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Using Reducing Agent TCEP to Alter the Rate of Mucociliary Transport

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A thesis submitted in partial fulfillment of the requirements for graduation with Honors in the Biology

Christopher Stipp
Thesis Mentor

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All requirements for graduation with Honors in the Biology have been completed.

Lori Adams
Biology Honors Advisor

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Abstract

Cystic Fibrosis (CF) as a genetic disease is characterized by a loss of function mutation in the Cystic Fibrosis transmembrane conductance regulator (CFTR). Without Chloride and Bicarbonate transduction the patient exhibits loss of host defense mechanisms and increased mucus viscosity, creating an environment that promotes chronic respiratory infection. We hypothesize that reducing agent tris(2-carboxyethyl)phosphine (TCEP) will break down intermolecular bonds within the mucus, thus increasing the rate of mucus clearance by mucociliary transport. It was shown that TCEP has no effect on the ciliary beat frequency of ethmoid epithelial cells, thus, not altering the rate of transport. *In vivo* computed tomography (CT) scans of non-CF pigs show a significantly increased clearance rate of microdisks applied to the airway after TCEP treatment. TCEP application caused no difference in viscosity of mucus samples with 6% and 12% mucin, but a slight decrease in the viscosity may occur with 3% mucin samples. In the future we hope to investigate these same questions using CF pig models rather than non-CF. Ease of mucus detachment has been hinted to significantly affect the rate of clearance. This research will prove important to developing newer and more effective treatments for Cystic Fibrosis.
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Introduction

Cystic fibrosis (CF) is an autosomal recessive disease that is caused by mutations in the gene responsible for encoding the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel, all of which result in loss of CFTR expression and/or function (Welsh, Ramsey, Accurso, Cutting, 2001). In many tissues, including the respiratory tract, functional CFTR conducts HCO₃⁻ and Cl⁻ across the apical membrane of the epithelium (Poulsen, Fischer, Illek, Machen, 1994). With loss of CFTR mediated HCO₃⁻ secretion, the airway surface liquid becomes acidic. The decreased pH of the airway surface liquid decreases the activity and efficiency of antimicrobials in the respiratory tract (Pezzulo et al., 2012). In addition, loss of CFTR-mediated HCO₃⁻ and fluid secretion affect mucus biophysical characteristics. As a result, CF mucus becomes more viscous and significantly difficult to clear or transport (Tang et al., 2016). The combination of reduced mucus transport by the ciliated epithelium, mucociliary transport (MCT), and inhibited antimicrobial activity leads to chronic respiratory infections and a general decline in lung function.

Mucus contains a mix of lipids, salts, proteins, macromolecules, and cellular debris. Mucin is a large glycoprotein with a bulky oligosaccharide domain (Lai, Wang, Hanes, 2008). Two gel-forming mucins are common to the respiratory system, MUC5AC and MUC5B. MUC5B is secreted by submucosal glands in the form of strands and MUC5AC is localized to goblet cells and creates a thin sheet of mucus. Both mucins contribute to MCT in distinct ways.
Mucin glycoproteins interact with each other by forming disulfide bridges between cysteine residues located in areas of low glycosylation. Mucus properties such as viscosity and elasticity are dependent on the disulfide bonds and physical entanglement between the mucin glycoproteins (Hsein, Garrait, Beyssac, Hoffart, 2015). CF mucus has an increased viscosity which acts as a barrier to gene therapy. The increased viscosity leads to a large decrease in MCT, this provides an optimal environment for bacteria to colonize.

We know that mucus contains many disulfide bonds that give it an increased viscosity, but we don’t know if reducing the sulfides that bond mucins together will change MCT. It has been reported that treating sputum with N-acetylcysteine, a reducing agent and antioxidant, decreases the viscosity of the mucus (Sadowska, Verbraecken, Darquennes, De Backer, 2006). To determine the effect of reducing agents on MCT we used tris(2-carboxyethyl)phosphine (TCEP) and analyzed its treatment on the different aspects of MCT, ciliary beat frequency (CBF) and mucus viscosity.

Our hypothesis was that TCEP treatment would increase the CBF of the airway lining. To evaluate this, we washed apical surface of pig ethmoid epithelium cultures with a phosphate buffered saline (PBS)++ vehicle. The cells are treated with 10mM TCEP for 10 min, and then were imaged. Images of the beating cilia undergo fast Fourier transform and the data from a representative sample size was plotted. The treated cells were tested against a PBS control.
We analyzed the effect of TCEP on mucus viscosity by tracking the Brownian motion of fluospheres in a synthetic CF mucus analog to test our hypothesis that TCEP would disrupt the mucus structure and decrease its viscosity. The Brownian motion, or “random walk” of particles through a fluid allows us to calculate the mean square displacement (MSD) of the particle and calculate its velocity. The CF sputum model was created from methods described in Dawson et al. (2004) and varying mucin concentrations were tested. The mucus samples were treated with 10mM TCEP. Samples were paired with a PBS treated control.

We hypothesized that TCEP treatment increases MCT. To test this hypothesis, methods described in the supplementary materials listed for Hoegger et al. (2014) were used. Non-CF piglets were anesthetized and underwent a control computed tomography (CT) scan to track the clearance of microdisks from the airways. 10mM TCEP aerosol was administered and the CT scan was done again. The clearance and transport of the particles for the control and TCEP treatment were compared.

It was observed that the loss of functioning CFTR correlates to the impaired mucus detachment from submucosal glands and leads to significantly reduced MCT (Hoegger et al. 2014). Although not tested, we believe that TCEP treatment can improve the detachment of mucus strands and restore MCT to CF airways.
Materials and Methods

Cell Culture Preparation and Imaging

This experiment was performed on porcine ethmoid epithelia cultures. Cells are well-ciliated and form a layer of mucus, providing an accurate reflection of lower airway epithelial cells (Chang, Zabner, 2015). The ex vivo cell cultures were washed of mucus with a phosphate buffered saline supplemented with calcium, magnesium and glucose (PBS+/+) five days and immediately before imaging. The apical surface of the cells was treated with 10mM TCEP in PBS+/+ for 10 minutes prior to imaging. A Nikon confocal microscope was used for imaging the cells. Using a 25x objective and reflected laser light at a wavelength of 488nm, a short video of the cilia in action was taken. 10 videos, each from a different field of view, were obtained from each sample. Ciliary beat frequency (CBF) was measured by transforming the data from time-space into a frequency-space using fast Fourier transform. Each sample was paired with a PBS+/+ treated control. A total of 6 culture donors were used for this experiment. The mean and median frequency for each culture was taken by averaging the data from the fields of view.

CF Mucus Viscoelasticity

Methods from Dawson et al. (2004) were used to create a CF sputum model with 3%, 6%, and 12% mucin at a pH of 7.4 using type II porcine gastric mucin. Mucus was mixed 1:10 with a 0.1µm solution of molecular probes-F8816 (Fluorospheres, 1.0µm, crimson- 625/645) in PBS. Mucus was treated to a concentration of 10mM TCEP. Some samples had the TCEP treatment added before the beads while some were treated after the beads were added. Each sample was paired with a PBS treated control. A Nikon confocal microscope with a 50x objective and 638nm wavelength laser light was used to image the movement of the particles in
the mucus samples. 10 videos of the beads at 226.4fps from different fields of view were taken from each sample. MATLAB was used to calculate the complex viscoelastic modulus derived from the MSD.

**Pig CT Scan**

Non-CF pigs were treated with 10mM aerosols of TCEP and compared to a saline control. Mucociliary transport rates (mean velocity) *in vivo* were obtained using a CT scan as described in the supplementary materials listed for Hoegger et al. (2014). We correlated the viscoelastic properties measured above to the mucociliary transport rates *in vivo*. 
Results

Ciliary beat frequency

Both the mean and median CBF of the porcine ethmoid cultures was taken, because the frequencies were skewed left (Fig. 1, Fig. 2). A two-tailed t-test with a significance at P<0.05 was used to measure the significance of differences between the TCEP and PBS treated cultures. Both the mean and median CBF of the cultures was not significantly different between the treatments.

Non-CF pig CT scan

Microdisks were tracked through the pig’s airways using a particle tracking software applied to the images taken from the CT scan (Fig. 3). The TCEP aerosol treatment was observed to increase the average rate of MCT by 32% (Fig. 4).

CF Mucus Viscoelasticity

The shear storage and loss modulus of the mucus models were measured to examine the biomechanical properties under the treatment of TCEP. Under all conditions and treatments for the mucus treated after fluorospheres were added, the shear storage began to exceed the shear loss once an omega of 1 was passed (Fig. 5). A similar pattern was observed with the mucus samples treated before fluorospheres (Fig. 6) The order of treatment was shown to have no effect on the viscoelasticity of the samples. The 6% and 12% mucin samples showed no difference between the TCEP and PBS treatments. 3% mucin samples showed a slight decrease in the shear storage when treated to 10mM TCEP before and after adding fluorospheres.
Figure 1: The mean ciliary beat frequency of the ethmoid epithelial cultures (n=12) treated with 10 mM TCEP was not significantly different (P>0.05) from the PBS+/+ vehicle control. TCEP treatment has no effect on the mean rate of ciliary beat frequency.
Figure 2: The median ciliary beat frequency of the ethmoid cultures (n=12) showed no significant difference (P>0.05) between the 10mM TCEP treatment and the PBS+/+ vehicle control. TCEP has no effect on the median rate of ciliary beat frequency.
Figure 3: Computed tomography scans of a non-CF pig administered with tantalum microdisks at $t=0$ min (A), $t=5$ min (B), and $t=10$ min (C). The microdisk labeled with a circle (○) is transported quickly out of the respiratory system and the square (□) labels a particle that does not move. Between $t=5$ and $t=10$, the circled particle leaves the field of view.
Figure 4: The mean particle velocity due to mucociliary transport in non-CF pigs (n=12) was significantly reduced (P<0.05) following 10mM TCEP aerosol treatment compared to the saline baseline. The mean velocity (mm/min) was reduced by 32% in TCEP treated pigs.
Figure 5: The viscoelastic moduli of the 3%, 6%, and 12% mucin CF mucus models treated to 10mM TCEP or PBS after fluorospheres were added. Shear storage (Gp) and shear loss (Gpp) are shown in red and blue, respectively, on a double log scale of omega (frequency of strain in Hz) and Pascals.
Figure 6: The viscoelastic moduli of 3%, 6%, and 12% mucin CF mucus models treated to 10mM TCEP or PBS before fluorospheres were added. Shear storage (Gp) and shear loss (Gpp) are shown in red and blue, respectively, on a double log scale of omega (frequency of strain in Hz) and Pascals.
Discussion

The CBF experiments resulted in the conclusion that TCEP had no significant effect on the mean (Fig. 1) or median (Fig. 2) CBF of ethmoid epithelial cultures. Thus, our hypothesis that TCEP would yield an increase in CBF was rejected. It has been shown that another reducing agent, dithiothreitol (DTT), can stabilize the motility of cilia and keep CBF relatively constant (Hard, R., Cypher, C. and Schabtach, E., 1988). Given this information, it makes sense that TCEP, as a reducing agent, would cause no significant change in CBF. A possible future experiment would be to investigate to what extent reducing agents are able to keep the CBF of cells constant using DTT and a compound that is known to reduce or enhance the rate of cilia motility.

The CT scans of non-CF pigs show particles that move at vastly different rates, ranging from clearing the trachea in minutes to completely stationary (Fig. 3). This follows closely with the hypothesis that TCEP and other reducing agents could increase mucus detachment from submucosal glands. The particles that were transported quickly could have been on a strand of MUC5B that broke away early on and the stationary microdisks may have been attached to strands that never broke free. The administration of TCEP causes a significant, and large, increase in the rate of airway MCT (Fig. 4). Our hypothesis that MCT is increased is supported, but it is unclear thus far whether it is due to increased mucus detachment or a general increase in the rate mucus can be transported by cilia. Something to consider is labeling the MUC5B so it is visible via CT scanning and observe the behavior of the MCT after TCEP has been administered.

These results are significant because we now know that TCEP reduces MCT in non-CF pigs, while having no effect on CBF. It is uncertain whether TCEP would be able to aid in the transduction of genes into the airway epithelium. Results from the mucus viscoelasticity
experiment will tell us more information about how host defense works to keep the lungs and airways clear and healthy, and how it can be modified.

The viscoelasticity of the mucus was measured using beads added before and after the TCEP in respective samples. The idea was to investigate whether the beads would become stuck in the mucus before applying the reducing agent, leading to an increased viscoelasticity. The measured variables were shear storage and shear loss. Shear storage is the ability for a material to absorb stress and recoil back to its original form, and shear loss is the risk of deformation of a material under stress (Toledano et al., 2017). The moduli of the mucus samples with the beads added before the treatment only showed a decrease in shear storage when TCEP was given at 3% mucin (Fig. 5). All other concentrations of mucin experienced no change in between treatments. The samples with fluorospheres added after treatment showed a similar result (Fig. 6). The order in which the fluorospheres and treatments were added had no significant effect on the viscoelastic properties of the samples. These results indicate that the reducing properties of TCEP can decrease the viscoelasticity of the mucus at lower mucin concentrations. The mucus of CF individuals may have a higher concentration of mucin because of ion deficiencies, thus decreasing the efficacy of TCEP to alter the viscoelasticity. Although the TCEP treatment seems to slightly decrease the shear storage of a low concentration mucus, it is inconclusive whether it is truly effective at decreasing viscoelasticity.

The future directions for this research are vast and promising. Considering in vivo CT scans of CF pigs treated with TCEP would allow us to determine how effective the therapy is to regain normal respiratory function. This will potentially create a significant advancement to developing more effective CF treatments. Using harvested mucus from both CF and non-CF individuals for viscoelasticity testing would prove useful to compare the results from the analog
mucus and organically harvested mucus. Our results would determine if our initial results could potentially be translated onto a living model, and if the mucus model used is viable for accurately mimicking mucus taken from living organisms. The mucus detachment experiment was mentioned, but not conducted using TCEP as a treatment. Applying TCEP to the trachea samples and measuring the rate of mucus detachment \textit{ex vivo} should be done to confirm results from the mucus clearance experiment. This can also help us to understand the role of MUC5B and MUC5AC in mucus clearance from the lungs.
References


