Investigations of pulmonary epithelial cell damage due to air-liquid interfacial stresses in a microgravity environment

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Pulmonary airway closure is a potentially dangerous event that can occur in microgravity environments and may result in limited gas exchange for flight crew during long-term space flight. Repetitive airway collapse and reopening subjects the pulmonary epithelium to large, dynamic, and potentially injurious mechanical stresses. During ventilation at low lung volumes and pressures, airway instability leads to repetitive collapse and reopening. During reopening, air must progress through a collapsed airway, generating stresses on the airway walls, potentially damaging airway tissues. The normal lung can tolerate repetitive collapse and reopening. However, combined with insufficient or dysfunctional pulmonary surfactant, repetitive airway collapse and reopening produces severe lung injury. Particularly at risk is the pulmonary epithelium. As an important regulator of lung function and physiology, the degree of pulmonary epithelial damage influences the course and outcome of lung injury. In this paper, we present experimental and computational studies to explore the hypothesis that the mechanical stresses associated with airway reopening inflict injury to the pulmonary epithelium.

Experimental Investigations

Experiments were performed in a parallel plate chamber lined with pulmonary epithelial cells, which was constructed as an idealized model of a collapsed segment of an airway where the walls are held in opposition by a viscous fluid. These experiments were conducted to determine whether air-liquid interfacial stresses can cause damage to epithelial cells, and to provide response behavior that can be correlated to the mechanical stimuli determined from computational investigations (below).

In a first set of experiments, a fetal rat pulmonary epithelial cell line (CCL-149, ATCC) was cultured to confluence on a small (1 cm²), square region of the upper plate. The narrow channel was filled a model airway lining fluid. Phosphate buffered saline including 0.1 mg/mL CaCl₂ and MgSO₄ (PBS) was used to model a surfactant-deficient airway lining fluid. A surfactant-containing airway lining fluid was approximated using Infasurf® (ONY, Inc.) diluted to 1 mg/mL phospholipid concentration in PBS. Airway “reopening” was generated by the steady progression of a semi-infinite bubble of air down the length of the channel using a constant rate infusion pump (7 or 70 ml/min). A digital camera mounted above the channel collected sequential overhead images of the progressing bubble, which were used to calculate bubble velocity. Once removed from the apparatus, the slide was incubated with 1.2 μM Ethidium homodimer-1 (Eth-1) and 1.2 μM calcein AM (Molecular Probes). For each slide, the number of injured cells was recorded as the average number of Eth-1 stained nuclei counted in fluorescence microscopic images.

In a second experimental study, we attempted to discriminate the stress magnitude from the stimulus duration. To do so, the stress magnitude is modified by varying the viscosity of the
occlusion fluid while fixing the reopening velocity across experiments. This approach causes the stimulus duration to be inversely related to the magnitude of the pressure gradient. We also explore the mechanism for acute damage and demonstrate that repeated reopening and closure is shown to damage the epithelial cell layer even under conditions that would not lead to extensive damage from a single reopening event.

**Fluid Dynamic Simulations**

The bubble and parallel-plate flow chamber was modeled as a semi-infinite bubble progressing within a Hele-Shaw cell. In this model the walls were separated by a distance $2H$, with the semi-infinite bubble progressing in the $x$-direction with tip velocity $U$. The surface tension, $\gamma$, was constant. The capillary number, $Ca = \mu U/\gamma$, representing the relative importance of viscous to surface tension effects on the bubble determines the dynamic response of the system. Stokes equations, $\nabla \cdot \mathbf{P} = \mu \nabla^2 \mathbf{u}$ and $\nabla \cdot \mathbf{u} = 0$, were solved using the boundary element method. The interfacial stress condition applied at the air-liquid interface was $|\sigma \cdot \mathbf{n}| = \gamma \kappa \mathbf{h}$, where $\sigma = -P \mathbf{I} + \mu (\nabla \mathbf{u} + (\nabla \mathbf{u})^\top)$ was the stress tensor, $\mathbf{n}$ was the unit normal, and $\kappa$ was the interfacial curvature. For a given $Ca$, the system was simulated until a steady-state meniscus had developed and the stress-field and bubble geometry were determined.

Three potentially injurious components of the stress cycle associated with bubble progression — the shear stress, the shear stress gradient, and the pressure gradient — were analyzed. Regression relationships describing the behavior of these components as a function of $Ca$ were determined for very small $Ca$ ($5 \times 10^{-4} \leq Ca \leq 2 \times 10^{-3}$). Additionally, the thickness of the thin film deposited by bubble progression was estimated. Dimensionless values for the experimental flow conditions were extrapolated from the regression equations and redimensionalized.

**Results and Discussion**

For each condition the average number of injured cells per square centimeter was measured. For the saline-occluded channels, bubble progression at both velocities produced significantly increased numbers of injured cells when compared to the control. The slow velocity resulted in a 66-fold increase in the number of injured cells and the fast velocity produced a 20-fold increase. The addition of Infusurf to the occlusion fluid reduced the number of injured cells to a level similar to the control. These results support the hypotheses that mechanical stresses associated with airway reopening injure pulmonary epithelial cells and that pulmonary surfactant in the normal lung protects the epithelium from injury due to airway reopening.

The stress component that best agrees with the experimentally observed trauma is the maximal pressure gradient. Pressure gradients create a force imbalance on the cell membrane over the length of the cell. In addition, cell damage remains directly correlated with the pressure gradient, not the duration of stress exposure. For a low profile predominately flat cell (or region of a cell), the non-uniformly distributed load can depress the cell and stretch the membrane. For high profile cells or regions of a cell, such as the protrusion cause by the nucleus, where the normal forces of the cell surface are nearly opposite, the pressure gradient will pinch that region. The pinching can tear the membrane at the base of the protrusion or force fluid upward rupturing the top surface of the cell. The present study thus provides additional evidence that the magnitude of the pressure gradient induces cellular damage in this model of airway reopening.
Investigations of Pulmonary Epithelial Cell Damage Due to Air-Liquid Interfacial Stresses in a Microgravity Environment

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Critical Path RoadMap

**Cardiovascular Alterations**
- Impaired Response due to Modified Orthostatic Mechanical Stress
- Diminished Cardiac Function
- Impaired Response to Exercise Stress

**Pulmonary Alterations**
- Airway Closure Becomes more Homogeneous
- Potentially Impaired Pulmonary Function
- Impaired Response to Exercise Stress
Gravity Effects on Ventilation Distribution

(A) At FRC the lower region is less expanded, but more compliant so it receives larger portion of ventilation

(B) At RV the lower lung regions experience airway closure

Airway Closure in Microgravity

MICROGRAVITY CAUSES:

• Regional Modification of Ventilation
• Changes of Blood Perfusion
• Variation in Lung Capacity

• ‘Patchy’ regions of airway collapse
Related Terrestrial Syndromes

- Infant Respiratory Distress Syndrome
- Acute Respiratory Distress Syndrome
- Ventilator-Induced Lung Injury
Pulmonary Multiscale Interactions
Motivation

Our goal is to determine the cause of reopening-induced damage, and the surfactant properties and airway reopening strategies that will allow pulmonary airways to be opened with minimal damage to the lung.
Stresses in Airway Reopening


Direction of Bubble Progression

Air Bubble

Collapsed Airway
Mechanisms of Cell Mechanotransduction and Damage

3-D Surface Topography Influences Stress Distribution

Luminal Transmembrane Proteins

Cell-cell proteins

Force Transmission

Nuclear Membrane

Focal Adhesion Sites

Matrix

Nucleus

Adapted from Davies, *Physiol. Rev.*, 1995
Lung epithelial cells were:
- Cultured in an idealized model of small airways,
- Exposed to a moving finger of air under reopening conditions,
- Examined for cellular trauma.
Methods – Variable Velocity

- Lung epithelial cells (CRL-149, ATCC) cultured to confluence on glass microscope slides.

- The channel dimensions were 2.5 x 7.0 x 0.17 cm.

- **Two velocities** (0.27 and 2.7 cm/s) were assessed.

- Two occlusion fluids were assessed:
  - phosphate buffered saline (PBS) and
  - 1 mg/mL Infasurf (ONY, Inc., Buffalo, NY) in PBS.

- Cellular trauma was quantified using fluorescent staining (Live/Dead Kit, Molecular Probes).
Stress Field – Rigid Channel

Direction of Bubble Progression

Air Bubble

Fluid Occlusion
Injury by a Single Bubble Progression
(L2 cells, Live/Dead Kit)

Bubble Velocity

Control

PBS

Occlusion Fluid

Infasurf

0.27 cm/s

2.7 cm/s
Injury by a Single Bubble Progression
(L2 cells, Live/Dead Kit)

PBS Infasurf (1 mg/mL in PBS)

<table>
<thead>
<tr>
<th>Bubble Velocity [cm/s]</th>
<th>Injured Cells [cells/mm²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.27</td>
</tr>
<tr>
<td>0.27</td>
<td>500</td>
</tr>
<tr>
<td>2.7</td>
<td>100</td>
</tr>
</tbody>
</table>

n = 5
* p < 0.01
Mechanisms of Cell Membrane Wounding

- Shear Stress
- Shear Stress Gradient
- Pressure
- Pressure Gradient
The Flow Model

Steady Flow of a Semi-Infinite Bubble in a Channel

Surface Tension, $\gamma_{eq}$

$Q = \text{Constant}$

Newtonian Fluid, $\mu$

2H

Governing Parameter: $Ca = \frac{\mu U}{\gamma_{eq}}$
### Predictions

**Fluid** | **Speed** | $\tau_s$ (dyn/cm²) | $\Delta \tau_s$ (dyn/cm²) | $\Delta P$ (dyn/cm²) | $f$ (µm)
---|---|---|---|---|---
Saline | Slow | 15.5 | 9.2 | 340 | 1.4
Saline | Fast | 34.3 | 10.1 | 170 | 6.0
Infasurf | Slow | 7.9 | 3.4 | 89 | 2.7
Infasurf | Fast | 17.5 | 3.8 | 44 | 11.6

**Control** Bubble Velocity [cm/s] 0.27 2.7

Different speeds and fluids show varying effects on $\tau_s$, $\Delta \tau_s$, $\Delta P$, and $f$. The diagram illustrates the impact of these variables on cell damage.
Predictions of Cell Normal Stresses

1. Film Thickness decreases with decreasing velocity
2. The pressure gradient on the cell surface increases with decreasing velocity
The variable velocity experiments induce stresses on cells that are not of constant duration.

Hypothesis:
The slow velocity experiments may induce greater damage because of the increased exposure time.
Methods – Constant Velocity

- Human Pulmonary Epithelial Cells (A549, ATCC) cultured to confluence on glass microscope slides.

- The channel dimensions were 2.5 x 7.0 x 0.17 cm.

- A single velocity (0.34 cm/s) was applied.

- Two viscosities were used
  \[ \mu = 8 \times 10^{-3} \text{ g/(cm s)} \text{ (PBS)} \]
  \[ \mu = 8 \times 10^{-2} \text{ g/(cm s)} \text{ (PBS + 14% Dextran)} \]

- Cellular trauma was quantified using fluorescent staining (Live/Dead Kit, Molecular Probes).
Traveling-Wave Behavior

Lubrication Film

Film Thickness Increases as Ca Increases

Ca > 0

Ca = 0

Pressure Field Near Contact Line

Pressure Gradient Decreases as Ca Increases

\[ Ca = \frac{\mu U}{\gamma} \]
Traveling-Wave Behavior

\[ \Delta P \sim \gamma / H \]

|dP/dx| Decreases as Ca Increases

\[ \Delta t_{\text{exp}} = \frac{L_{\text{wave}}}{U} \propto \frac{H\mu^{0.29}}{U^{0.71}\gamma^{0.29}} \]

\[ Ca = \frac{\mu U}{\gamma} \]
Pressure Gradient, not Exposure Duration, Determines Damage

\[ U = 0.34 \text{ cm/s}, \mu_{\text{Dextran}} = 10\mu_{\text{PBS}} \]

Kay et al., JAP, 2004
Our system is modeled to isolate the influence of epithelial topography on the following components of the stress cycle during airway reopening:

– shear stress and shear stress gradient
– normal stress and normal stress gradient
Computational Model

Geometric Parameters: $\varepsilon = a/H$, $\Lambda = \lambda/H$
Computational Model

Boundary Element Method

Interfacial Stress

\[ \tau^* = \gamma \kappa^* n \]

Stokes Flow

\[ \nabla^* P^* = \mu \nabla^2 u \]

Kinematic Boundary Condition

\[ \frac{\partial Y^*}{\partial t^*} n = u^* n \]

Lubrication Theory

Governance Parameter: \[ \text{Ca}_Q = \frac{Q^* / 2H}{\gamma / \mu} \]
Normal Stress Distribution

\( \lambda/H = 2, \ Ca = 0.01 \)

Increasing cell height

\( \epsilon = a/H = 0.00 \)
\( \epsilon = a/H = 0.05 \)
\( \epsilon = a/H = 0.10 \)
Normal Stress Distribution

\[ a/H = 0.1, \lambda/H = 2, Ca = 0.01 \]
Tangential Stress Distribution

$\lambda/H = 2, \text{ Ca } = 0.01$

Increasing cell height

$\epsilon = a/H = 0.00$
$\epsilon = a/H = 0.05$
$\epsilon = a/H = 0.10$
Tangential Stress Distribution

\( a/H = 0.1, \lambda/H = 2, Ca = 0.01 \)
\( \text{Ca}_Q \) vs. Tangential Stress and Stress Gradient \( \frac{\varepsilon}{\Lambda} = 0.05 \)

**Tangential Stress**

\[
(\tau_t)_{\text{max}} = \left( \frac{Q^*}{2H} \right) / (\gamma / \mu)
\]

**Tangential Stress Gradient**

\[
(\frac{d\tau_t}{dx})_{\text{max}} = \left( \frac{Q^*}{2H} \right) / (\gamma / \mu)
\]

Increasing \( \frac{\varepsilon}{\Lambda} \)

\( \text{Ca}_Q = \left[ \frac{Q^*}{2H} \right] / (\gamma / \mu) \)

Increasing \( \frac{\varepsilon}{\Lambda} \)

**Graphs**

- Red line: \( \frac{\varepsilon}{\Lambda} = a/\lambda = 0.05 \)
- Orange line: \( \frac{\varepsilon}{\Lambda} = a/\lambda = 0.00 \)
Ca_Q vs. Normal Stress Gradient

\[ \varepsilon/\Lambda = 0.05 \]

\[ \text{Ca}_Q = \frac{[Q^*/(2H)]/(\gamma/\mu)}{} \]

Normal Stress Gradient

\[ (d\tau_n/dx)_{\max} \]

increasing \[ \varepsilon/\Lambda \]

\[ \varepsilon/\Lambda = a/\lambda = 0.05 \]

\[ \varepsilon/\Lambda = a/\lambda = 0.00 \]
Surfactant Effects
Equilibrium Equation of State (Infasurf)

Influence of Surfactant Concentration

(A549 cells, Live/Dead Kit, 0.25 cm/s)

Infasurf Concentration [mg/mL]

Population Injury [cells/cm²]

Infasurf Concentration [mg/mL]

CBC
## Correlation of Stress and Injury

<table>
<thead>
<tr>
<th>Infasurf Speed (mg/mL)</th>
<th>Speed (cm/s)</th>
<th>Injury (cells/cm²)</th>
<th>( \tau_s ) (dyn/cm²)</th>
<th>( \Delta \tau_s ) (dyn/cm²)</th>
<th>( \Delta P ) (dyn/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.25</td>
<td>++</td>
<td>13.1</td>
<td>4.8</td>
<td>163</td>
</tr>
<tr>
<td>0.01</td>
<td>0.25</td>
<td>++</td>
<td>12.8</td>
<td>4.6</td>
<td>154</td>
</tr>
<tr>
<td>0.1</td>
<td>0.25</td>
<td>++</td>
<td>7.1</td>
<td>1.9</td>
<td>48</td>
</tr>
<tr>
<td>1</td>
<td>0.25</td>
<td>-</td>
<td>6.7</td>
<td>1.8</td>
<td>44</td>
</tr>
</tbody>
</table>

- ✔️: Significant
- ?: Indeterminate
Influence of Non-Equilibrium Behavior of Infasurf

Non-equilibrium behavior:
- produces dynamic surface tensions that are greater than the equilibrium surface tension,
- creates non-equilibrium surface tension that causes film-thinning.
Dynamic Surface Tension of Infasurf

Conclusions

• Combined experiments and computational investigations allows us to estimate the mechanical stresses that damage epithelial cells during reopening.

• The damaging effects from reopening are likely to be due to a large pressure gradient from the traveling air-liquid interface.

• Topological effects can increase the magnitude of deleterious stresses.

Non-equilibrium surface-tension effects may increase damage unless concentrations are large.

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