10 nM SRB
10 cmH2O PEEP, 10 nM SRB
Average FIO2
0.8
0.6
0.4
0.2
*
0
1
2
3
4
Time, hrs

Figure 2

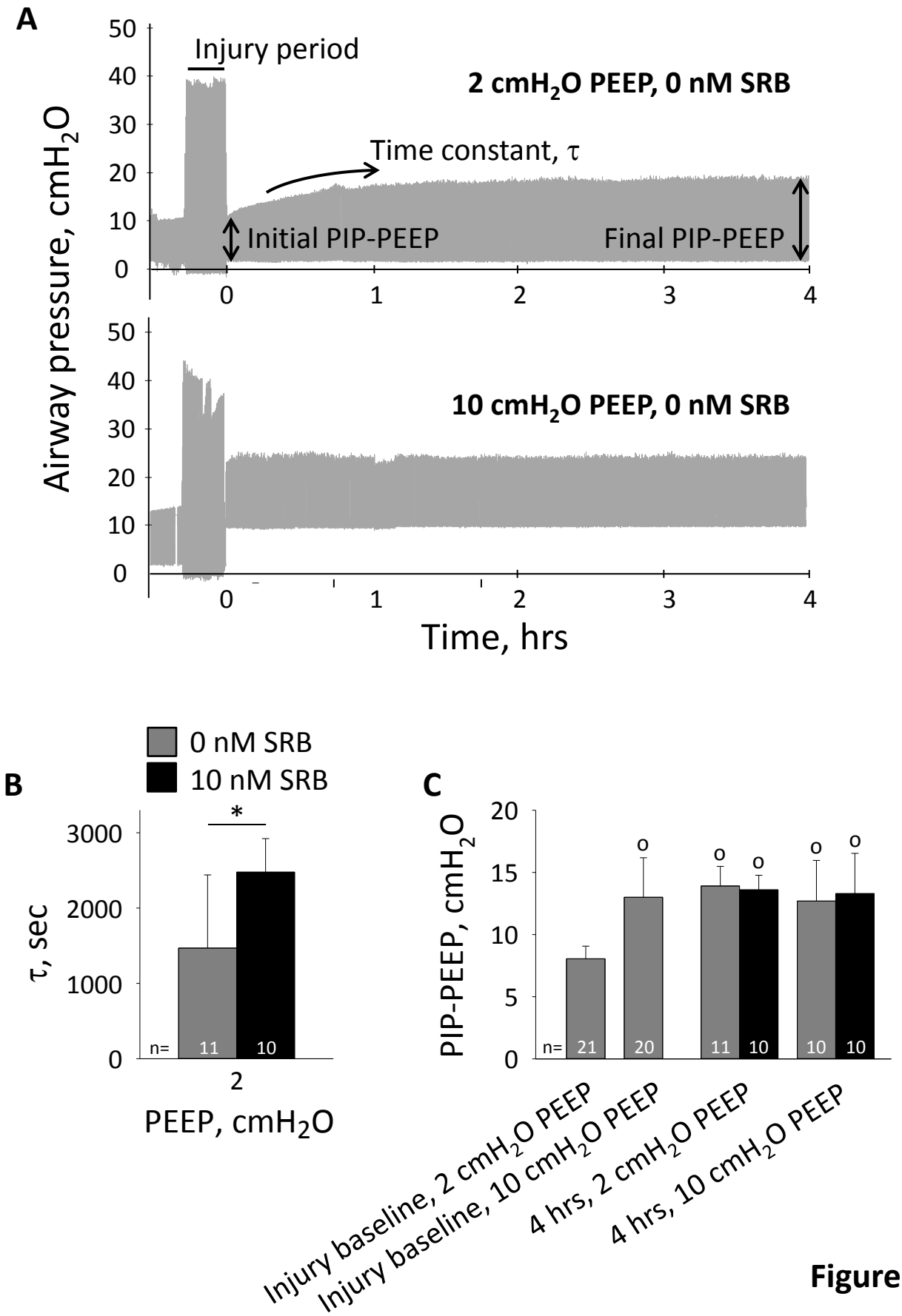

A
Injury period
2 cmH2O PEEP, 0 nM SRB
Time constant, τ
Initial PIP-PEEP
Final PIP-PEEP
10 cmH2O PEEP, 0 nM SRB
Airway pressure, cmH2O
Time, hrs
0 nM SRB
10 nM SRB
B
τ, sec
PEEP, cmH2O
n=
C
PIP-PEEP, cmH2O
Injury baseline, 2 cmH2O PEEP
Injury baseline, 10 cmH2O PEEP
4 hrs, 2 cmH2O PEEP
4 hrs, 10 cmH2O PEEP

Figure 3

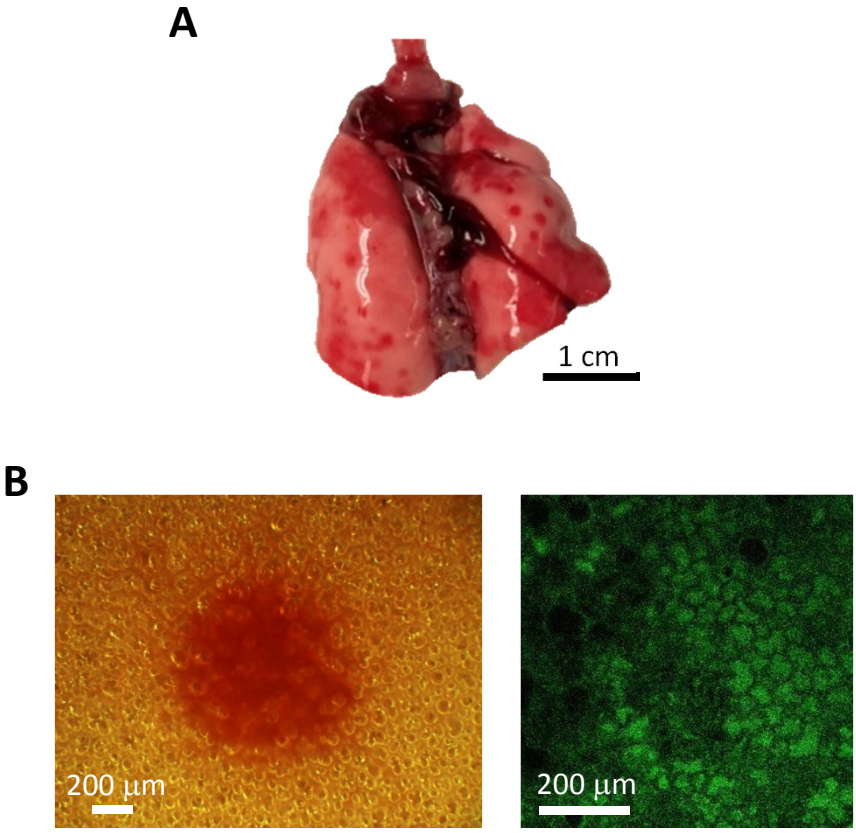
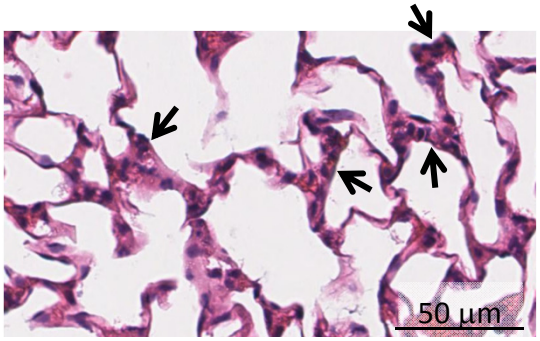
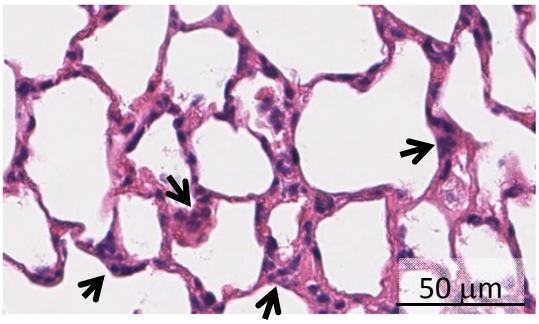


Figure 4

A

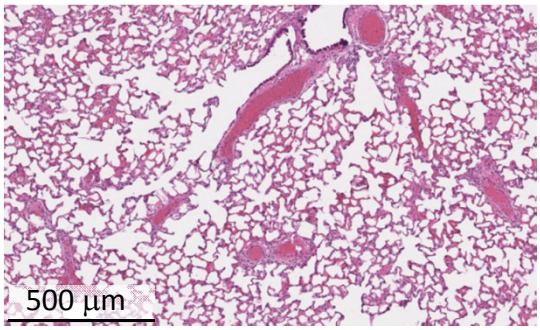


Healthy control

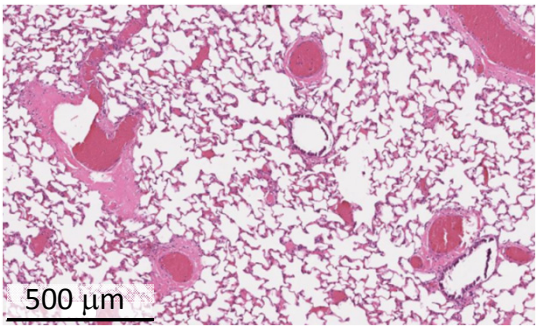


4 hrs, 2 cmH_2O PEEP/0 nM SRB

B

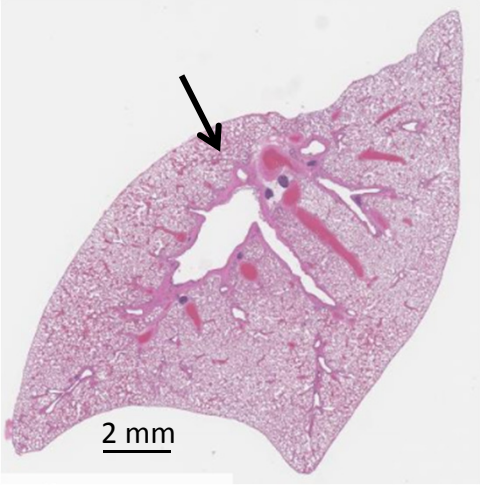


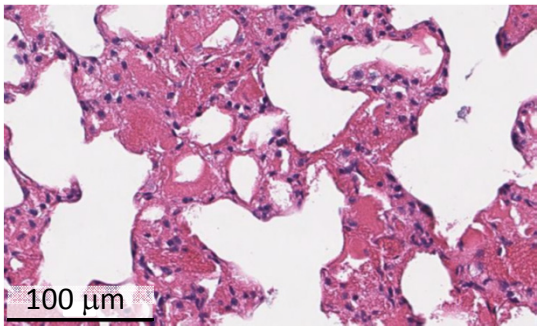
4 hrs, 2 cmH_2O PEEP/0 nM SRB



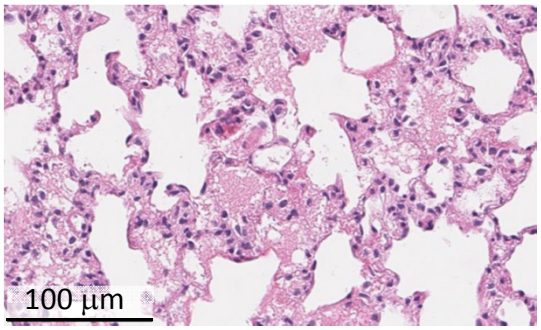
4 hrs, 10 cmH_2O PEEP/0 nM SRB

C





Hemorrhage



Edema

Figure 5

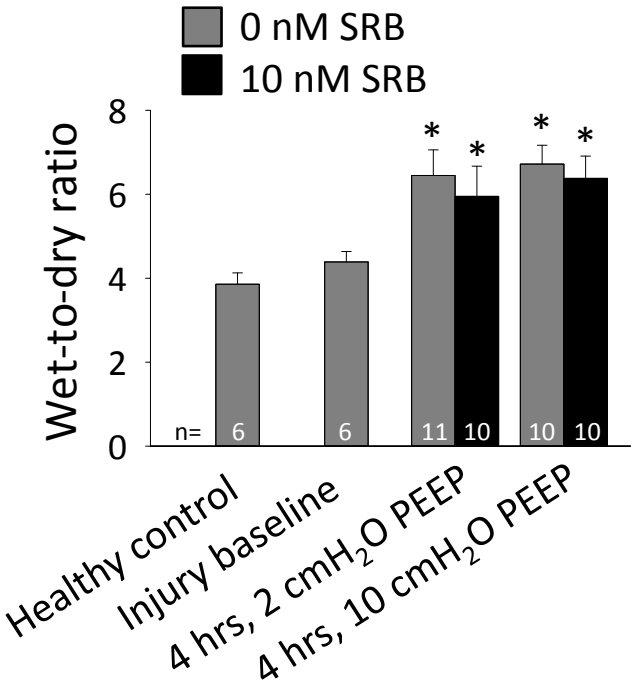

0 nM SRB
10 nM SRB
Wet-to-dry ratio
8
6
4
2
0
n=
6
6
11
10
10
10
*
*
*
*
Healthy control
Injury baseline
4 hrs, 2 cmH2O PEEP
4 hrs, 10 cmH2O PEEP

Figure 6

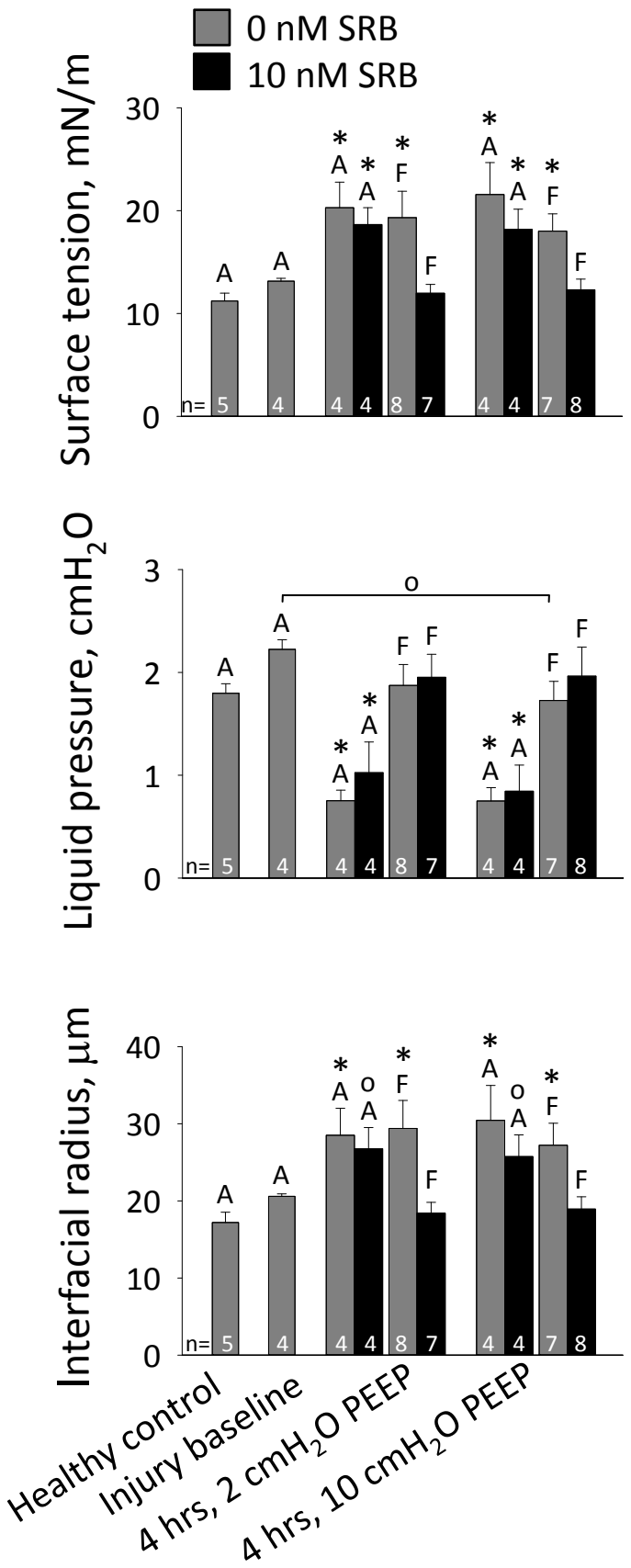

0 nM SRB
10 nM SRB
Surface tension, mN/m
Liquid pressure, cmH2O
Interfacial radius, µm
n= 5 4 4 4 8 7 4 4 7 8
Healthy control
Injury baseline
4 hrs, 2 cmH2O PEEP
4 hrs, 10 cmH2O PEEP

Figure 7

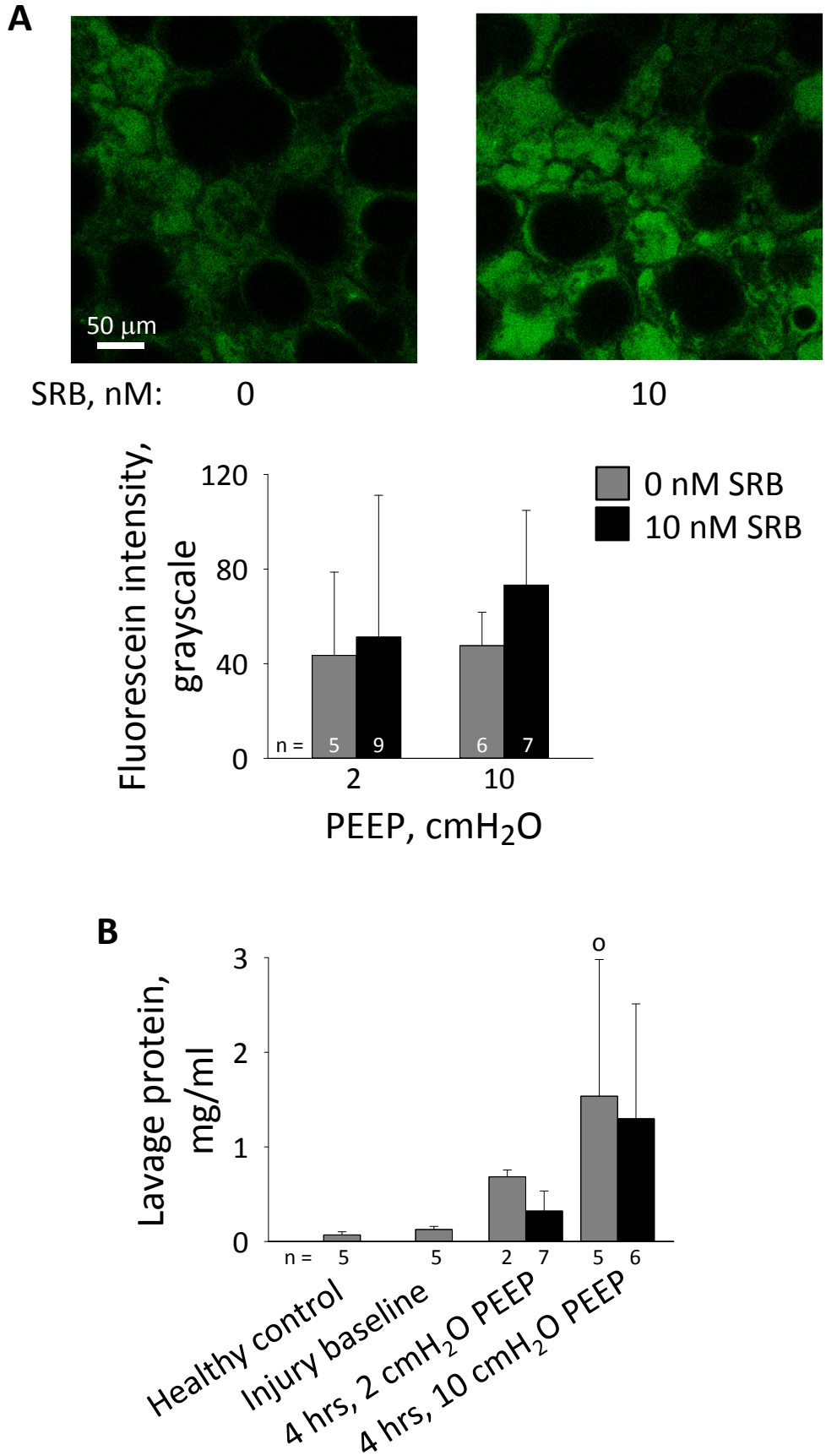

A
50 µm
SRB, nM: 0 10
Fluorescein intensity, grayscale
120
80
40
0
n = 5 9 6 7
2 10
PEEP, cmH2O
0 nM SRB
10 nM SRB
B
Lavage protein, mg/ml
3
2
1
0
o
n = 5 5 2 7 5 6
Healthy control
Injury baseline
4 hrs, 2 cmH2O PEEP
4 hrs, 10 cmH2O PEEP

Figure 8

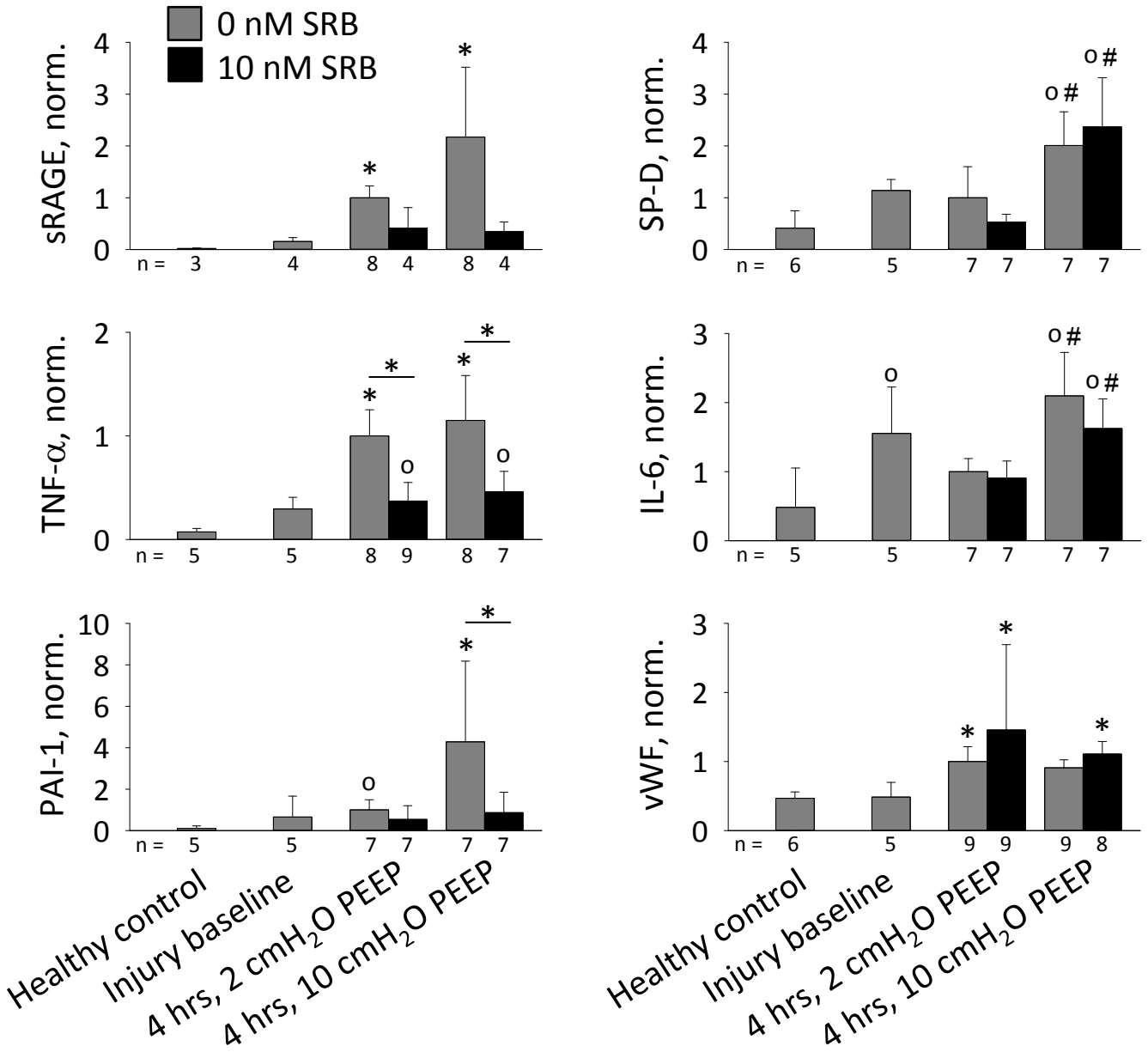

0 nM SRB
10 nM SRB
sRAGE, norm.
SP-D, norm.
TNF-α, norm.
IL-6, norm.
PAI-1, norm.
vWF, norm.
n = 3 4 8 4 8 4
n = 6 5 7 7 7 7
n = 5 5 8 9 8 7
n = 5 5 7 7 7 7
n = 5 5 7 7 7 7
n = 6 5 9 9 9 8
Healthy control
Injury baseline
4 hrs, 2 cmH2O PEEP
4 hrs, 10 cmH2O PEEP

Figure 9