Title: Sulforhodamine B and exogenous surfactant effects on alveolar surface tension in acute respiratory distress syndrome models

Authors: Tam L. Nguyen and Carrie E. Perlman

Affiliation:
Department of Biomedical Engineering
Stevens Institute of Technology, Hoboken, NJ

Correspondence to:
Carrie E. Perlman, Ph.D.
Stevens Institute of Technology
Department of Biomedical Engineering
Castle Point on Hudson
Hoboken, NJ 07030
201-216-8779
cperlman@stevens.edu

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Summary: In the acute respiratory distress syndrome (ARDS), surface tension, *T*, is believed to be elevated and surfactant therapy has failed to reduce mortality. Here, we test whether various substances suggested to contribute to elevated *T* in ARDS in fact raise *T* in the lungs. And we test the ability of exogenous surfactant and of a potential alternative therapeutic, sulforhodamine B (SRB), to reduce *T*. We find exogenous surfactant unable to lower *T* in the presence of cell debris but SRB, which can be administered intravascularly, a candidate for lowering *T* in non-aspiration ARDS.

Abstract

BACKGROUND: In the acute respiratory distress syndrome (ARDS), elevated alveolar surface tension, *T*, may increase ventilation-induced lung injury. Exogenous surfactant therapy has not reduced ARDS mortality. Sulforhodamine B (SRB), which acts with albumin to improve native lung surfactant efficacy, could be an alternative *T*-lowering therapeutic. We test whether substances suspected of elevating *T* in ARDS raise *T* in the lungs – where, unlike in most *in vitro* tests, the surfactant monolayer is intact – and test the abilities of exogenous surfactant and SRB to reduce *T*.

METHODS: In isolated rat lungs, we micropuncture a surface alveolus and instill a solution containing a substance purported to raise *T* in ARDS: control saline, cell debris, secretory phospholipase A_2 ($sPLA_2$), acid or mucins. We test each substance alone; with albumin, to model proteinaceous edema liquid; with albumin and subsequent exogenous surfactant; or with albumin and SRB. We determine *T* by combining servo-nulling pressure measurement with confocal microscopy, and applying the Laplace relation.

RESULTS: In the control group, saline, albumin and Infasurf do not alter *T*; SRB reduces *T* below normal. With albumin, the experimental substances raise *T*. With cell debris, surfactant does not alter *T;* SRB normalizes *T*. With $sPLA_2$, surfactant normalizes *T*; SRB reduces *T*. With acid or mucins, neither surfactant nor SRB alters *T*.

CONCLUSIONS: The inability of surfactant to counter cell debris may contribute to the failure of surfactant therapy for ARDS. For non-aspiration ARDS, SRB, which can be delivered intravascularly to target injured lung regions, holds promise as a treatment.

Introduction

Acute respiratory distress syndrome (ARDS) can occur following a direct pulmonary insult, such as gastric aspiration or corona virus-induced pneumonia, or a systemic insult, such as sepsis or acute pancreatitis (47, 49). In ARDS, alveolar edema impedes gas exchange. Mechanical ventilation supports gas exchange but exacerbates lung condition and causes ventilation-induced lung injury (VILI) (5, 6). Evidence suggests that alveolar surface tension, *T*, is elevated in ARDS and that lowering *T* should reduce VILI (15, 38, 50). However, surfactant therapy has failed, in clinical trials, to reduce ARDS mortality (4).

The cause of elevated *T* in ARDS is not well understood. While plasma proteins in edema liquid are believed to raise *T*, we and others have shown that plasma proteins do not alter *T* in the presence of an intact surfactant monolayer as exists in the lungs (22, 25, 27, 34). Other possible causes include cell debris contamination of the alveolar liquid phase (24); increased activity of phospholipases, particularly secretory phospholipase A_2 ($sPLA_2$) (17, 40); and gastric aspiration, which may lower alveolar pH or wash *T*-raising airway mucins to the alveoli (33).

In ARDS, more than one of the above substances are likely present. And while the failure of surfactant therapy may be attributable to ineffective dosage or delivery strategy (11), the ability of exogenous surfactant to lower *T* in the presence of the above-mentioned suspected *T*-raising substances and an intact surfactant monolayer has not been assessed. Further, we found that the non-toxic dye sulforhodamine B (SRB), in combination with albumin, lowers surface tension 27% below normal in healthy lungs (28) and wish to test the efficacy of SRB in the presence of the above-mentioned substances. Thus we use our technique for determining *T in situ* in surface alveoli of isolated lungs (27, 33) to determine *T* under ARDS conditions without and with exogenous surfactant or SRB.

Results

In isolated rat lungs, we micropuncture a surface alveolus and instill normal saline (NS) alone (control) or with a substance purported to raise *T* in ARDS: red blood cell (RBC) debris or debris fraction (Fig. 1); one of the $sPLA_2$ forms most likely to be present in ARDS – group IB or IIA $sPLA_2$ (19, 23, 40); hydrochloric acid (HCl); or mucins. To model the ARDS disease state in which plasma proteins are present in the alveolar edema liquid, we include albumin. To test the effect of exogenous surfactant or SRB, we administer a subsequent injection of Infasurf or include SRB in the *T*-raising substance solution, respectively. For visualization, we include non-*T*-altering fluorescein or sulforhodamine G (SRG) (27, 33). Combining servo-nulling pressure measurement and confocal microscopy, we apply the Laplace relation to determine *T in situ* in the lungs (27, 33).

Control saline. As in previous studies (27, 28, 33), alveolar injection of NS or NS + albumin does not alter *T*. Likewise, NS + albumin followed exogenous surfactant does not alter *T* (Fig. 2). However, NS + albumin + SRB lowers *T* below normal. Alveolar septa appear black, indicating that fluorescein administered with the instilled solutions remains in the alveolar liquid, (Fig. 3A).

Cell debris. Homogenized whole-RBC debris raises *T* marginally and whole cell debris + albumin raises *T* significantly (Fig. 2). Exogenous surfactant fails to counter the increase in *T*. Sulforhodamine B normalizes *T*.

From a 6x-volume lysate of RBCs, supernatant, ghost solution and ghost lipid extract fractions all raise *T* (Fig. 4A). With inclusion of albumin, lysate supernatant further raises *T*, ghost solution tends further to raise *T* and lipid extract fails to raise *T*.

Supernatants from 1x-, 6x- and 30x-volume lysates of RBCs all raise *T* (Fig. 4B). However, only for the most concentrated 1x supernatant does a solution of the same concentration of methemoglobin (metHb; Table 1) raise *T*. Thus, for the 6x and 30x supernatants, solutes other than Hb must raise *T*.

Phospholipase A_2. Group IB or IIA $sPLA_2$ solution, without or with albumin, raises *T* (Fig. 2). Subsequent surfactant normalizes *T*. Sulforhodamine B reduces (IIA) or fully normalizes (IB) *T*. Fluorescein concentration in cells suggests hydrolysis of epithelial plasma membranes and hydrolysis of macrophage plasma membranes or macrophage activation (Fig. 3B).

Hydrochloric acid and mucins. Hydrochloric acid solution causes regional damage evident in bright-field images as visible discoloration (not shown) and in confocal images as fluorescein concentration in cells (Fig. 3C), and marginally raises *T* (Fig. 2). (This *T* result is from and was significant in our previous study (33). With greater total variance, significance is lost in the present multiple comparison.) Hydrochloric acid solution buffered by albumin, despite a pH of 5.3 (Table 2), causes a similar pattern of damage (Fig. 3D) and likewise marginally raises *T*. Subsequent surfactant or inclusion of SRB further elevates *T*.

Mucin solution without or with albumin significantly raises *T* (Fig. 2). Subsequent surfactant tends to normalize *T*. Inclusion of SRB does not alter *T*.

Discussion

We consider, below, the mechanisms through which the substances we introduce into the alveolus may alter *T*.

Control Saline. Our present results with albumin (Fig. 2) are consistent with our past report that neither albumin nor blood plasma raises alveolar *T* (27). Plasma proteins can increase *T* by adsorbing faster than surfactant to a clean interface (with constant interfacial area or cyclic 50% area compression) or re-adsorbing faster following monolayer collapse (caused by 80% area compression *in vitro* or 1-30 cmH_2O transpulmonary pressure, P_L, variation in isolated lungs). However, plasma protein addition to the subphase does not alter *T* in the presence of an intact surfactant monolayer – *in vitro*, even with 50% compression applied after monolayer formation, or in isolated lungs, with physiologic P_L (22, 25, 27, 48). With functional native surfactant, additional surfactant is not beneficial but SRB + albumin, via an as-yet-to-be-determined mechanism, improves the efficacy of native surfactant and lowers *T* below normal.

Cell debris. In ARDS, the epithelium is damaged and alveolar hemorrhage is sometimes present (1, 12, 32). Our finding that surfactant fails to counter the *T*-raising effect of cell debris (Fig. 2) suggests that surfactant – even if it could be delivered to the alveolus without accumulating *T*-raising airway mucins (33) – may not be capable of reducing *T* in ARDS. Sulforhodamine B, in contrast, normalizes cell debris-elevated *T*.

To investigate the components of cell debris responsible for elevating *T*, we lyse RBCs and fractionate the lysate. The effect of albumin differs between fractions (Fig. 4A).

Extending the previous finding that RBC membrane lipids (extracted from whole sonicated cells) interfere with surfactant adsorption (24), we show lipid extract to interfere with an intact surfactant monolayer. Red blood cell membrane lipids comprise 54% phospholipid (PL), of which 6.5% is lysophosphatidylcholine (lysoPC) and 4% is disaturated PC (24). Thus membrane lipids are more fluid than and may interfere with the tight packing of surfactant phospholipids. LysoPC, in particular, has been shown to interfere with – suggested to intercalate into – intact surfactant monolayers (23, 25), though whether our lipid extract contains sufficient lysoPLs to raise *T* (20) is not known. Addition of albumin abolishes the *T*-raising effect of lipid extract. Although we use physiologically-relevant fatty acid-replete albumin, albumin likely acts by sequestering additional lipids.

Ghost solution is a homogenate of a complex mixture of RBC shells comprising cytoskeleton plus plasma membrane; organelles with their own bilayer membranes; and some whole RBCs. The presence of whole RBCs in ghost solution, which we obtain from 6x lysate, is attributable to incomplete hemolysis. During hemolysis in water, osmolarity rises quickly and retards further hemolysis. Multiplying ghost solution Hb concentration by hemolysis dilution factor, we obtain an effective 1x Hb concentration that is less than the Hb concentration of whole cell debris (Table 1), indicating incomplete hemolysis. In ghost solution, membranes may be the ghost solution component that raises *T*. This possibility is supported by the lack of effect of albumin –membranes are too large to be sequestered by albumin.

Our comparison of lysate supernatants to Hb solutions (Fig. 4B) indicates that, in lysate supernatant, there are components other than Hb that raise *T*. A difference between lysate supernatant and Hb solution is that the former contains Fe^{2+}-carrying fresh Hb whereas the latter contains Fe^{3+}-carrying metHb. That fresh Hb is less likely than metHb to disrupt the surfactant monolayer (42), however, supports the likelihood that in, 6-30x supernatant, a component other than Hb raises *T*. The *T*-raising component and the mechanism through which albumin further raises *T* (Fig. 4A) remain to be determined.

When Hb does increase *T*, one possible mechanism is an ionic interaction between Hb and negatively charged surfactant phospholipid heads that could destabilize the surfactant monolayer (42). Previously, Hb concentrations of 2.5-20 g/dl were tested during co-adsorption with surfactant and shown to raise *T* (24). Here, we show that 2.0 g/dl Hb is required to raise *T* of an intact monolayer (Fig. 4B).

Secretory PLA_2. How elevated $sPLA_2$ activity may elevate *T* in ARDS has not been fully explained. First, the mechanism could be phospholipid depletion, but >80% hydrolysis might be required to raise *T* (20, 23). As (i) the hydrolysis product lysoPL associates with, but does not completely compose, small aggregates and (ii) small aggregates contain 72% of ARDS bronchoalveolar lavage fluid (BALF) phospholipids (39, 40), this threshold is not likely met. Second, hydrolysis of plasma membranes (Fig. 3B) might release cell debris that elevates *T*. Third, the surfactant- and plasma membrane-hydrolysis products lysoPL and fatty acid (FA) could disrupt surfactant monolayer packing. LysoPLs, at 10-20% hydrolysis, raise *T* of an intact monolayer, raise *T* following co-adsorption with surfactant and, subsequent to co-adsorption, raise minimum *T*, T_{MIN}, following cyclic 50% area compression (20, 23, 25). While lysoPLs often comprise <10% of ARDS BALF phospholipids, lysoPC in one case comprised ~18% (39, 40). Fatty acids, at 10-20% hydrolysis, raise *T* during co-adsorption with surfactant and subsequently, albeit inconsistently and to a lesser degree than lysoPLs, raise T_{MIN} following cyclic 50% area compression (20, 23). Fourth, the activity of $sPLA_2$ IIA, which cleaves phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) but not PC, is elevated in ARDS BALF and the fraction of PG is reduced in ARDS BALF (21, 39, 40). Degradation of PG, which interacts with surfactant protein B, may have an outsized effect on *T* (40).

We find that albumin does not alter or marginally increases the $sPLA_2$ effect on *T* (Fig. 2). These results fit within a range of reports of interdependent albumin and $sPLA_2$-product effects on $sPLA_2$ activity. Plucthun and Dennis (35) showed that FAs stimulate $sPLA_2$ IB hydrolysis of PC/ or PE/Triton-X 100 micelles. Also for $sPLA_2$ IB, Conricode and Ochs (8) showed that cleavage products inhibit and ≤1% albumin stimulates hydrolysis of PC liposomes; and <0.1% albumin stimulates and 0.1-0.3% albumin inhibits hydrolysis of PC/cholate micelles. (Conricode and Ochs presented no data at a higher, physiologic ~5% albumin concentration.) Conricode and Ochs surmised that, as FAs stimulate $sPLA_2$ IB, likely by remaining almost entirely associated with and providing a negative charge at the lipid-water interface, (i) it must be lysoPLs, a greater fraction of which dissociate, that inhibit hydrolysis of PC liposomes and (ii) the lysoPLs must be sequestered by albumin. Conricode and Ochs further speculated that, as lipid-water interface charge affects membrane affinity for FAs, different substrate affinities for

FAs explain the different albumin effects under different conditions. Our observation that albumin does not alter the $sPLA_2$ IB effect on *T* suggests that FA stimulation and lysoPL inhibition of $sPLA_2$ IB may be relatively balanced for native surfactant, perhaps due to charge at the native surfactant-water interface, and may remain so in the presence of albumin. Although we do not know of data investigating product stimulation/inhibition of $sPLA_2$ IIA, our observation that albumin tends to increase *T* suggests the possibility of product inhibition and albumin sequestration of product.

In our experiments, Infasurf fully counters *T* elevation by either $sPLA_2$; SRB fully counters $sPLA_2$ IB and partially but significantly counters $sPLA_2$ IIA. The efficacy of Infasurf suggests that any cell debris released should be different in composition or concentration than homogenized whole cell debris, against which Infasurf is ineffective, and that the mechanism of $sPLA_2$ *T* elevation more likely depends on hydrolysis of phospholipids, whether those of surfactant or the plasma membrane. As SRB acts by interacting with albumin and native surfactant, sufficient functional surfactant must be present for SRB to lower *T*. Our results suggests that SRB may counter *T* elevation by $sPLA_2$ in ARDS.

Acid aspiration. In aspiration, pH 2.2-4.5 gastric contents (7) enter the airways and, *en route* to the alveolus, mix with airway liquid that should buffer pH but contribute *T*-raising mucins (29, 33). If there is little mixture with airway liquid then low pH liquid, which should aggregate mucins and block their *T*-raising effect (26, 33), may reach the alveolus. As pH 2 solution does not alter surfactant function *in vitro* (16), raises alveolar *T* progressively over time (33) and damages the epithelium (Fig. 3C), low pH may raise *T* indirectly via generation of cell debris. If there is significant mixture with airway liquid, then it may be a higher-pH liquid that reaches the alveolus. If pH exceeds 4, mucins should be un-aggregated (26) and may raise *T* just as, following tracheal saline instillation, mucins reached the alveolus and raised *T* by 42% (33). As tracheal saline instillation does not cause lung injury, however, the physiological significance of mucin-elevated *T* is not known.

That albumin-buffered HCl, with a pH of 5.3 (Table 2), injures the epithelium (Fig. 3D) is surprising. We speculate that solution pH may decrease upon injection into the alveolus, where phospholipid head group phosphates may induce H^+ dissociation from albumin.

Neither Infasurf nor SRB reduces the *T* elevation caused by acid or mucins. As discussed above, acidic solution may raise *T* by generating cell debris. We speculate that acid, unlike $sPLA_2$, may damage the plasma membranes in a way that results in release of cell debris similar to that of our homogenized whole cell debris, against which Infasurf is ineffective (Fig. 2). And we previously showed Infasurf ineffective against mucins – tracheal Infasurf instillation, by accumulating mucins, raises alveolar *T* by 45% (33). We speculate that the large, non-glycosylated hydrophobic domains of mucins (41, 44) interfere with surfactant by hydrophobic interaction. The inefficacy of SRB against HCl could be attributable to a pH effect on SRB-albumin interaction. Albumin Sudlow site I attracts the xanthene rings of SRB (30). As discussed above, alveolar injection may reduce the pH of HCl + albumin solution. And pH <4.3 causes compaction of albumin (3, 10), which might interfere with albumin-SRB binding. Alternatively, the SRB response to pH is likely similar to that of rhodamine WT, which has a net charge of -1 at pH >4.7 and becomes a zwitterion below (46). The lost charge, which is not on the xanthene rings, might not prevent interaction with albumin but might alter the interaction of an albumin-SRB complex with native surfactant. In the presence of mucins, we again suspect hydrophobic interaction – the hydrophobic domains of mucins may sequester the xanthene rings of SRB (30).

Study limitations. One limitation of our study is that we do not test all purported mechanisms of *T* elevation in ARDS. We do not investigate possible cholesterol contamination of the surfactant monolayer (14, 31). While blood plasma is a major source of cholesterol, cell membranes also contain significant cholesterol (45). That SRB normalizes *T*-elevation caused by cell debris (Fig. 2) suggests that SRB might be effect against *T*-elevation caused by cholesterol. Another possible mechanism is oxidative

damage to surfactant phospholipids or proteins by leukocyte- or macrophage-released reactive oxygen species (ROS). As discussed elsewhere (34), the principal effect of phospholipid oxidation is likely to be via generation of lysophospholipids (36). As SRB reverses $sPLA_2$-elevated *T*, which is likely attributable to lysophospholipid generation, SRB would likely reduce *T* elevated by phospholipid oxidation. Oxidation of surfactant protein B, however, may also contribute to *T* elevation in ARDS (36). Whether SRB would reduce such *T* elevation remains to be determined. Another limitation is that, for the substances we test, we do not know the pathophysiologic concentration in ARDS edema liquid. Thus, we select concentrations that raise *T* to levels comparable to those we observed in the lungs in other models of lung injury (27, 33, 51). To determine whether SRB can reduce *T* under ARDS conditions, testing in animal models of ARDS will be required.

Conclusion. We show that cell debris, $sPLA_2$, acid and mucins raise *T* of an intact surfactant monolayer. In acid-aspiration ARDS, neither surfactant therapy nor SRB is likely to reduce *T*. And against cell debris, exogenous surfactant is ineffective, which may help to explain the inefficacy of surfactant therapy in treating ARDS.

Sulforhodamine B normalizes cell debris-induced *T*-elevation and reduces $sPLA_2$-induced *T* elevation. Sulforhodamine B could be administered via the trachea or the vasculature. Tracheal instillation would be unadvisable given that it washes *T*-raising mucins to the alveolus (33). Tracheal nebulization would avoid collecting airway mucins but is inefficient and delivers a spatially variable dose (9, 13, 18, 43), likely concentrated in healthy lung regions. Intravenous administration, in contrast, capably deliver SRB, at a controllable concentration, directly to injured lung regions (52). Intravenous SRB might effectively lower *T* in non-aspiration ARDS.

Methods

We perform this study in isolated rat lungs.

Isolated lung preparation

As described previously (33), we anesthetize a male or female Sprague-Dawley rat (225-325g, n = 37) with 3.5% isoflurane in 100% oxygen. We puncture the heart through the chest wall (21G needle) and euthanize by withdrawal of ~10 ml blood into a syringe with 1 ml of 1000 units/ml heparin. We cannulate the trachea (blunted 15G needle connected to a stopcock), perform a midline thoracotomy, inflate the lungs (2 ml air), close the stopcock and excise the heart and lungs *en bloc*. We place the lungs on a microscope stage and connect an air source and pressure transducer to the tracheal stopcock. We increase P_L to 30 cmH_2O and then decrease P_L to a constant 5 cmH_2O, thus maintaining lung volume above functional residual capacity.

Model ARDS solutions

We model ARDS edema liquid using NS (pH 5.0) as the solvent. We include 23 µM fluorescein (00297-17, Cole Parmer, Vernon Hills, IL) in all solutions excepting Infasurf (ONY Biotech, Amherst, NY), in which we include 5 µM SRG.

Cell debris. We start by washing the collected RBCs (Fig. 1). We centrifuge the heparinized blood (5000 x g, 4 °C, 20 min) and discard the supernatant. We suspend the pellet in pH 7.5 NS to the heparinized-blood volume, centrifuge and discard the supernatant, and repeat the process two more times. Next, to obtain whole-cell debris we homogenize the RBCs or to obtain cell debris fractions we lyse the RBCs.

1. To obtain whole-cell debris, we suspend the pellet in pH 5.0 NS to the heparinized blood volume. We homogenize the solution on ice (VDI12, VWR, Radnor, PA; maximum speed; 5min) and filter (3-μm pore) the solution.

2. To obtain cell debris fractions, we modify the methods of Rosenberg *et al*. (37). We lyse the RBCs by suspending the pellet in 1, 6 or 30x the heparinized blood volume of Milli-Q-purified water ("water," pH unadjusted, room temperature, 2 hrs). We centrifuge the lysate (20,000 x g, 4 °C, 40 min), separate the supernatant and pellet and follow one of three protocols:

2a. Lysate supernatant. We add NaCl (0.9%) to the supernatant from the 1x-, 6x-, or 30x-volume lysate. The Hb-containing solutions are translucent and vary from red (1x) to pink (30x).

2b. Ghost solution. We wash the 6x lysate pellet. We suspend the pellet in the 6x lysate volume of pH 7.5 NS (more Hb is removed at neutral than acidic pH), centrifuge the suspension (20,000 x g, 4 °C, 40 min) and discard the Hb-containing supernatant, and repeat the process two more times. We suspend the pellet in pH 5.0 NS to the 6x lysate volume, homogenize the solution on ice (maximum speed, 5 min) and filter (3-μm pore) the solution. The solution is light pink and cloudy.

2c. Lipid extract of RBC ghost. We wash the 6x lysate pellet as in 2b and then suspend it in 10:3 water:toluene, with the water volume that of the 6x lysate. To enable phase separation, we vortex the solution briefly, let it stand on ice (2 hrs) and then centrifuge it (13,000 x g, 4 °C, 30 min). We collect the toluene layer, evaporate the toluene under nitrogen and obtain dry ghost lipids. We weigh some samples, prepared specifically for lipid quantification, on a microbalance. We prepare other samples for alveolar injection by suspending the dry lipids in 100 μl of pH 5.0 NS. The ghost lipid solution is clear, without color.

We quantify Hb concentration of whole cell debris, lysate supernatants and ghost solution by Drabkin's assay. We divide each sample for duplicate assay and use bovine metHb (H2500, Sigma Aldrich, St. Louis, MO) as a standard. For each solution, we calculate effective 1x Hb concentration by multiplying measured concentration by dilution factor. For each lysate supernatant, we prepare a solution of the same concentration of MetHb.

sPLA$_2$. We dissolve bovine pancreatic sPLA$_2$ IB (P8913, Sigma Aldrich; 0.1 mg/ml) or recombinant rat sPLA$_2$ IIA (Uniprot no. P14423 from ELISA kit LS-F23950, LS Bio, Seattle, WA; 2.5 ng/ml) in pH 5.0 NS.

Acid and mucins. To test low pH or mucins, we add 0.01 N hydrochloric acid (HCl) or 25 μg/ml porcine gastric mucin (M2378, Sigma Aldrich), respectively, to pH 5.0 NS.

Experimental groups

For control pH 5.0 NS and solutions of each potential *T*-raising substance, we test: (i) Solution alone. (ii) To model plasma-protein rich ARDS edema liquid, solution + 5% bovine serum albumin (A8327, Sigma Aldrich). (iii) To test exogenous surfactant, initial administration of solution + 5% albumin and subsequent administration, to the same region, of Infasurf. (iv) To test SRB (341738, Sigma Aldrich), single administration of solution + 5% albumin + 10 nM SRB. Table 2 shows pH values for select solutions.

Protocol

We hold P_L at 15 cmH_2O, puncture an aerated alveolus on the costal surface with a glass micropipette filled with a given solution and inject approximately 7-10 μl of solution, which floods a group of alveoli. In flooded alveoli, the air-liquid interface forms a meniscus (2, 27).

We determine *T* in a flooded alveolus as described previously (27, 33). For Infasurf groups, we determine *T* in an alveolus in which the flooding solution is labeled with both fluorescein and SRG. Briefly, after alveolar injection, we ventilate twice between P_L of 5 and 15 cmH_2O, then hold P_L at 15 cmH_2O. We determine alveolar air pressure from the tracheal transducer, alveolar liquid pressure by servo-nulling pressure measurement (Vista Electronics, Ramona, CA) and three-dimensional meniscus radius from a z-stack of fluorescent confocal images (x20 water immersion objective, 0.7 N.A., 0.2-mm working distance, plan apochromatic; SP5 confocal microscope, Leica Microsystems, Buffalo Grove, IL). We calculate *T* from the Laplace relation, obtaining one *T* value per solution injection.

Statistical analysis

We analyze data sets comprising experimental groups (i)-(iv), detailed above, plus an additional group for normal *T*, also at P_L of 15 cmH_2O, of the aerated alveolar liquid lining layer (data from (27)) by one-way ANOVA and post-hoc Tukey's analysis. We report group mean ± SD and accept $p<0.05$ as significant.

Author contributions

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

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FIGURE LEGENDS

Figure 1. Protocol for obtaining cell debris. Protocol for (1) homogenizing red blood cells (RBCs) to obtaining whole cell debris or (2) lysing RBCs to obtain (2a) lysate supernatant, (2b) ghost solution or (2c) lipid extract.

Figure 2. Solution effects on alveolar surface tension, *T*. Base solutions are: normal saline (control solution and solvent of experimental solutions); whole cell debris, with hemoglobin concentration of heparinized blood; secretory phospholipase A_2 ($sPLA_2$) IB, 0.1 mg/ml; $sPLA_2$ IIA, 2.5 ng/ml; hydrochloric acid, 0.01 N; and porcine gastric mucin, 25 µg/ml. Additives are as shown. Surface tension determined at 15 cmH_2O transpulmonary pressure over 10-15 min period following solution injection. Horizontal gray bar shows mean ± SD for *T* of normal liquid lining layer in aerated lungs, also at 15 cmH_2O transpulmonary pressure, from (27). Data for "no additive" groups with saline, hydrochloric acid and porcine gastric mucin are from previous study (33). Statistics: *$p < 0.05$ vs. normal *T* in aerated lungs; # $p < 0.05$ between indicated groups.

Figure 3. Solution effects on cells. Confocal images of alveoli after flooding with fluorescein (23 µM; green in images) in **(A)** normal saline, **(B)** 0.1 mg/ml $sPLA_2$ IB, **(C)** 0.01 N HCl or **(D)** 0.01 N HCl plus 5% albumin. In (A), airspace (labeled) and septa (dashed lines) are black. The latter indicates fluorescein exclusion by intact epithelium. In (B-D), fluorescein appears to be concentrated in macrophages (arrowheads, round morphology), alveolar epithelial type I cells (open-headed arrows, thin morphology lining septum) and, likely, alveolar epithelial type II cells (closed-headed arrows), indicating damage to cell membranes and possible macrophage activation. Image (A) is representative of images not only for saline injection but also for injection of saline + albumin, mucin and mucin + albumin. Image (B) is representative of images for all injections of $sPLA_2$ IB without and with albumin but only for 1/4 injections of $sPLA_2$ IIA without albumin and 2/4 injections of $sPLA_2$ IIA with albumin. Images of other $sPLA_2$ IIA injections, without or with albumin, look like (A). However, $sPLA_2$ IIA elevates *T* even without causing visible cellular injury. Images (C and D) are representative of images for all injections of HCl without and with albumin, respectively. Images (488 nm excitation, indicated laser power, 750 gain) taken ~2 min after solution injection, at 15 µm sub-pleural depth. Low pH in alveolar liquid quenches fluorescein. Due to saturation in (B) and (C) on left, lower-laser-power replicate images shown on right.

Figure 4. Red blood cell fraction effects on *T*. (A) Effects of 6x lysate fractions on *T*. Sets of fraction solutions (no additive, + 5% albumin) obtained from same blood sample. **(B)** Effects of all concentrations of lysate supernatant and solutions with matching concentrations of methemoglobin on *T*. Lysis water volume is multiple of original heparinized blood volume. Surface tension determination, horizontal bar and statistics as in Fig. 2.

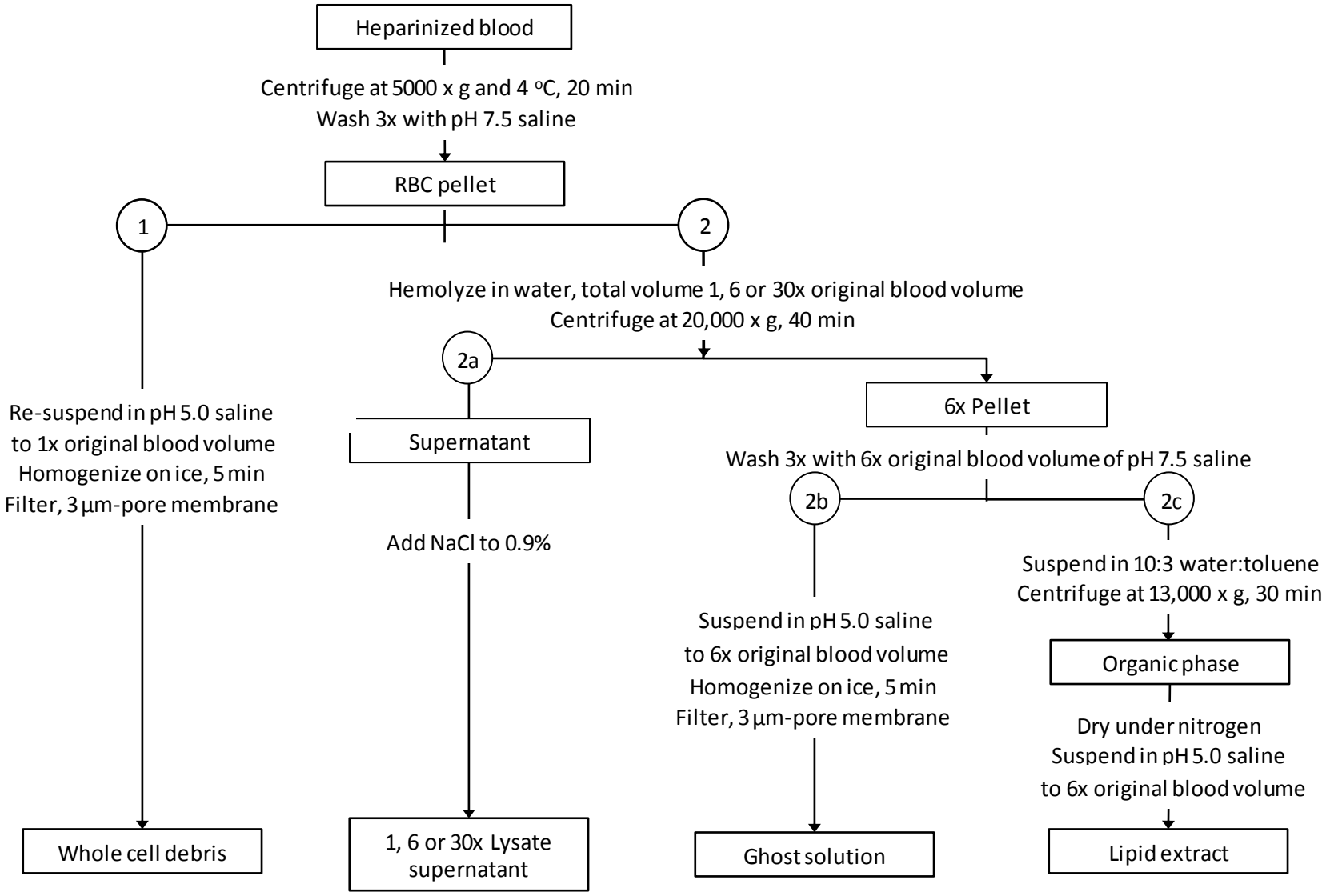
Heparinized blood
Centrifuge at 5000 x g and 4 °C, 20 min
Wash 3x with pH 7.5 saline
RBC pellet
1
2
Re-suspend in pH 5.0 saline
to 1x original blood volume
Homogenize on ice, 5 min
Filter, 3 µm-pore membrane
Whole cell debris
Hemolyze in water, total volume 1, 6 or 30x original blood volume
Centrifuge at 20,000 x g, 40 min
2a
Supernatant
Add NaCl to 0.9%
1, 6 or 30x Lysate
supernatant
6x Pellet
Wash 3x with 6x original blood volume of pH 7.5 saline
2b
2c
Suspend in pH 5.0 saline
to 6x original blood volume
Homogenize on ice, 5 min
Filter, 3 µm-pore membrane
Ghost solution
Suspend in 10:3 water:toluene
Centrifuge at 13,000 x g, 30 min
Organic phase
Dry under nitrogen
Suspend in pH 5.0 saline
to 6x original blood volume
Lipid extract

Figure 1

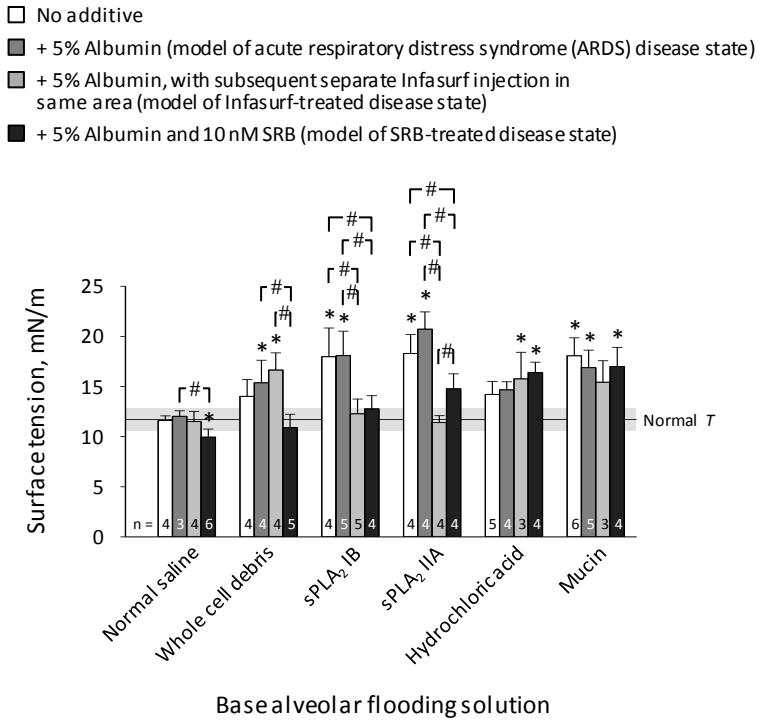
No additive
+ 5% Albumin (model of acute respiratory distress syndrome (ARDS) disease state)
+ 5% Albumin, with subsequent separate Infasurf injection in same area (model of Infasurf-treated disease state)
+ 5% Albumin and 10 nM SRB (model of SRB-treated disease state)
Surface tension, mN/m
25
20
15
10
5
0
Normal T
n = 4 3 4 6
4 4 4 5
4 5 5 4
4 4 4 4
5 4 3 4
6 5 3 4
Normal saline
Whole cell debris
sPLA2 IB
sPLA2 IIA
Hydrochloric acid
Mucin
Base alveolar flooding solution

Figure 2

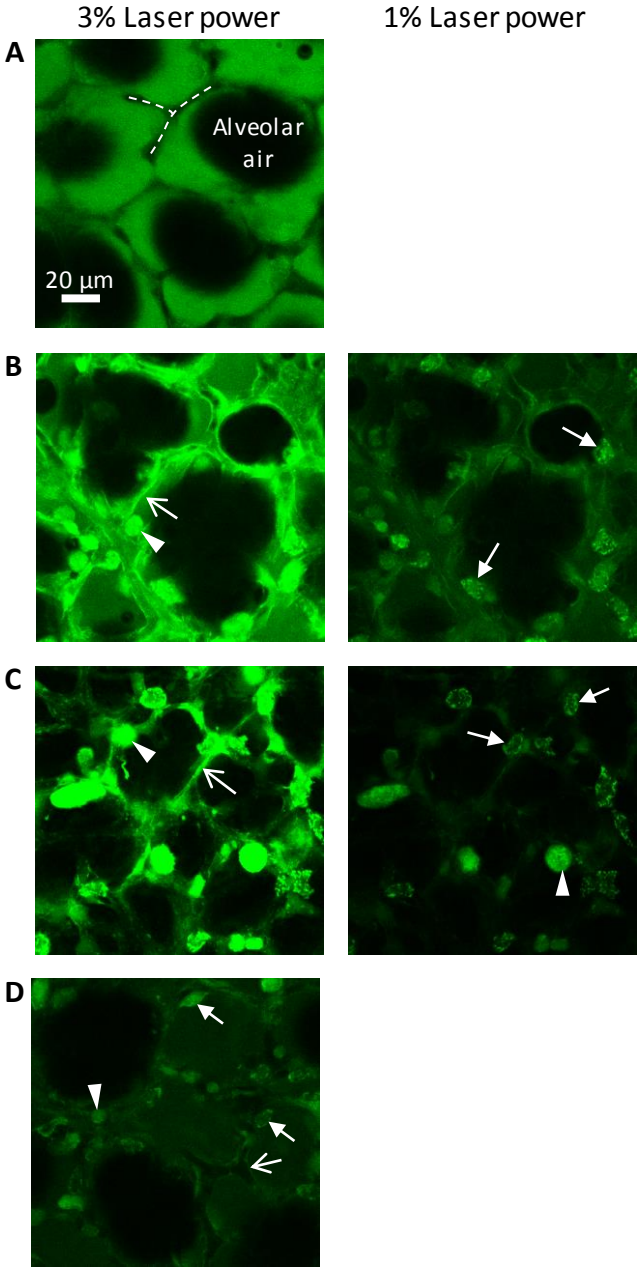

Figure 3

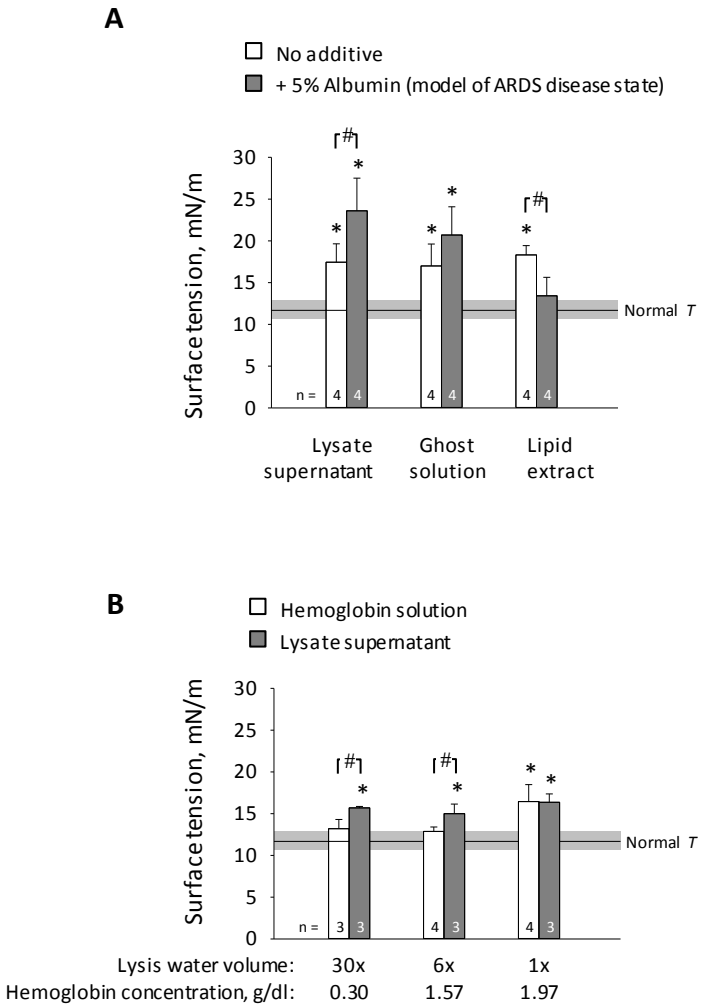
A
No additive
+ 5% Albumin (model of ARDS disease state)
Surface tension, mN/m
30
25
20
15
10
5
0
n = 4 4 4 4 4 4
Normal T
Lysate supernatant
Ghost solution
Lipid extract
B
Hemoglobin solution
Lysate supernatant
Surface tension, mN/m
n = 3 3 4 3 4 3
Normal T
Lysis water volume: 30x 6x 1x
Hemoglobin concentration, g/dl: 0.30 1.57 1.97

Figure 4

Table 1: Concentrations of hemoglobin (Hb) and lipids in whole red blood cell (RBC) debris and RBC lysate fractions
Solutions obtained from heparinized blood as depicted in Fig. 1 and described in Methods. Effective 1x concentration is product of measured concentration and dilution factor.

	Hb concentration, g/dl (n = 3/group)	**Lipid concentration, g/dl** (n = 3/group)	**Effective 1x Hb concentration, g/dl**
Whole cell debris from homogenization	13.3 ± 0.34	—	13.3
Supernatant from 1x-volume lysate	1.97 ± 0.27	—	1.97
Supernatant from 6x-volume lysate	1.57 ± 0.13	—	9.42
Supernatant from 30x-volume lysate	0.30 ± 0.01	—	9.00
Ghost solution from 6x-volume lysate	0.12 ± 0.02	—	0.72
Lipid extract from 6x-volume lysate ghost	—	0.13 ± 0.02	—

Table 2: Albumin effects on pH of select solutions

Solution pH measured on benchtop at 22°C before injection into alveoli. *p < 0.05 vs. 0% albumin/0 nM SRB group for same solution.

	0% Albumin 0 nM SRB (n=3/group)	**5% Albumin 0 nM SRB** (n=3/group)	**5% Albumin 10 nM SRB** (n=3/group)
Normal saline	5.03 ± 0.02	6.83 ± 0.07	6.88 ± 0.05
0.01 N HCl	1.98 ± 0.02	5.31 ± 0.08	5.23 ± 0.02