In situ enhancement of pulmonary surfactant function using temporary flow reversal

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Glindmeyer HW 4th, Smith BJ, Gaver DP 3rd. In situ enhancement of pulmonary surfactant function using temporary flow reversal. J Appl Physiol 112: 149–158, 2012. First published October 13, 2011; doi:10.1152/japplphysiol.00643.2011.—Acute respiratory distress syndrome is a pulmonary disease with a mortality rate of ~40% and 75,000 deaths annually in the United States. Mechanical ventilation restores airway patency and gas transport but leads to ventilator-induced lung injury. Furthermore, surfactant replacement therapy is ineffective due to surfactant delivery difficulties and deactivation by vascular proteins leaking into the airspace. Here, we demonstrated that surfactant function can be substantially improved (up to 50%) in situ in an in vitro pulmonary airway model using unconventional flows that incorporate a short-term retraction of the air-liquid interface, leading to a net decrease in cellular damage. Computational fluid dynamic simulations provided insights into this method and demonstrated the physicochemical hydrodynamic foundation for the improved surfactant microscale transport and mobility. This study may provide a starting point for developing novel ventilation waveforms to improve surfactant function in edematous airways.

microfluidic cell culture; pulmonary surfactant; respiratory distress syndrome; airway reopening; physicochemical hydrodynamics

ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) and infant respiratory distress syndrome (IRDS) are pulmonary diseases with mortality rates of ~40%, resulting in 75,000 deaths in the United States annually (26). Disruption of the mechanical environment in the lung by these diseases plays a large role in the viability of sensitive pulmonary tissue and may elicit pathological responses from the epithelial cell lined-lumen of the respiratory tract. For example, the reopening of obstructed airways, which is necessary for allowing gas transport to the alveoli, may cause cell necrosis (3, 8, 13, 22, 23, 38), increased permeability (29), and pulmonary surfactant dysfunction that can have a long-term consequence to pulmonary function (7, 9). Treatment with mechanical ventilation restores airway patency and gas transport; however, this is a double-edged sword that may lead to ventilator-induced lung injury (VILI). VILI is characterized by damage resulting from high lung volume ventilation (barotrauma), the repeated closure and reopening of fluid-filled or collapsed airways (atelectrauma), and mechanically induced systemic inflammatory responses (biotrauma) (36). The mechanical stresses that occur during the reopening of occluded airways may introduce or exacerbate the lung injury caused by ARDS and IRDS and contribute to VILI (12, 25).

Figure 1A shows the reopening of an atelectic airway. This is conceptualized as a finger of air that displaces the occlusion fluid and imposes hydrodynamic stresses on the airway wall, the magnitudes of which are determined by the dynamic surface tension and geometry of the air-liquid interface. Computational fluid dynamic simulations have been used to investigate the magnitudes of these stresses, as shown in Fig. 1B (11, 15, 20, 40). These studies predict that cells far downstream of the air bubble experience only a small shear stress, whereas cells near the bubble tip experience a combination of shear and normal stresses and cells in the reopened region are exposed primarily to a normal stress (i.e., pressure). Most importantly, the cells near the bubble tip experience significant spatial and temporal gradients in shear and normal stress that may contribute to VILI.

Pulmonary surfactant dynamically modifies the surface tension of the lining fluid in the lung and is critical to adequate pulmonary function. However, surfactant is deficient in IRDS (2) and ARDS, resulting in elevated surface tension. This amplifies the magnitude of the mechanical stresses during airway reopening and contributes to VILI. Surfactant replacement therapy reduces the severity of IRDS through the exogenous delivery of surfactant. Unfortunately, delivery of surfactant for the treatment of ARDS has not been shown to be effective, likely due to delivery obstacles and competitive adsorption by blood plasma components such as albumin and cholesterol that infiltrate the airspaces with edema fluid (37). This problem is amplified over time. For example, a recent study by Seah et al. (30) has shown in mouse models of VILI that continuous mechanical ventilation leads to the progressive accumulation of fluid into the lung. This results in a “vicious cycle” that leads to a commensurate rise in surface tension and progressive atelectrauma that causes the lung to be increasingly difficult to ventilate.

To investigate the specific mechanisms leading to airway damage during reopening, several studies have propagated a semi-infinite finger of air across a confluent monolayer of pulmonary epithelial cells in a constant-height flow chamber (3, 13, 23), whereas another study (22) investigated the damage associated with the propagation or rupture of liquid plugs. Reopening with a semi-infinite finger of air induces an increase in cellular membrane wounding with decreasing reopening velocity. By correlating these experimental results with theoretical stress field predictions, it is possible to implicate the large normal stress gradient near the bubble tip as the mechanical stimulus that causes cell damage. Damage to epithelial cells was reduced when pulmonary surfactant was introduced to the occlusion fluid. This occurs because the surfactant adsorbs to the air-liquid interface and lowers its surface tension, thereby decreasing the normal stress gradient that sweeps across the surface of the cell (3).

In the present work, our goal was to investigate the hypothesis that the transport and efficacy of endogenous surfactant may be substantially improved through the use of novel ven-
tillation waveforms that will promote microscale flow fields that will induce physicochemical interactions leading to surfactant enrichment at the air-liquid interface. To do so, we investigated the use of unsteady flows in a cylindrical tube model of a pulmonary airway (Fig. 1C) that is lined with a confluent monolayer of human lung epithelial cells (Fig. 1D).

Experimental studies (9, 21) have already demonstrated that interfacial compression (as occurs during exhalation) can cause the formation of a surfactant multilayer with an ultra-low metastable surface tension. This mechanism is demonstrated by the surfactant surface tension versus area hysteresis loop shown in Fig. 2A that occurs during purely oscillatory volume cycling of a nearly spherical bubble (6, 24). We hypothesized that airway reopening that is forced by a constant flow with a short-term superimposed flow reversal may be used to incorporate interfacial compression during reopening, thereby dynamically reducing the surface tension in the vicinity of the bubble tip, which may protect the airway epithelium from atelectrauma. While a multilayer may only be initiated during the first cycle, with repetitive cycling it is expected that this multilayer may grow and enhance the protective effects associated with reduced surface tension by providing a substantial reservoir of surfactant to the bubble tip (28).

Interactions between interfacial flows and surfactant transport are highly coupled. In steady forward flow, theoretical and experimental investigations in model airway systems have demonstrated that a nonuniform surfactant distribution on the bubble tip will lead to surface tension gradients that rigidify the interface by inducing a shear stress that opposes the direction of the microscale flow in the vicinity of the interface (Marangoni stress) (14, 34). Therefore, microscale surfactant transport limitations can directly result in increased reopening pressures and mechanical stresses during interfacial expansion. In contrast, interfacial compression can enhance the surfactant concentration, forcing the surfactant molecules into a multilayer formation with ultra-low surface tension, as exists in the base region of the surface tension hysteresis loop shown in Fig. 2A.

By combining the forward flow with temporary flow reversal that results in a net forward motion, we hypothesized that the average surface tension will be decreased and that this will protect epithelial cells. Benchtop experiments by Pillert and Gaver (28) have explored the potential of multiple cycles of this type of flow (pulsatile flow) to reduce the average reopening pressure. Specifically, these experiments investigated a range of cycling frequencies (0 Hz ≤ frequency ≤ 1 Hz), oscillation amplitudes, and waveforms (fast forward/slow reverse vs. symmetric vs. slow forward/fast reverse) on macroscale measures of surfactant function. This study (28) demonstrated that a frequency = 1 Hz fast forward/slow reverse pulsatile flow is capable of reducing the macroscale pressure by ~30% compared with steady flow of the same average velocity. While this macroscale investigation demonstrated the potential for pulsatile flow to reduce mechanical stresses associated with atelectrauma, flow reversal has not been tested for reducing cell damage in controlled biological studies. The goal of this study was therefore to investigate whether bubble retraction, as would occur in pulsatile flow, may protect cell layers.

To examine the effect of surface tension reduction (possibly related to multilayer formation and redistribution) during un-
steady flow in a benchtop airway reopening scenario, we dynamically drove the air-liquid interface after a trajectory, as shown in Fig. 2B. This trajectory, based on the finding that a reopening velocity combined with a slower retraction velocity may be the best method for lowering the surface tension on the interface (28), contained a 25-mm/s steady reopening event that was superimposed with a single ~0.84-s withdrawal that temporarily retracted the bubble at a velocity of ~5 mm/s. Figure 2B shows stages 1, 2a, 2b, and 3 of the waveform and relates these stages to the different phases of the flow field that are shown in Fig. 2C.

Our quantitative understanding of microscale transport processes and surfactant redistribution as a function of the flow waveform is based on computational simulations by Zimmer et al. (41) and also the computational/experimental work by Smith et al. (33). These studies, as well as the experimental work of Yamaguchi et al. (39), show that particulates redistribute onto the interface in different locations based on whether the bubble is moving in the forward or reverse direction. Finally, the macroscopic experimental work by Pillert and Gaver (28) has shown that the pressure drop across the air-liquid interface changes substantially as a function of pulsatility. Since the pressure drop is largely determined by the surface tension and interfacial curvature at the tip, this provides evidence for surfactant sorption and redistribution during flows with temporary reverse flow. It should be noted that we do not have experimental evidence for multilayer formation and that an alternative hypothesis could simply be based on the residence time for surfactant sorption and a redistribution based on the convection field shown in Fig. 2. This residence time is substantially increased in the retrograde motion region.

Surfactant physicochemical interactions occur when microscale fluid streamlines convect surfactant to different regions of the interface, and the resulting surfactant concentration gradients influence the flow field. Locations of accumulation occur at converging stagnation points (+), and depletion occurs at diverging stagnation points (−; Fig. 2C). With intermittent flow reversal, both forward (Fig. 2C, 1 and 3) and reverse (Fig. 2C, 2a and 2b) phases exist: this leads to regions of surfactant redistribution sweeping across the interface. This potentially has two primary benefits: 1) it may distribute surfactant more uniformly across the tip of the interface tension versus area for a cyclically oscillated air-liquid interface of a solution doped with pulmonary surfactant. Static adsorption at high concentrations led to an equilibrium surface tension of 22 dyn/cm and a surface concentration of \( \Gamma_m \). A reduction in area compressed the interface and enriched the surfactant concentration \( (\Gamma_{\text{max}}) \), resulting in a metastable ultra-low surface tension and multilayer formation. Reexpansion of the interface reduced the surfactant concentration and increased the surface tension. Hysteresis occurred from dynamic transport processes during compression and expansion (24, 27). B: velocity trajectory of the reopening finger of air as it propagated through the system with a pulsatile forcing. The different stages of the flow are marked as 1, 2a, 2b, and 3, with a negative (retrograde) velocity occurring near the center of the tube length during stages 2a and 2b. C: microscale flow field and surfactant distribution surrounding the bubble tip as it propagated through a tube during stages 1, 2a, 2b, and 3. Dashed lines with arrowheads represent fluid streamlines that transport surfactant toward the bubble tip and create nonuniform surfactant distributions on the air-liquid interface during the pulsatile trajectory described in B. Surface-tension gradients on the air-liquid interface create Marangoni stresses (\( \tau_m \)) that can redistribute surfactant.

Fig. 2. Physicochemical hydrodynamic interactions during pulsatile reopening. A: surface tension versus area for a cyclically oscillated air-liquid interface of a solution doped with pulmonary surfactant. Static adsorption at high concentrations led to an equilibrium surface tension of 22 dyn/cm and a surface concentration of \( \Gamma_m \). A reduction in area compressed the interface and enriched the surfactant concentration \( (\Gamma_{\text{max}}) \), resulting in a metastable ultra-low surface tension and multilayer formation. Reexpansion of the interface reduced the surfactant concentration and increased the surface tension. Hysteresis occurred from dynamic transport processes during compression and expansion (24, 27). B: velocity trajectory of the reopening finger of air as it propagated through the system with a pulsatile forcing. The different stages of the flow are marked as 1, 2a, 2b, and 3, with a negative (retrograde) velocity occurring near the center of the tube length during stages 2a and 2b. C: microscale flow field and surfactant distribution surrounding the bubble tip as it propagated through a tube during stages 1, 2a, 2b, and 3. Dashed lines with arrowheads represent fluid streamlines that transport surfactant toward the bubble tip and create nonuniform surfactant distributions on the air-liquid interface during the pulsatile trajectory described in B. Surface-tension gradients on the air-liquid interface create Marangoni stresses (\( \tau_m \)) that can redistribute surfactant.
bubble and 2) it may create a multilayer that provides a reservoir of surfactant in the high resistance region near the wall, as shown in Fig. 2C. This reservoir would create a Marangoni stress that reduces the effect of interfacial rigidification. While not studied here, proper manipulation of a repetitive pulsatile reopening waveform may therefore enhance multilayers that are created during flow reversal (stages 2a and 2b) that will significantly reduce the surface tension at the interface and hence decrease the magnitude of the detrimental normal stress gradient along the airway wall.

Without surfactant, the reverse flow stages (stages 2a and 2b) are likely to be detrimental because they will create enormous normal stress gradients (32) that would damage human lung cells; therefore, this allows the use of a surfactant-free control to investigate the efficacy of surfactant physico-chemical interactions for airway protection. Additionally, we incorporated albumin into a subset of our experiments to investigate competitive adsorption effects that may occur during ARDS due to increased vascular permeability. In summary, the goal of this report is to describe experiments that investigated the biological effects of temporary reverse flow during airway reopening so as to decrease the damage that occurs during a single reopening event.

MATERIALS AND METHODS

Overview. We quantify the damaging stresses caused by reopening scenarios through analysis of cell viability in a 1.6-mm-inner diameter silicone tube with a fully confluent monolayer on the inner surface. To prepare these flow chambers, tubes were first coated with 0.15 mg/ml fibronectin (Invitrogen, Carlsbad, CA) and then rotated 180° every 6 h over a single 24-h period. Cells from human lung airway adenocarcinoma epithelial cell line NCI H441 (HTB-174, American Type Culture Collection, Manassas, VA) were then seeded at a density of 5 \times 10^5 cells/ml. Six hours after the initial seeding, tubes were rotated 180° to ensure uniform cell coating, and 6 h later, 200 μl fresh media was pipetted into the culture. Tubes reached a confluent state 24 h after this media replacement and were then ready to be used for experiments (Fig. 1D). At confluence, live/dead cell viability (Invitrogen) tests showed ~1% cell death and 97% confluence throughout the tube. As a control condition, tubes were filled with PBS and attached to the system to rest in the 37°C water bath for 5 min. These controls thus accounted for possible confounding elements that could damage the H441 cells, including stresses that arose during the assembly and disassembly processes or through the addition of PBS to the system. We demonstrated that handling did not affect the amount of cell injury (as described in MATERIALS AND METHODS) (Fig. 1D). It should be noted that the cells shown in the middle bottom and bottom images of Fig. 1D are indeed fully confluent; the procedure for combining the fluorescent images resulted in a deep blue background of cells while emphasizing the damaged red cells (normally, the tube would look like the two bright-field images).

In these reopening scenarios, we investigated the effectiveness of pulmonary surfactant (Infasurf, ONY, Buffalo, NY) using the following four different occlusion fluids, which were placed into the model airways:

1. 0.0 mg/ml surfactant (surfactant free)
2. 0.1 mg/ml surfactant
3. 1.0 mg/ml surfactant
4. 1.0 mg/ml surfactant with an addition of 5.0 mg/ml albumin

We investigated the 0.0 and 0.1 mg/ml surfactant concentrations as a lower range to identify concentration-dependent behavior in our system, which may be most appropriate for surfactant deficient lungs (such as in ARDS) without surfactant replacement therapy. Since Infasurf is conventionally delivered at a concentration of 35 mg/ml but becomes diluted by native liquid in the lung, it is reasonable to expect that concentrations in this situation will be 1 mg/ml or higher. In IRDS, vascular permeability (which causes protein leakage) is not the root cause of surfactant insufficiency, and so we considered surfactant alone to be physiologically relevant for modeling initial reopening events. In cases of ARDS, we additionally doped the lining fluid with albumin at a concentration of 5 mg/ml to mimic the leakage of vascular fluid and proteins into the airspaces. The albumin concentration was chosen to be consistent with the work of Braun et al. (4). The low concentrations in our experiments are thus most relevant to cases of surfactant deficiency/deactivation that would exist in IRDS or ARDS due to flooding of the airspaces or delivery limitations.

Reopening was initiated by a custom actuator-based (LinMot, Spreitenbach, Switzerland) syringe pump that pushed air into the occluded airway (Fig. 1C). As shown in Fig. 2B, a reverse flow occurred as the bubble tip progressed halfway through the tube. After the liquid obstruction was cleared, a staining procedure was administered to quantify the damage to the cell layer. This design allows the damaging stresses caused by reopening to be mapped across the length of the tube, since after the scenario a live/dead stain was placed into the tubes and a damage profile can be recovered through analysis with a fluorescent microscope.

The reopening waveform was created by the following flow rates \((Q_0 - Q_3)\) that were programmed to our custom actuator-based syringe pump:

\[
Q(t) = \begin{cases} 
Q_0 & t < t_0 \\
Q_1 + Q_2 \cos(2\pi f(t - t_0)) & t_0 \leq t \leq t_1 \\
Q_3 & t > t_1
\end{cases}
\]

where \(Q_0 = Q_1 = 5 \times 10^{-2} \text{ cm}^3/\text{s}, Q_1 = 2 \times 10^{-2} \text{ cm}^3/\text{s}, Q_2 = 3 \times 10^{-2} \text{ cm}^3/\text{s}, \) frequency \(f = 0.24 \text{ Hz}, \) initial time \(t_0 = 0.84 \text{ s}, \) and final time \(t_1 = 5 \text{ s} \). This resulted in a regime with a nearly constant velocity, followed by a segment with reverse flow in the middle of the tube, and finally with a nearly constant velocity area in the postretrogade region. For the graphs of velocity versus position or cell death (Fig. 3), we approximated the meniscus tip velocity \((U)\) as \(U(t) = Q(t)/(\pi R^2)\), where \(R\) is the radius. This approximation neglected the minor effects of cell height (~5 μm) and residual film thickness (~20 μm), as estimated by the classical work of Bretherton (5). Since the bubble displaces nearly all of the fluid in the tube, this velocity approximation has an error of ~5%.

The average velocity in the segment was 12.4 mm/s, which is approximately the convective air velocity in the region spanning the transition between the conducting airways and respiratory bronchioles in the patent lung. It should be noted that reopening velocities in the lung are likely to deviate substantially from airflow velocities due to complex interfacial instabilities (19) that may result in avalanche-like reopening scenarios (35).

Cell culture. Cells from human lung airway epithelial cell line NCI H441 (HTB-174, American Type Culture Collection, Manassas, VA) were seeded on a 20-cm-long silicone tube (Bio-Rad, Hercules, CA) with an inner diameter of 1.6 mm and outer diameter of 1.8 mm. To accomplish this, silicone tubes were autoclaved, coated with 0.15 mg/ml fibronectin (Invitrogen), and incubated for 24 h under standard culture conditions (humidified, 37°C, 5% CO2-95% air). During that period, the tube was rotated 180° every 6 h. Once the silicone tubes were prepared, H441 cells were suspended within the tissue culture flask using 0.25% trypsin with EDTA (Invitrogen), removed to a 15-ml conical tube, and centrifuged at 130 rpm for 5 min. The supernatant was discarded, and the remaining cell pellet was resuspended in RPMI-1640 growth media with L-glutamine, 10% FBS, and 1% antibiotic-antimyotic solution (Invitrogen) at 5 \times 10^6 cells/ml. Suspended solution (200 μl) was then gently pipetted into one of the prepared silicone tubes, which resulted in the ejection of the fibronectin solution. It is necessary to prevent air bubbles from entering the system as this interferes with the results. Six hours after initial
seeding, tubes were rotated 180° to ensure even cell coating, and 6 h later, 200 μl of fresh media were pipetted into the culture. Tubes reached a confluent state 24 h after the media replacement and were then ready to be used for experiments.

Apparatus. Each reopening experiment was performed using a custom actuator-based (LinMot) syringe pump that introduced air into the system using the waveform described in Eq. 1. This was guided by the LinMot talk r1.3.14 program, which sends a signal to a controller...
that guides the oscillatory motion of the pump. The plunger of a 250-μl glass syringe was attached to the actuator, and its lower tip was connected by a stopcock with a 1/8-in. luer fitting (Cole-Parmer) to a 50 cm length of the silicone tubing. At the other end of this attachment was a 1/8-in. straight barbed connector to which the 20-cm-long silicone tubes coated with a fully confluent H441 cell layer were attached for experimentation. Finally, to the outlet of these tubes was attached an additional 30 cm length of silicone tube by another 1/8-in. straight barbed connector, which emptied into a waste beaker. The 20-cm-long portion of tubing coated with cells was submerged in a warm saline bath at 37°C.

Reopening scenario. Four occlusion fluids were examined. Phosphate-buffered saline including 0.1 mg/ml CaCl2 and MgSO4 (PBS) was used to model a surfactant-deficient lung. Pulmonary surfactant experiments were performed using Infasurf (ONY), a biologically derived pulmonary surfactant. This solution was delivered at a 35 mg/ml phospholipid concentration, which had been warmed to 37°C and mixed by three gentle inversions. Two different dilutions were prepared for experimentation with an Infasurf concentration of 0.1 or 1.0 mg/ml, in addition to a third solution containing 1.0 mg/ml Infasurf along with 5.0 mg/ml albumin (Invitrogen), which is a concentration that has used in another pulmonary study (4). These three preparations modeled low surfactant, high surfactant, and surfactant deactivation by serum protein scenarios, respectively.

Before experimentation, 200 μl of the desired occlusion fluid were added to a fully confluent silicone tube, which was then fit to the reopening apparatus. The actuator-based syringe pump was programmed to follow Eq. 1 and introduced 200 μl of air into the system, thereby displacing the liquid occlusion and simulating a reopening event. The bubble tip velocity was in the range of ~5 mm/s ≤ bubble tip velocity ≤ 25 mm/s, with a single bubble tip retraction occurring in the middle of the silicone tube (Fig. 2A). Once the air infusion was complete, the silicone tube was removed from the saline bath for subsequent analysis.

As a control condition, tubes were filled with PBS and attached to the system to rest in the 37°C water bath for 5 min. This controls thus accounted for possible confounding elements that could damage H441 cells, including stresses that arose during the assembly and disassembly processes or through the addition of PBS to the system. We quantified the resulting cell injury (using the methods described in Quantification of cellular injury) with no increase in injury compared with the nonhandled control.

Quantification of cellular injury. Once removed from the apparatus, the entire length of tube with attachments was connected to a constant-rate syringe pump (KD Scientific, New Hope, PA), at which point a staining solution was introduced to the tubing at 25 mm/s. This solution contained 5 μM Hoechst (Invitrogen), which stains the nuclei of all cells in the system, and 5 μM ethidium homodimer-1 (Invitrogen), which stains the nuclei of cells with plasma membrane damage, in PBS. Pulmonary surfactant (1.0 mg/ml) was also incorporated to minimize the potentially damaging effects of reintroducing liquid into the system during the staining procedure.

Each tube was placed onto the stage of a Nikon Eclipse TE2000-E inverted epifluorescence microscope (Nikon, Melville, NY) 5 min after the initial staining procedure. The cell layer was examined at ×10 magnification using a red (excitation: 510–560 nm) or blue (excitation: 325–375 nm) emission filter with identical exposure times for each type of image acquired. The bottom-most portion of the entire length of tubing was imaged, resulting in 200 images/tube, including 2 images on each length for red and blue emission filters. However, 1 cm from each end, where the straight barbed connector protruded into the system, was disregarded. These images were captured and stored using a microscope-mounted, cooled digital 12-bit charge-coupled device camera system (Sensicam QE, COOKE, Romulus, MI) and IPLab imaging software (Becton Dickinson, Franklin Lakes, NJ).

The percentage of red-emitting cells relative to the entire quantity of blue-emitting cells in each image was counted using a custom MATLAB program (Mathworks, Natick, MA). Automatic counting ensured the absence of viewer bias that may arise during manual counting. The curvature of the tube resulted in ~40% of an image being out of focus, and measurements were therefore taken in the central 60% of the image. Statistical analysis of the results was established using one-way ANOVA with specific statistical differences between each experimental group identified using a Tukey post hoc test. In addition, four-way linear regressions were computed for the four experimental groups using the regression analysis system in SigmaPlot 11 (Systat Software, San Jose, CA) and added to the data plots. Live/dead images were combined to form a single image using ImageJ (National Institutes of Health, Bethesda, MD), after which an autoadjust of brightness was performed for each image to maximize the contrast between live and dead cells; nevertheless, not all live cells were viewable through this process. In Fig. 4, the normalized dead

![Fig. 4. Comparison of cell death before and after retrograde motion. Cell death versus velocity using the death before (blue) and after (red) retrograde motion is shown. A: surfactant-free group. B: 0.1 mg/ml Infasurf group. C: 1.0 mg/ml Infasurf group. D: 1.0 mg/ml Infasurf + 5.0 mg/ml albumin group. Area values are the differences in the area under the curves, with area > 0 corresponding to a decrease in cell death as a result of retrograde flow.](http://jap.physiology.org/)

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values represent the five-point moving average of the data shown in Fig. 3. We noted that during the quantification of cell injury, there was insignificant cell detachment. While cell detachment was not quantified, we can estimate that 3–5 h after experimentation, ~25–50% of cells were detached in and around the region of retrograde motion. These are similar in values to experiments with three airway reopening events before the quantification of cell injury (38).

**Finite-time Lyapunov exponent analysis.** Velocity information was computed using the boundary element method, as described in Smith and Gaver (32). An initially regular grid of tracer particles (spacing height: 0.01) was advected with fourth-order Runge-Kutta integration computed using the boundary element method, as described in Smith and Gaver (32). These are similar in values to experiments with three airway reopening events before the quantification of cell injury (38).

The finite-time Lyapunov exponent from the initial time \( t_0 \) was advected with fourth-order Runge-Kutta integration computed using the boundary element method, as described in Smith and Gaver (32). Following Shadden et al. (31), the finite-time Cauchy-Green deformation tensor was determined as follows:

\[
\Delta(x) = \left[ \frac{d}{dx} \phi_t^{i+1}(x) \right] \times \left[ \frac{d}{dx} \phi_t^{i}(x) \right]
\]

so that the finite-time Lyapunov exponent (FTLE) field was as follows:

\[
\sigma_{i}^{T}(x) = \frac{1}{T_i} \log \sqrt{\lambda_{\text{max}}[\Delta(x)]}
\]

where \( \lambda_{\text{max}}[\Delta(x)] \) is the maximum eigenvalue of the symmetric matrix \( \Delta(x) \).

**RESULTS**

Figure 3A shows the damage profile that exists in the surfactant-free case. Damage was quantified as normalized fraction of cells with plasma membrane wounds (\( \eta \)), where the normalization factor equalled the fraction of cells that were wounded in surfactant-free steady-state reopening at 25 mm/s (12%). In this case, velocity reduction clearly increased damage (\( P < 0.01 \)). Consistent with the normal stress gradient as the damaging stimulus (3, 23); however, the most damaging stimulus occurred during retrograde motion (2a and 2b). In this region (demarcated by the vertical lines in Fig. 3), the normal stress gradient was increased (32), and the bubble propagated three times across the cell layer, increasing damage substantially (\( \eta = 2.5; P < 0.01 \)).

When only a slight concentration of surfactant was incorporated (0.1 mg/ml), the damage profile changed substantially (Fig. 3B). In this case, \( \eta = 0.3 \) in the forward phase, and thus membrane wounding was reduced by 70%. Furthermore, bubble retraction no longer caused a substantial increase in damage; instead, the level of damage was held nearly constant (\( \eta = 0.3 \)), which was a reduction in damage of 88% from the peak damage in the surfactant-free case. Since a constant surface tension interface would result in an increase in damage during retraction (see above), this result demonstrates that retrograde motion does not maintain a constant surface tension and results in a decrease in surface tension. This occurred due to an interfacial compression that increased surfactant surface concentrations and a modified flow field that recruited surfactant to the bubble tip region (41), as shown schematically in Fig. 2C. This reduced the surface tension and protected the cell layer from the extraordinarily large normal stress gradients that would exist during the retrograde stages (stages 2a and 2b) in a constant surface tension system.

Strikingly, while the 1.0 mg/ml surfactant group demonstrated equivalent protection as the 0.1 mg/ml surfactant group during the forward steady flow phase (1), it also afforded extraordinary protection during the retrograde flow stages (stages 2a and 2b; \( P < 0.01 \)), as shown in Fig. 3C. This resulted in a reduction to \( \eta = 0.15 \) from a level of \( \eta = 0.3 \) in the steady flow region, indicating that retrograde flow further protected the epithelial layer and reduced the damage by 50% compared with steady flow with surfactant (and by 94% from the surfactant-free retrograde flow case). We also observed that the “protective” effect of the retrograde motion led to a decrease in damage that extended ~1.5 cm past the region of reverse flow. While full multilayers were likely to develop after multiple cycles, the extended region of protection indicated that a reservoir of surfactant may be developed through the initiation of a multilayer formation that could exist on the interface in the high resistance region during the retrograde motion (Fig. 2C).

Finally, we added 5 mg/ml of albumin to the high concentration (1 mg/ml) surfactant system to evaluate the scenario of competitive adsorption between albumin and surfactant, since this has been hypothesized to lead to surfactant deactivation in ARDS (41). Figure 3D shows this deactivation, with damage increasing to \( \eta = 0.9 \) in the upstream forward phase from \( \eta = 0.3 \) without albumin. This shows that albumin-based deactivation increases the dynamic surface tension to nearly that of the surfactant-free case (\( \eta = 1.0 \)). Interestingly, these albumin experiments did not demonstrate the extraordinary damage that was observed in retrograde flow in the surfactant-free experiments. This lack of increased damage during retrograde flow indicates the presence of protective surface-associated molecules at the air-liquid interface when albumin was introduced, even though competitive adsorption had deactivated much of the surfactant function. After retrograde motion, the damage was substantially reduced (\( \eta = 0.7 \)). This 23% reduction of damage was significant (\( P < 0.05 \)) and indicates an increase in surfactant function that persisted for an extended length (~3.5 cm) as the bubble progressed down the tube. Evidently, retrograde flow entices pulmonary surfactant to incorporate onto the interface or excludes albumin from the surface, which diminishes the ability of albumin to competitively absorb on the forward stroke, potentially as a result of solution-capillary-induced Marangoni stresses (16).

**DISCUSSION**

This study demonstrates that the dynamic motion of an air-liquid interface can significantly affect the viability of the epithelial cell layer. In our study, we found that air-liquid interfaces introduced into an idealized model of pulmonary airway reopening with flow reversal can have either a positive or negative effect depending on the surface-active properties of surfactant in the system.

We observed that when there was no surfactant (providing a constant surface tension interface), retrograde motion led to a large increase in cellular damage. However, this effect was negated with the introduction of only a small concentration of surfactant (0.1 mg/ml). With the addition of higher concentrations of surfactant, retrograde motion protected epithelial cells (concentration: 1.0 mg/ml), leading to a decrease in damage during and after the oscillation. Finally, we found evidence that pulmonary surfactant that is contaminated by albumin can be partially “reactivated” by retrograde motion.
The measurements in our system were conducted to evaluate lethal damage to epithelial cells. Since the measurement of membrane damage occurred several minutes after insult, we cannot report the magnitude of cell wounding and subsequent repair if it occurs on the timescale of <1 min. Therefore, we may underestimate the level of acute cell damage that occurs directly after the passage of a bubble. Additionally, the introduction of the air-liquid interface may increase cell damage; however, this insult was uniform across all samples. We note that during the quantification of cell injury, there was insignificant cell detachment (for an example, see Fig. 3A,2). Detachment of cells occurred well after measurements were conducted, although we did not quantify the level of cell detachment.

Prior experiments on the relationship between damage, mucus velocity, and exposure duration have been reported by Kay et al. (23). This study (23) demonstrated that velocity, and not exposure duration, correlated with the magnitude of damage to epithelial cells. In our current study, the time course of the reopening events was equivalent for each trial. While the surfactant-free case exhibited increased damage during retraction, this was not the case in situations where surface-active substances were incorporated. This provides further evidence in support of the local mechanical environment being the primary cause of damage. The measure of inertia versus viscous stresses in the system is provided by the Reynolds number ($Re = \rho UR/\mu$), where $\rho$ is the density and $\mu$ is viscosity. In our system, $Re << 1$, and therefore inertia is negligible compared with viscous effects. Likewise, the capillary number (capillary number $= \mu U/\gamma$, where $\gamma$ is the surface tension) relates the magnitude of viscous to surface tension effects. Since the capillary number was $<<1$, surface tension dominated viscous effects (and hence inertia as well). The present study therefore indicates that interfacial mechanics determine the level of epithelial cell damage and that this effect can be modulated locally by surfactant physicochemical interactions.

To quantify the change in damage before and after retrograde motion (phases 1 and 3, respectively), we graphed the average cell death versus velocity (Fig. 4). Since repeated cycling was not explored, these are not precisely hysteresis loops; however, the relative difference between damage during phase 1 and 3 curves provides insights into the protection afforded by flow reversal (phase 2). To quantify this trend, we calculated the difference in the area under each phase. A phase 1 and 3 curves provides insights into the protection afforded by flow reversal (phase 2). To quantify this trend, we determined the level of epithelial cell damage and that this effect can be modulated locally by surfactant physicochemical interactions.

Fig. 5A shows the FTLE for forward steady flow with the LCS demarcated by a solid red line extending from the diverging stagnation point (−) on the bubble. Two particle trajectories are shown on either side of the LCS. A particle originally located (or “seeded”) radially outside of the LCS (yellow) propagated upstream to the thin film, whereas a neighboring particle seeded inside the LCS (red) advected downstream of the LCS (red) advected downstream ahead of the advancing bubble (Fig. 5A). So, in steady forward flow, the residence time for surfactant in the bubble tip region is small, although the converging stagnation point at the tip (+) may lead to surfactant accumulation (39). In contrast, during steady reverse bubble motion (Fig. 5B), the LCS divided the domain at the centerline. Here, the convection field separates particles that were initially seeded on opposite sides of the neighborhood of a point over a finite time interval (17, 31). The maximal regions of the color map (shown in red) describe Lagrangian coherent structures (LCS) that represent boundaries between qualitatively different regions of the flow. The LCS demarcates the boundary between the regions of the fluid that become separated as the bubble progresses down the airway. Note that Fig. 5 shows a two-dimensional representation of a three-dimensional structure; therefore, the off-center LCS is a surface of revolution around the centerline axis.

To quantify the transport dynamics associated with the protective effects related to flow reversal, we computationally simulated this system (32). As shown in Fig. 5, we visualized the Lagrangian convective transport characteristics of fluid in the neighborhood of the bubble tip using a FTLE field. This FTLE represents the maximum rate of fluid stretching in the
centerline, since the flow transports a particle initialized adjacent to the bubble tip (red) radially outward along the interface towards the wall. So, this analysis confirmed that a temporary flow reversal will allow surfactant accumulated at the bubble tip to redistribute along the interface, as shown in Fig. 2C.

Figure 5C shows the FTLE and LCS for pulsatile flow similar to that of our experiments. In this simulation, the trajectory of a single particle seeded inside the LCS (red dot) was followed as the bubble propagated through phases I–IV (as shown in Fig. 2C). The combination of forward and reverse flows caused this particle to migrate into the thin film and then reverse direction to migrate along the cap region into the downstream bulk. This flow pattern significantly increased the residence time for surfactant adsorption to the interface and will lead to surfactant accumulation, redistribution, and potentially the creation of multilayers, as schematically shown in Fig. 2C. Thus, this computational fluid dynamic simulation provided support for the physicochemical interactions that motivated this study. For further analysis of Lagrangian transport properties in this system, we refer to Smith et al. (33). This report used the FTLE method to analyze Lagrangian transport properties using experimentally determined velocities in surfactant-doped solutions.

Conclusions. In summary, ARDS and IRDS are two of the most severe forms of acute lung injury, with significant mortality rates. Edema results in obstructive airway closure (15), and the reopening of these airways is associated with traumatic stresses that damage the pulmonary epithelium (3). The present study combined experimental and computational methods to investigate surfactant physicochemical hydrodynamic interactions that may protect the lung from physical insult during the inflation of atelectic airways.

We observed that flow reversal in the presence of endogenous surfactant substantially reduced the deleterious mechanical stresses associated with a semi-infinite bubble propagating through a tube lined with a confluent monolayer of human lung airway epithelial cells, whereas in surfactant-free situations the damage was substantially increased. We also found that cell protection may persist beyond the retrograde portion of the waveform, demonstrating the possible existence of an enriched surface-associated phase that can be used to propagate surfactant further into the airway.

We showed that these interactions can mobilize very small quantities of endogenous surfactant to significantly increase concentrations at the bubble interface. While these experiments did not investigate waveform properties [as studied by Pillert and Gaver (28)], we nevertheless demonstrated a substantial reduction in airway damage using retrograde flow. This reduction may result from dynamic surfactant multilayer interactions due to temporary interfacial compression.

The present study therefore suggests an approach akin to “endogenous surfactant therapy,” a term coined by Arold et al. (1). Their research suggests that surfactant secretion can be significantly enhanced through the use of variable ventilation rates. They hypothesized that variable rate ventilation induces stochastic resonance that increases the flux of surfactant from alveolar type II cells, thereby increasing the concentration of surfactant in the lining fluid and leading to a reduction of atelectrauma.

Instead, we propose that flow reversal may provide a means for endogenous surfactant delivery. Here, the dynamic flow field takes advantage of native surfactant to target delivery specifically to regions of the lung where airway recruitment is occurring. This provides a reservoir of surfactant to areas that are difficult to reach with exogenous surfactant that are at high risk for damage. The use of exogenous surfactant delivery may reduce the need for exogenous surfactant delivery, which is a difficult and inefficient process (18).

While surfactant has long been recognized as fundamental to defining the macroscale properties of the lung, our study demonstrated a microscale protective mechanism that relates to surfactant function that has heretofore not been recognized. Flow reversal uses this protective mechanism to reduce the damage that might otherwise occur during the propagation of an air-liquid interface through an edematous airway.

Since heterogeneous distributions of surfactant may exist in the diseased lung, a cautious approach toward reverse flow ventilation should be implemented in regions of the lung that are entirely devoid of surfactant since bubble retraction is damaging in the surfactant-free system. However, the present study shows that only a very small amount of surfactant (concentration: 0.1 mg/ml) is sufficient to prevent additional damage during retrograde flow. Furthermore, higher concentrations of surfactant (1.0 mg/ml) result in enhanced protection with retrograde flow, most likely due to an increased concentration of surfactant at the tip of the bubble and along the high resistance region near the vessel wall following the dynamics shown in Fig. 2C. Additionally endogenous surfactant transport to the bubble tip region may provide a reservoir that will allow surfactant to penetrate into surfactant-free areas and thereby reduce the damage to sensitive tissues.

Exhalation will result in long durations of reverse flow and the potential for reclosure of segments of the lung. The present study and those of Fujioka and Grotberg (10) and Smith and Gaver (32) show that long segments of backward flow create regions with very high mechanical stresses near the wall. Surfactant transport may ameliorate this effect, but it is also likely that short pulsations of forward flow during exhalation could be helpful in this scenario as well.

We acknowledge that this study is highly idealized because it involves the investigation of one relatively inflexible airway with a single reverse flow regime. Since the protection extended beyond the reverse flow region, it is likely that a pulsatile flow could be used to extend the region of protection. The development of strategies that implement ventilation management in heterogeneous branching airways will be a major challenge, as it will require the opening of a network of airways while preventing the overinflation of patent airways and alveoli. Nevertheless, it is our hope that these observations may contribute to the design of novel treatments for ARDS and IRDS that could reduce the severity of VILI.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
REFERENCES


