Nebulized Hypertonic Saline-Triggered Increase in Mucociliary Clearance Rate is Mediated by the Nervous System

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ABSTRACT

Inhaled nebulized hypertonic saline (HTS) is a commonly prescribed mutation-agnostic therapy used in the treatment of CF-related lung disease. It is currently well-understood that HTS functions, in part, to increase the volume of airway surface liquid (ASL) by generating an osmotic gradient that draws water into the airway lumen and, thus, increasing mucociliary clearance (MCC). However, recent research has also demonstrated that HTS-triggered increase in ASL height is mediated by the nervous system, whereby HTS stimulates sensory neurons to promote active secretion of ASL via airway epithelia. It has been shown that there is an approximate 50% reduction in the effects of HTS on ASL height in the presence of neural blockers. To study how this relationship translates into potential differences in MCC, this thesis seeks to test whether neurogenic component of HTS influences HTS-triggered increase in MCC *ex vivo* in excised swine trachea. Using a MCC assay based on tracking the clearance of tantalum microdisks by the ciliated epithelium of the trachea, we were able to observe that the HTS-mediated increase in MCC is mostly eliminated following preincubation with the neural blocker tetrodotoxin (TTX) in wild-type swine. To further demonstrate the utility of the nervous system in promoting MCC, we used sensory agonists capsaicin and menthol in combination with HTS or isotonic saline (ITS), and found them to cause substantial improvements on MCC in wild-type swine. Lastly, our experiments using CFTR-/- piglets demonstrated a surprising trend, whereby preincubation with TTX potentiated the effects of HTS, rather than decreasing them. We conclude that HTS-triggered increase in MCC is heavily dependent on the nervous system in swine. The next step will be to test the effects of neuromodulators *in vivo* using swine, or possibly even in human CF patients.

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DEDICATION

This dissertation is dedicated to the memory of my mother, whose determination and unwillingness to give up in the face of overwhelming adversity have forever shaped me as a person. May I one day use what you have taught me to improve the lives of people just like you.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AMP	Adenosine monophosphate
ASL	Airway surface liquid
CaCC	Ca^{2+}-activated chloride channel
Ca^{2+}	Calcium
CF	Cystic fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CFTR-/-	CFTR-knockout
CFTRinh-172	Pharmacological inhibitor of CFTR channel
Cl^-	Chloride
ENaC	Epithelial Na^+ Channel
FEV1	Forced expiratory volume in one second
HCO_3^-	Bicarbonate
HTS	Hypertonic saline
ITS	Isotonic saline
MCC	Mucociliary clearance
MCT	Mucociliary transport
Na^+	Sodium
NO	Nitric oxide
NOS	Nitric Oxide Synthase
PCL	Periciliary liquid
SP	Substance P
TTX	Tetrodotoxin
VIP	Vasoactive Intestinal Peptide

CHAPTER 1: INTRODUCTION

1.1 Summary

Cystic fibrosis (CF) is the most common lethal chronic genetic disorder in Canada, with 1 in 3600 Canadians born with the disease, and more than 70,000 patients worldwide (Cutting, 2015). Though the condition is fatal, recent advancements in both the diagnosis and treatment of the disease have improved the median age of survival for patients significantly. As of 2018, the estimated median age of survival for CF in Canada was 52.1 years (Cystic Fibrosis Canada, 2018), and 47.4 years in the United States (Cystic Fibrosis Foundation, 2018). The median age of Canadian CF patients listed in the 2018 Cystic Fibrosis Canada Patient Registry was 23.5 years, up from 11.7 years in 1987, further illustrating improvements in patient care (Cystic Fibrosis Canada, 2018).

CF is caused by mutations in the gene encoding for the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) anion channel, leading to progressive decline in the health of the patient (Regard et al., 2018). The primary organ system affected is the respiratory tract, but the presence of this channel across multiple organ systems means that CF may cause complications in numerous other organs as well (Cutting, 2015; Regard et al., 2018). While death typically results from respiratory complications, complications within the gastrointestinal tract, kidneys, pancreas, and other organs cause significant morbidity in patients, leading to severe complications if left untreated. With the availability of effective treatments for non-respiratory complications of the disease, research on CF has largely turned toward treating the effects in the respiratory tract (Davis, 2006). CFTR mutations result in dehydration of the airway surface liquid layer which, consequently, impairs mucociliary clearance of inhaled particles, thus diminishing the innate defense against pathogenic organisms. In turn, this causes the mucus accumulation and subsequent airway obstruction that is characteristic of CF (Kreda et al., 2012). Thus, there has been an interest in developing therapies aimed at restoring the airway hydration. One such treatment is nebulized hypertonic saline (HTS), aimed at improving airway hydration and increasing mucociliary clearance, which will be the focus of this thesis.

HTS nebulization has been shown to improve mucociliary clearance and the beneficial effects on lung health through airway rehydration is undisputed (Elkins et al., 2006; Tildy & Rogers, 2015). However, its beneficial effect on lung function, as measured by spirometry, can be small and the treatment is not always tolerated by patients; thus there is a need for improved rehydration treatments. Unfortunately, the mechanisms of action of HTS nebulization remain somewhat unclear. Due to these knowledge gaps, it is difficult to improve upon the current treatment to increase its effectiveness and tolerability. Traditionally, HTS nebulization was believed to increase airway hydration through an osmotic effect, which would draw water from the tissue into the lumen of the airway. Recent experiments have shown that, in addition to the osmotic effect, HTS-triggered increase in airway hydration is partially mediated by stimulation of sensory neurons in the airway, which stimulate active fluid secretion by the airway epithelia. Thus, the effect of HTS on airway hydration is partially blocked in the presence of neural blockers lidocaine, tetrodotoxin, and atropine (Luan et al., 2019).

In this thesis, I seek to test the hypothesis that HTS-triggered increase in mucociliary clearance are also mediated by stimulation of the nervous system. This research will provide an improved understanding of HTS at a basic scientific level, while also informing potential novel therapies or modifications to improve existing therapies.

1.2 Genetic Basis of Cystic Fibrosis

CF is an autosomal recessive disorder, caused by a variety of mutations in the CFTR gene located on chromosome 7 in humans (Rommens et al., 1989). The gene encodes the CFTR anion channel, a member of the ATP-binding cassette transmembrane superfamily. This protein consists of six alpha-helices, a regulatory domain, two membrane-spanning regions, two cytoplasmic domains, and multiple phosphorylation consensus sequences (Farkas et al., 2020; Higgins, 1992). The CFTR protein embeds in the lipid membrane of epithelial cells in multiple organs, where it plays a major role in ion and water balance (Farkas et al., 2020; Riordan et al., 1989). Primarily, it is responsible for transporting chloride and bicarbonate anions across the apical membrane of multiple cell types, including those of the epithelial cells lining the airways (Denning et al., 1992; Trezíse & Buchwald, 1991).

Roughly 2000 disease-causing CFTR gene mutations have been described to date, and can be classified with respect to their effect on protein structure and function (Cutting, 2015). One commonly cited expanded classification system places mutations of the CFTR gene into six distinct classes, organized with respect to their effect on the CFTR protein production and function. Class I mutations involve mutations to the protein production, arising from nonsense mutations predominately, as well as frameshift and splicing mutations. These mutations prevent the messenger RNA from being properly translated, thus leading to severely reduced or completely absent expression of the CFTR protein (Veit et al., 2016). Class II mutations include the most common mutation, a deletion of a phenylalanine in position 508 (i.e., F508del), which affects more than 70% of CF patients (Gentzsch & Mall, 2018). Class II mutations result in misfolding, premature degradation, and impaired protein production. These result in an inability for the protein to properly reach the cell surface (Marson et al., 2016; Veit et al., 2016), thus impairing function. Class III mutations are characterized by impaired CFTR channel regulation due to abnormal gating properties; in such mutations, there is a reduction in the open probability of the channel (Veit et al., 2016). Class IV mutations lead to reduced channel conductance, thereby reducing the ability of ions to pass through the ion pore. Such mutations lead to reduced current through the channels, and consequently cause a milder disease phenotype in comparison to the first three mutation classes (Sheppard et al., 1993; Veit et al., 2016). Class V mutations involve reduced expression of an otherwise functional protein (Marson et al., 2016), often involving splice site mutations (Highsmith et al., 1997; Marson et al., 2016) or promoter abnormalities (Veit et al., 2016). Lastly, Class VI mutations lead to proteins that are less stable (Marson et al., 2016) and are thus prone to accelerated rates of protein turnover (Silvis et al., 2003). Mutation classes I, II, and III lead to more severe disease phenotypes, while classes IV, V, and VI are typically milder (Marson et al., 2016).

1.3 Airway Physiology and Cystic Fibrosis

1.3.1 Basic Airway Physiology

The primary function of the respiratory system is to facilitate gas exchange between the external environment and the circulatory system. In humans, there are approximately 300 million alveoli that accomplish gas exchange between the respiratory and circulatory systems, and these

alveoli receive inspired air from 23 bifurcations. This creates an extensive cumulative surface area that facilitates efficient gas exchange with pulmonary circulation (Widdicombe & Wine, 2015).

Lining the airway are epithelial cells, the majority of which are ciliated (Bustamante-Marin & Ostrowski, 2017; Widdicombe & Wine, 2015), with roughly 200 cilia per cell (Rhodin, 1966). Separating the airway lumen and the epithelium is a thin film of surface liquid commonly known as airway surface liquid (ASL). This liquid barrier can be separated into two layers: the periciliary liquid (PCL) layer and a mucus gel layer lying directly on top of the PCL (Sturgess, 1977; Widdicombe & Wine, 2015; Yoneda, 1976). The PCL is a 7 μm thick fluid solution which contains a dense mesh of tethered mucins that project from the epithelial cilia and microvilli, and it plays an important role in excluding large particles from the cilia; in doing so, this enables the continuous beating of the cilia to propel mucus layer (Hattrup & Gendler, 2008; Sheehan et al., 2008).

Inspired air often contains small particles and pathogens. While a minority of these inhaled particles and pathogens will be exhaled, most require other mechanisms for removal. In the upper airways, the accumulation of larger particles and pathogens over time may cause significant issues that interfere with normal respiratory function. The mucus layer lying on top of the PCL will bind to both particles and pathogens, at which point ciliary beating propels the mucus, a process called mucociliary clearance, out of the airway (Widdicombe & Wine, 2015).

Airway surface liquid (ASL) is produced by the epithelial cells lining the lumen of the airway: the airway surface epithelia and submucosal glands (Widdicombe & Wine, 2015). In the larger airways, approximately 90% of the ASL is produced by the submucosal glands, and their distribution in the airways mimics deposition patterns of the largest inhaled particles (Widdicombe & Wine, 2015). As such, submucosal glands are most prevalent in the upper airways, which encounter the greatest rate of inhaled particle impacting the airway wall due to the turbulent nature of air flow in those anatomical regions. Below 2 mm diameter airways, the airflow becomes laminar, and the submucosal glands become more infrequent (Widdicombe & Wine, 2015).

Optimal mucociliary clearance requires and appropriate airway hydration. The volume of ASL and mucus hydration are highly influenced by epithelial secretions (Widdicombe & Wine,

2015), and studies have been performed investigating the relationship between gland secretions and mucus clearance. Gland secretion is stimulatory of mucociliary clearance (Widdicombe & Wine, 2015), with one study showing that, following treatment of excised porcine tracheas with acetylcholine (ACh) to induce glandular liquid secretion, there was an approximate threefold increase in MCT (Ballard et al., 2002). Similarly, it was found that, in ferrets, there was an increased rate of mucus transport in response to mediators that promoted glandular secretions (Jeong et al., 2014). Lastly, it was found that stimulation of glandular secretions via cholinergic agonist methacholine in pigs caused greater clearance of tantalum microdisks *in vivo* (Fischer et al., 2019).

In CF airways, it has been found that submucosal glands behave differently in comparison to normal glands. For example, CF glands respond abnormally to secretagogues (Joo et al., 2002; Nam et al., 2006; Salinas et al., 2005; Verkman et al., 2003; Wine & Joo, 2004), cytokines (Baniak et al., 2012), and inhaled pathogens (Luan et al., 2017), thus suggesting their involvement in CF pathogenesis (Ianowski et al., 2008; Salinas et al., 2005; Stoltz et al., 2015). Interestingly, the submucosal glands are innervated by the nervous system, which may promote liquid secretion (Ianowski et al., 2008; Wine, 2007). As a result of the vital role submucosal glands play in airway hydration, and their abnormal behaviour in CF airways, the ability to stimulate submucosal glands to rehydrate CF airways has enormous therapeutic potential.

1.3.2 Neural Control of Airway Submucosal Glands.

It has been suggested that the airway submucosal glands are involved in two main functions (Wine, 2007). The first proposed function is a housekeeping innate mucosal defense against basal levels of inhaled particles, whereby low-level ASL secretions promote constitutive airway defense, in part through MCC, to keep the airways clear of pathogens and particles (Knowles & Boucher, 2002; Wine, 2007). In this function, paracrine signals, released by epithelial and immune cells, as well as neural circuit intrinsic to the airway (i.e., networks of neurons within the airway), are responsible for ASL secretion in response to inhalation of small numbers of particles, as well as control of muscle tone and perfusion of the respiratory tract. These responses occur in the background, without the host being aware or perceiving its function. The housekeeping innate

immune response is highly effective, since most individuals rarely become sick or cough, despite inhalation of thousands of potentially noxious particles per day. In CF patients, this housekeeping function of the airway epithelia is compromised, increasing the probability of infection and inflammation (Wine, 2007).

The second proposed function is an emergency response to large and immediately life-threatening insults, such inhalation of large particles, water, or irritating aerosols. This response is triggered by the central nervous system as a reflex to sensory activation in the airway and is mediated through the vagus nerve (Wine, 2007). The emergency response triggers reflexive glottal closure, airway constriction, pulmonary vessel dilation, cough, and copious glandular secretions (Wine, 2007). Glandular secretions via this reflex are primarily caused by cholinergic input, and it is characterized by large volumes of fluid and mucin secretion. This response is intact in CF (Wine, 2007), and is relevant to HTS treatment in CF patients. HTS activates this emergency response, causing high level secretion and, in some patients, triggers unwanted side effects of this emergency response, such as cough, airway constriction, and inflammation, reducing the tolerability of the treatment (Umeno et al., 1990; Wine, 2007).

Submucosal glands receive significant innervation from nerve terminals containing vasoactive intestinal peptide (VIP), ACh, substance P (SP), and nitric oxide synthase (NOS) (Wine, 2007). VIP, a strong secretagogue that acts through elevation of cAMP, causes considerable secretions from submucosal glands in non-CF subjects (Joo et al., 2002; Peatfield et al., 1983), an effect which is absent from CF airways (Joo et al., 2002). Furthermore, submucosal glands respond well to SP (Coles et al., 1984; Shimura et al., 1987). SP is released by sensory fibres and is another potent secretagogue, which induces glandular secretion, likely in a CFTR-dependent manner (Trout et al., 2001). Within the airway wall are sensory neurons, C-fibres and Aδ-fibres, which terminate within the surface epithelium (Hunter & Undem, 1999; Widdicombe, 2003). These sensory pathways activate sympathetic and parasympathetic centrally-mediated reflexes, as well as local anterograde release of SP and other neuropeptides (Ianowski et al., 2008; McDonald, 1987; Németh et al., 2003; Widdicombe, 2003). Local deposition of irritants or pathogens may trigger central reflexes if the insult is large enough, or activate these sensory pathways and promote local glandular secretions without involvement of the central nervous system (Ianowski et al., 2007).

Additionally, the ability of these sensory neurons to respond to hypertonic solutions (Fox et al., 1995; Pedersen et al., 1998; Pisarri et al., 1992) makes the involvement of both the airway intrinsic neural circuitry and the central autonomic nervous system in HTS treatment extremely interesting.

1.3.3 Airway Defense: Mucociliary Clearance and Cystic Fibrosis

A key goal of cystic fibrosis research is understanding why a mutation in an anion channel (i.e., CFTR) results in the collapse of the innate immune system in the lung, with subsequent advent of chronic infection, inflammation, tissue remodeling and, ultimately, lung failure (Stoltz et al., 2010). For several decades, the most significant hypothesis has been that CFTR is necessary to ensure mucociliary clearance of inhaled pathogens (Bustamante-Marin & Ostrowski, 2017; Hoegger et al., 2014; Sheehan et al., 2008), and thus CF patients suffer from abnormal bacteria clearance. However, the reasons why mucociliary clearance fails in CF is still a matter of debate.

As mentioned above, the surface epithelium of the airways is covered in cilia, which interact directly with ASL. Recall that ASL contains a PCL layer, which is a low viscosity fluid layer that acts as a lubricant to facilitate ciliary beating, and resides underneath a mucus layer. The physical characteristics of ASL are crucial to proper MCC and airway health, and its abnormalities in CF explain the hallmark respiratory pathogenesis of the condition (Bustamante-Marin & Ostrowski, 2017). The composition of the mucus layer plays a substantial role in its capacity to be transported through the respiratory tract (Bustamante-Marin & Ostrowski, 2017). Currently, there are two major hypotheses to explain the reduced mucociliary clearance observed in CF airways.

For more than 20 years, the most prevalent hypothesis was that CF airways are dehydrated, and thus the PCL volume is reduced, inhibiting ciliary movement (Puchelle et al., 1995; Tarran et al., 2005). Airway surface liquid and PCL hydration depend on transepithelial secretion of fluid. Fluid production is largely driven by active anion transport into the lumen via CFTR and Ca^{2+}-activated Chloride Channels (CaCC), and fluid reabsorption is mediated by active Na^{+} absorption through a process involving Epithelial Na^{+} Channel (ENaC) (Tarran et al., 2005).

In CF airways, elevated sodium reabsorption due to hyperactive ENaC reduces volume of PCL, which leads to disruptions in MCC (Tarran, 2004; Tarran et al., 2005). For maintenance of effective MCC, the PCL must be maintained at an adequate height to separate the mucus layer from the cilia enough to allow effective ciliary beating and mucus propulsion through the airways (Puchelle et al., 1995; Tarran, 2004). The PCL is maintained at an ideal volume through a homeostatic process that regulates CFTR and ENaC activity. Part of that regulation is proposed to include the CFTR inhibition of ENaC activity. Thus, in CF airways, ENaC becomes hyperactive due to the lack of CFTR, and the PCL volume collapses, causing the cilia to become immobile. The end result is that mucociliary clearance fails, predisposing the CF lung to infection and lung failure (Joo et al., 2016; Nam et al., 2006; Zhou et al., 2011).

This hypothesis has been challenged by evidence that ENaC is not hyperactive in CF, and that PCL is normal in CFTR-/- swine (Chen et al., 2010). Other experiments have also shown that defects in submucosal gland activity may be most significant in initiating CF-related lung disease, including issues with pH (Gustafsson et al., 2012; Quinton, 2008), poor secretions, and mucus tethering (Hoegger et al., 2014).

The second hypothesis proposes that MCC is reduced in CF due to failure of airway submucosal glands to release healthy mucin (Wine & Joo, 2004) in response to inhaled pathogens (Luan et al., 2014, 2017). The glandular secretion in CF airways is acidic, which reduces the antimicrobial properties of the ASL (Berkebile & McCray, 2014). Moreover, the mucus secreted by the glands in CF airway fail to detach from the gland opening, thus reducing mucociliary clearance (Hoegger et al., 2014).

Regardless of the underlying reason for the failure of MCC, the mucus layer becomes relatively static. This facilitates the accumulation of pathogens, ultimately leading to frequent occurrences of respiratory infections (Puchelle et al., 1995); therefore, therapies often aim to improve airway hydration and rheological properties of the ASL layer. Enzymatic therapies, ion transport modulators, and treatments that break up mucus or enhance airway hydration are commonly prescribed therapies (Donaldson et al., 2006; Puchelle et al., 1995).

1.4 Inhaled Treatments to Facilitate Airway Clearance

Since airway dehydration appears to be at the core of CF lung pathology, great efforts have been directed at developing treatments to improve airway hydration. Several treatments are currently available to CF patients, including dornase alfa, dry powder mannitol inhalation, and hypertonic saline nebulization.

Dornase alfa, a treatment that hydrolyzes the DNA present in sputum/mucus, seems to be effective in improving lung function and decreasing the frequency of pulmonary exacerbations, but with no apparent improvement in patient quality of life (Yang & Montgomery, 2018). Similarly, a Cochrane review was performed for inhaled dry powder mannitol, a treatment aimed at improving hydration by creating an osmotic gradient. Nolan et al. found that there was an apparent increase in lung function when compared with controls, but again, did not find any improvement in patient quality of life (Nolan et al., 2015). Lastly, I will discuss inhaled nebulized hypertonic saline, which will be the focus of this thesis.

The evidence indicates that HTS causes an absolute increase in lung function when compared with isotonic controls, as well as a reduction in the frequency of pulmonary exacerbations (Elkins et al., 2006). Another study found that patients receiving nebulized 7% NaCl HTS solution had improvements in MCC and forced expiratory volume in one second (FEV1) (Donaldson et al., 2006), further supporting the role HTS may play in treatment of CF lung disease. Unlike the other treatments mentioned, HTS may also increase adult patient quality of life (Wark & Mcdonald, 2018). Although HTS is generally well-tolerated (Dellon et al., 2008; Rosenfeld et al., 2011; Wark & Mcdonald, 2018), it is important to recognize the limitations and unwanted side effects of this treatment. For some patients, the beneficial effects may be marginal and, due to the emergency reflex that HTS activates, some may experience coughing and airway constriction. For these reasons, some patients may struggle to tolerate HTS (Wine, 2007). Thus, there is a need for improved treatments with better health outcomes and reduced side effects.

This dissertation will explore the role of the nervous system to HTS nebulization-triggered increase in MCC. The results may contribute to the development of new HTS formulations that

would modulate the contribution of the nervous system to the response to HTS to produce a more efficacious treatment, with longer duration and reduced negative side effects.

1.5 Nebulized Hypertonic Saline

It is well understood that HTS improves MCC (Robinson et al., 1996; Tildy & Rogers, 2015), though the mechanisms are not fully characterized. The predominant idea is that HTS functions to rehydrate the airways through osmotic forces, drawing fluid across the epithelium due to its hypertonicity, i.e., forcing water into the airway lumen by osmosis (Goralski et al., 2018; Reeves et al., 2012). Additionally, the induction of coughing may play a role in clearing mucus (Dellon et al., 2008). However, recent studies from our laboratory have explored the relationship between HTS and the nervous system. It has been proposed that HTS, in addition to its osmotic effect, also stimulate sensory neurons in the airways, thus promoting active ion transport and fluid secretion by airway epithelia via the release of neuropeptides and thus improving airway hydration. The role of the nervous system was evidenced by an approximate 50% reduction in the effects of HTS on ASL height when combined with neural blockers (Luan et al., 2019). This suggests that it may be possible to target the nervous system to improve HTS treatment. However, it is currently unclear how the neurogenic response to HTS nebulization contributes to HTS-mediated improvements in MCC. This thesis seeks to answer this, by examining the role of the nervous system on HTS-mediated increases in MCC, the most clinically relevant outcome of the treatment.

1.6 Hypotheses

Main Hypothesis: *HTS-triggered increase in MCC is mediated by the nervous system.*

The primary goal of my research was to investigate the role that the nervous system plays in HTS-mediated increase in MCC. I hypothesize that blocking airway neurons prior to nebulized HTS treatment will cause a reduction in MCC when compared with HTS in the absence of neural blockage. I expect that the reduction in liquid secretions when HTS is preceded by neural blockers (Luan et al., 2019) will translate to a reduction in MCC, due to the relationship described between fluid secretions and MCC in previous publications (Jeong et al., 2014; Puchelle et al., 1995).

Objective 1: Develop a MCC assay that is sensitive to changes in MCC in response to nebulized saline treatments. Existing *ex vivo* MCC assays (Hoegger et al., 2014; Joo et al., 2016) are limited for the purposes of detecting differences in MCC in response to HTS nebulization treatments. In those assays, the tissue is submerged in fluid, which effectively washes out the contribution of the treatment to airway hydration. Thus, a new *ex vivo* technique was required for investigating HTS and its effects on MCC. Once a method for incubating the tissue with neural blockers and/or CFTR channel inhibitors was developed, as well as methods for standardizing the initial state of the tissue preparations (i.e., lumen hydration and contamination with debris), it was possible to explore the role of the nervous system in MCC.

Objective 2: Explore the effects of blocking the nervous system on HTS-mediated increases on MCC *ex vivo* in excised wild-type swine tracheas. The second portion of the thesis involved experiments on wild-type juvenile swine, whereby our assay was used to detect changes in MCC in response to various treatments. The overarching objective was to determine what effect, if any, blocking the nervous system has on HTS-mediated increases in MCC. My hypothesis was that the use of TTX prior to HTS nebulization would cause a significant reduction in MCC when compared with HTS alone. Additionally, I expected to see HTS combined with TTX to still be significantly better at promoting MCC than nebulization with isotonic saline (ITS), since the osmotic effect would remain.

Objective 3: Explore the use of neural agonists capsaicin and menthol combined with HTS or ITS in improving MCC *ex vivo* in excised wild-type swine tracheas. To do so, menthol or capsaicin was combined with either ITS or HTS in wild-type juvenile swine experiments. The goal was to demonstrate whether agents that stimulate sensory neurons in the airways could be used to potentiate the effects of nebulized HTS or ITS. I hypothesized that the use of either menthol or capsaicin alongside HTS would cause a small increase in MCC when compared with HTS alone. When combined with ITS, I expected the use of menthol or capsaicin to cause a significant increase in MCC over ITS, but less MCC than HTS formulations due to the osmotic effect.

Objective 4: Investigate the role of the nervous system in HTS treatment *ex vivo* in CFTR-/- swine and CFTRinh-172 treated wild-type swine. Differences in airway physiology in CF, including that

of the submucosal glands (Widdicombe & Wine, 2015), may affect the relationship between the nervous system and HTS-mediated MCC. Therefore, I performed experiments first using the CFTR inhibitor CFTRinh-172 to model the CF condition in wild-type juvenile swine, followed by experiments with transgenic CFTR-/- piglets. My hypothesis was that there would be a significant reduction in MCC when HTS was combined with TTX, when compared with HTS alone, in both CFTR-/- swine and juveniles treated with CFTRinh-172.

CHAPTER 2: MATERIALS AND METHODS

Ethics Statement: This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.1 Swine as a Model

The porcine model is an excellent model for CF lung disease, as their respiratory anatomy and physiology more closely resemble that of humans than other animal models. Of significant importance is the similar distribution of submucosal glands between pigs and humans, and the availability of CFTR gene knockouts (Cutting, 2015; Keiser & Engelhardt, 2011). Swine CF models have demonstrated similar disease progression to that of patients, including lung pathogenesis. It has been shown that CFTR-/- pigs possess a reduced ability to protect against pathogens, as well as a higher incidence of lung infections, comparable to what is observed in humans (Stoltz et al., 2010). Moreover, CFTR-/- swine exhibit airway mucus buildup, bacterial infection, inflammation, and obstruction within the first few months of life (Ostedgaard et al., 2011; Stoltz et al., 2010). Thus, the similarities in respiratory anatomy and physiology, and the availability of CFTR gene knockouts, make swine a highly practical model for the experiments performed for this dissertation. Additionally, there is a large body of published research on swine airway and lung disease that will allow us to better interpret our results, as well as compare and correlate findings with prior work performed in the Ianowski lab.

2.1.1 Wild-type Swine

We used female and male wild-type swine purchased from the Prairie Swine Center (University of Saskatchewan, Canada) to perform all juvenile pig (~5-week-old juvenile pigs, ~15kg) experiments, as well as control experiments for the CFTR-/- experiments. Juvenile pigs were used for all MCC experiments discussed in Chapter 3. Newborn and one-week-old wild-type piglets (~3kg) were used as controls for the CFTR-/- piglets in Chapter 4. The methods employed

will vary to some extent based upon the age of the animal, as described later. Euthanasia of wild-type animals was performed using captive bolt.

2.1.2 CFTR-/- Swine

We used gut-corrected CFTR-/- swine purchased from Exemplar Genetics (Iowa, USA). Exemplar Genetics provided a sow implanted with cloned embryos with the gut-corrected CFTR-/- genotype (CFTR-/- ; TgFABP > pCFTR pigs). Gut correction indicates that the CF piglets, which would otherwise be born with meconium ileus (i.e., a form of bowel obstruction), have CFTR function restored to the intestines, preventing this complication from occurring (Stoltz et al., 2013). The sow was transported to Canada from the United States and was housed at the Western College of Veterinary Medicine Animal Care Unit, University of Saskatchewan, approximately four weeks prior to its due date for quarantine and to give the sow time to adjust to the novel environment in an effort to reduce stress prior to and during delivery. Delivery of the piglets was performed using caesarean section through a specialized veterinary surgeon team. Caesarean section was used to minimize exposure of the piglets to the stress of normal delivery and transit through the birth canal. The procedure took ~10 minutes from onset of anaesthesia, until euthanasia of the sow.

Seven viable male piglets of normal weight were delivered, with one additional piglet that appeared to have died during gestation. A team of veterinary specialists and university researchers were on site to assist in getting the piglets to begin breathing; this included tactile stimulation to promote breathing, and removal of mucus in some subjects via suction tube. Each piglet was assigned a number and was monitored by a veterinarian or veterinarian technician for 24 h a day at the Western College of Veterinarian Medicine.

The piglets were carefully monitored for food intake, bowel movement frequency and consistency, urination, temperature, weight, respiratory function, oxygen saturation, and general behaviour and responsiveness. If the veterinarian supervising observed or expected that a piglet's health was declining, and would be expected to reach the End Humane Point in the proximate future, the animal was quickly destined for experimentation. Then, the animals were euthanized

via exsanguination under anesthesia, and the trachea was dissected out. Of the seven viable piglets, four had terminal complications during the first day. One survived to the second day, one survived five days, and one survived the full seven-day term of the experiment. It is for this reason that newborn and 1-week-old wild-type controls were necessary.

2.2 Experimental Procedure

2.2.1 Dissection of the Trachea

Within 30 minutes of death, the pigs were transported to the Lab Animal Services Unit (LASU) at the Health Science Building, University of Saskatchewan. At this point, an incision was made along the ribcage, as well as along the thoracic region to allow access to the chest cavity. Once the trachea and lungs were located, it was separated from the esophageal tissue, and a cut was made across the most distal portion of the larynx, above the trachea, and at the carina below the trachea, excising the trachea from the animal. Immediately, the clamped trachea was placed in ice-cold Krebs-Ringer solution and oxygenated with 95% O_2/95% CO_2 gas.

2.2.2 Tracheal Preparation and Cartilage Surgery in 5-Week-Old Swine.

A section of ~1-1.5 inches was removed from the trachea and rinsed in phosphate buffer solution (PBS) to remove debris or blood that may have been present in the lumen; some pigs had more blood or other matter in the tracheal lumen than others, and this initial cleaning allowed for a more standardized starting point. After 10-15 seconds of rinsing in PBS, the tissue was pinned flat at each corner, with the luminal surface adjacent to the trachealis smooth muscle facing upward. This was followed by dissection of the cartilage rings (Fig. 2-1) to increase the access of test drugs to mucosa, submucosal glands, and airway sensory neurons.

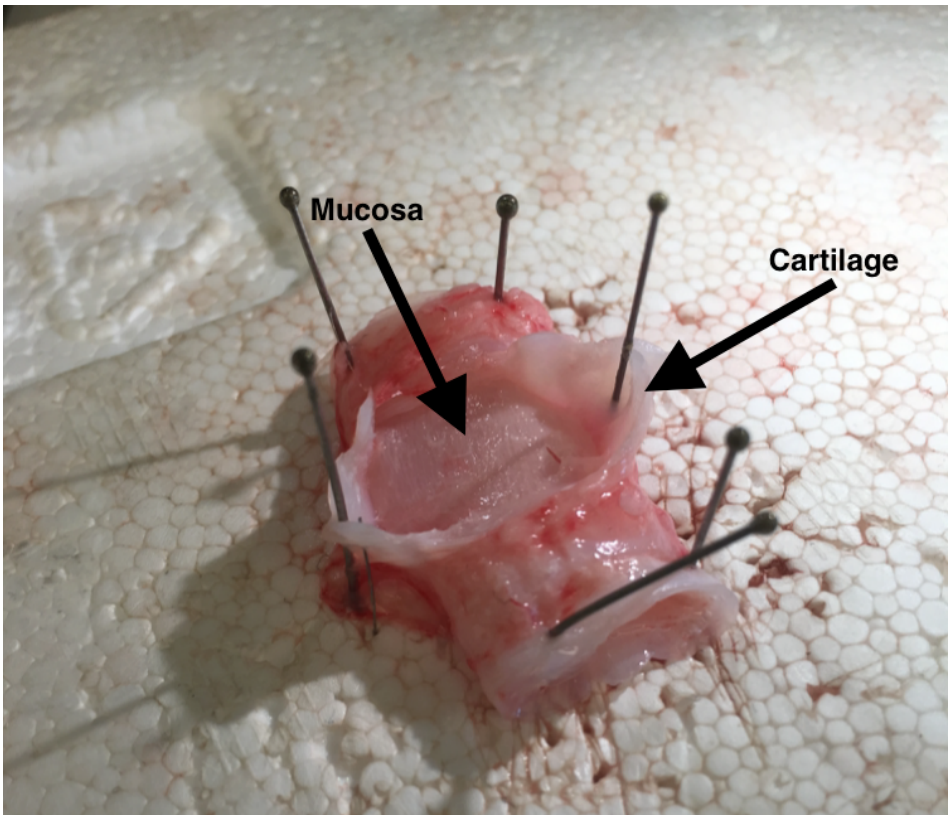


Figure 2-1: Section of juvenile swine trachea following cartilage surgery. Cartilage was cut open to allow the pharmacological agents TTX and/or CFTRinh-172 to access the mucosa, including the submucosal glands.

This was followed by dissection of the cartilage rings (see Fig. 2-1 above). A shallow incision was made across three or four cartilage rings in a longitudinal direction. Once a hole was made through the cartilage, a specialized round-tip elevator tool was used to create separation between the mucosa and the overlying cartilage. Using the elevator as a guide, the scalpel was used to cut through the remainder of the three to four cartilage rings. Forceps were then used to gently pull back the cartilage laterally along the entire length of the incision, while using the elevator to separate the cartilage from the mucosa and prevent tearing. Once a moderately sized (~0.5-0.75 inches in length, ~0.5 inches in width) window to the mucosa was established, the tissue was clamped off at both ends and placed within ice-cold Krebs-Ringer solution, alongside any required pharmacological agents for an incubation period of 15 minutes, as described below. At each point of this surgery, it was important to observe potential punctures or tears in the mucosa. In the event of a puncture, the tissue was discarded. This process took, on average, ~5 minutes. Krebs-Ringers solution was applied to the outside of the tissue periodically to prevent it from drying out; it was important that no fluid entered the lumen of the trachea during this process.

2.2.3 Tracheal Preparation in 1-Week- and 1-Day-Old Swine

The cartilage surroundings the trachea of young piglets is much less substantial than that of older animals, and thus it presents a much less severe barrier to the access of test drugs to the submucosa. Moreover, the size and fragile nature of the trachea necessitated forgoing the cartilage dissection. Therefore, young piglet tracheas went immediately to the incubation step described below. No other notable differences existed between juvenile and young swine experimental methods.

2.2.4 Trachea Incubation in Test Solutions.

Once the trachea preparation was ready, the next step was to incubate the tissue in Krebs-Ringer solution containing one of four drugs combinations: no drug; CFTRinh-172 only; tetrodotoxin (TTX) only; CFTRinh-172 and TTX. CFTRinh-172 is a CFTR channel inhibitor (Caci et al., 2008) that is selective for CFTR, with no apparent effects on non-CFTR chloride channels (Ma et al., 2002). The mechanism of action is currently being explored but is not yet fully

understood (Kopeikin et al., 2010). For this project, CFTRinh-172 was used to simulate CF conditions in wild-type pigs. CFTRinh-172 purchased from Cedarlane Labs (Burlington, ON, CA) and dissolved in DMSO to achieve a concentration of 100mM. This was then dissolved in Krebs-Ringer solution to achieve a final concentration of 100µM. The final concentration of DSMO was < 0.1%.

Tetrodotoxin was purchased from Alomone labs (Jerusalem, Israel). TTX was used to cause blockage of tetrodotoxin-sensitive (TTXs) sodium channels (Scholz et al., 1998). A 1mM stock solution of TTX was dissolved in Krebs-Ringer solution to obtain a final working concentration of 1µM, which has been shown to block the TTXs sodium channels (Scholz et al., 1998). It has been shown that TTX can reduce, but not eliminate, the synaptic activity of airway intrinsic neurons (Wine, 2007) without having an effect on epithelial ion secretion (Luan et al., 2017). While other experiments have benefitted from the use of lidocaine to block TTX-insensitive Na^+ channels (Luan et al., 2019; Scholz et al., 1998), lidocaine has been shown to decrease ciliary beat frequency (Ingels et al., 1994). This would be problematic, as changes in MCC could not be definitely attributed to the assigned nebulization treatment, due to potential changes in clearance resulting from abnormal ciliary behaviour. For the purpose of this experiment, complete blockage of neuronal activity was not necessary, as the goal of determining whether the nervous system was involved in HTS-mediated increases in MCC was achievable without complete blockage.

2.2.5 Nebulization

To study the effect of HTS and other treatments on MCC, the lumen of the trachea preparation was connected to a nebulizer (MedPro, IN, USA) immediately following the 15-minute incubation period. With the exception of the *no treatment* control group, all tissues received nebulization with either hypertonic (7% NaCl solution w/v) or isotonic (0.9% NaCl solution w/v) saline solutions, with some groups receiving menthol or capsaicin as well. Nebulization was performed for a 90-second period, delivering approximately 0.3 ml of liquid, much of which was allowed to freely flow out of the preparation through the open end of the trachea opposite to the nebulized end to best-simulate patient use of the treatment, whereby a patient inhales the nebulized product and then exhales the excess aerosols.

Six nebulized saline formulations were used for the experiments: HTS only; ITS only; HTS + Menthol; ITS + Menthol; HTS + Capsaicin; and ITS + Capsaicin. Experiments involving capsaicin were performed in a ventilated hood for safety. A 1M stock solution of menthol was added to either HTS or ITS to achieve a final concentration of 1mM (Oz et al., 2017). A 100mM stock solution of capsaicin was added to HTS or ITS to achieve a final concentration of 10μM (Matera et al., 1997).

2.2.6 Mucociliary Clearance Assay

After nebulizing the trachea, scissors were used to cut the trachea longitudinally to open the trachea. In 5-week-old pigs, scissors were used to cut lengthwise at two locations, with approximately one third of the trachea discarded; this was to allow the trachea to be opened and laid flat in the imaging chamber. An effort was made to standardize cuts between different experiments. For the 1-day-old and 1-week-old piglets, the smaller circumference of the trachea allowed for a single lengthwise incision, therefore opening the trachea without discarding any of the tissue.

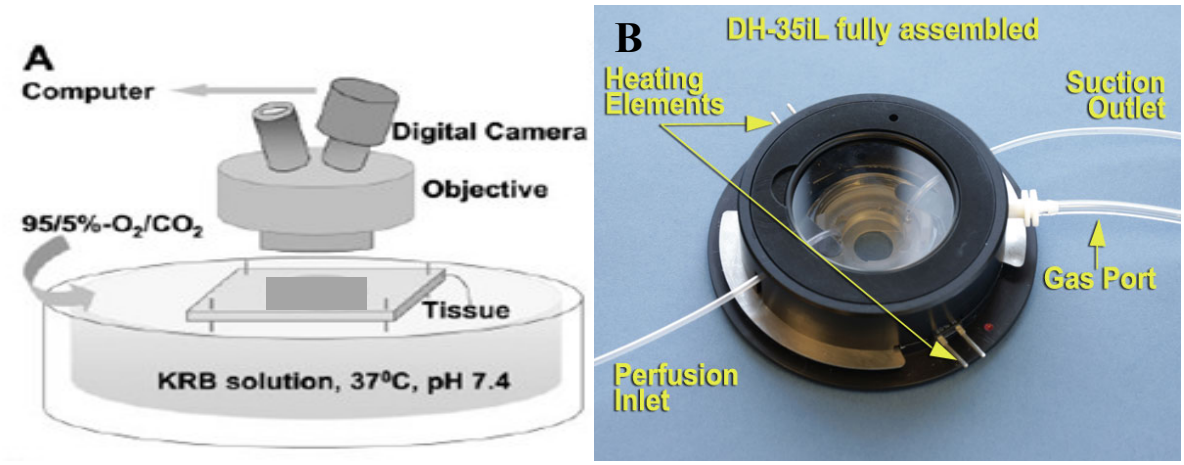
The tissue was then placed in an agarose plate with a layer of gauze lining the bottom. A small volume of Krebs-Ringer solution was used to soak the gauze; it was important to ensure the volume of solution used was small enough to avoid flooding the surface of the trachea during imaging. The purpose of this was to maintain humidity and tissue health during the experiment, prolonging the period during which the tissue could be imaged. The tissue was then pinned to the plate to provide support and maintain a relatively flat surface.

Then, tantalum disks (250 μm diameter and 25 μm thickness) were placed along the luminal surface, at the caudal end of the preparation, to allow adequate space for movement toward the proximal portion. For 5-week-old pigs, a minimum of eight particles were placed, with a maximum of twelve. The number placed would vary based upon the time taken to place them. The goal was to minimize time spent, while maximizing coverage of the luminal surface. For the smaller diameter 1-day-old and 1-week-old piglets, a minimum of six particles were used. An effort was made to place the particles in a conserved manner, with a standard two rows of four

particles. Areas of the tissue damaged from clamping were avoided during particle placement. Lastly, particles were not to be placed on the trachealis smooth muscle, due to poor observed movement in preliminary experiments. The process of opening the trachea and placing particles typically took ~2-3 minutes, during which the tissue was exposed to the surrounding environment.

2.2.7 Tracking Mucociliary Clearance

Once the tantalum particles were placed, the trachea was placed in the DH-35iL temperature control system (Harvard Apparatus, Saint-Laurent QC, Fig. 2-2). This chamber was temperature-regulated, allowing for a physiological temperature of 37 °C to be maintained (Fig. 2-2B). The chamber received humidified 95%O_2/5%CO_2 gas mixture to replicate physiological conditions. The position of the tantalum disks was recorded with a digital camera (MiniVid, GA, USA, Fig. 2-2A and Fig. 2-3) every 30 seconds for as long as the disks were mobile, with a minimum imaging period of 20 minutes (Fig. 2-4).



Obtained from https://www.warneronline.com/culture-dish-incubator-dh-35il

Figure: 2-2A shows simplified pictorial representation of imaging set-up excluding the DH-35iL chamber, modified from Joo et al (Joo et al., 2001). *2-2B is a fully assembled DH-35iL chamber used during the imaging period.*

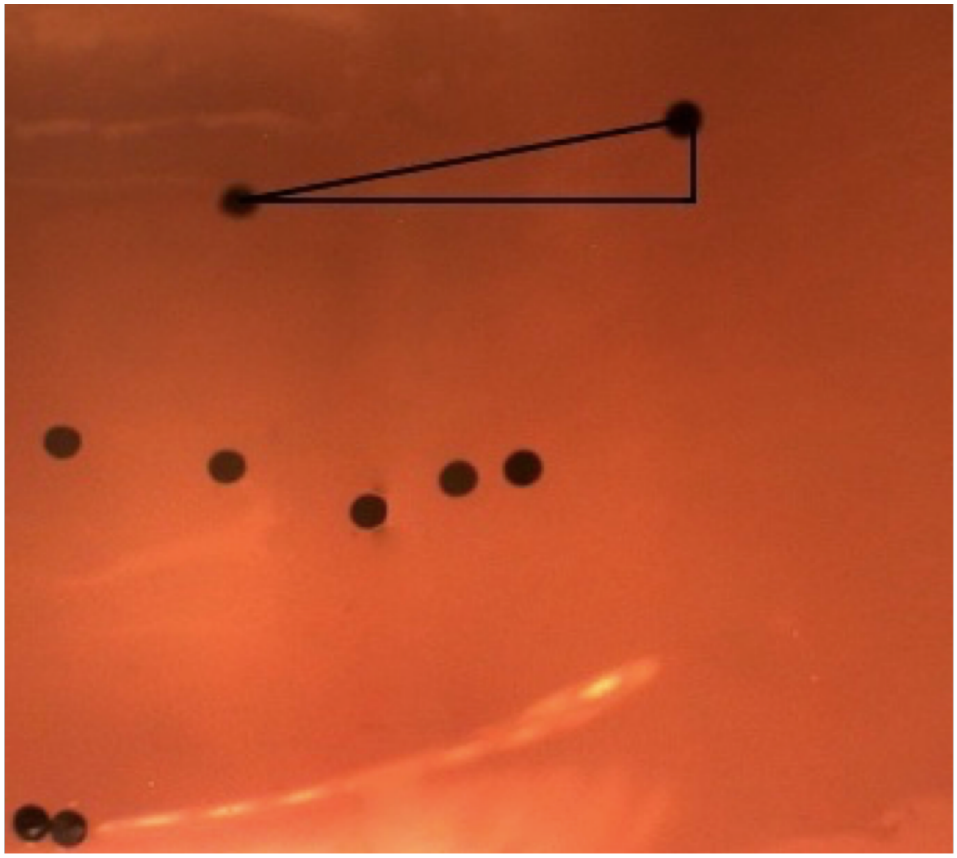


Figure 2-3. Subsequent images were used to analyze particle movement. This figure is composed of two consecutive images merged. The change in X-position and Y-position between consecutive images was measured, then used to calculate the hypotenuse of the particle's movement. From this, various parameters of MCC could be generated.

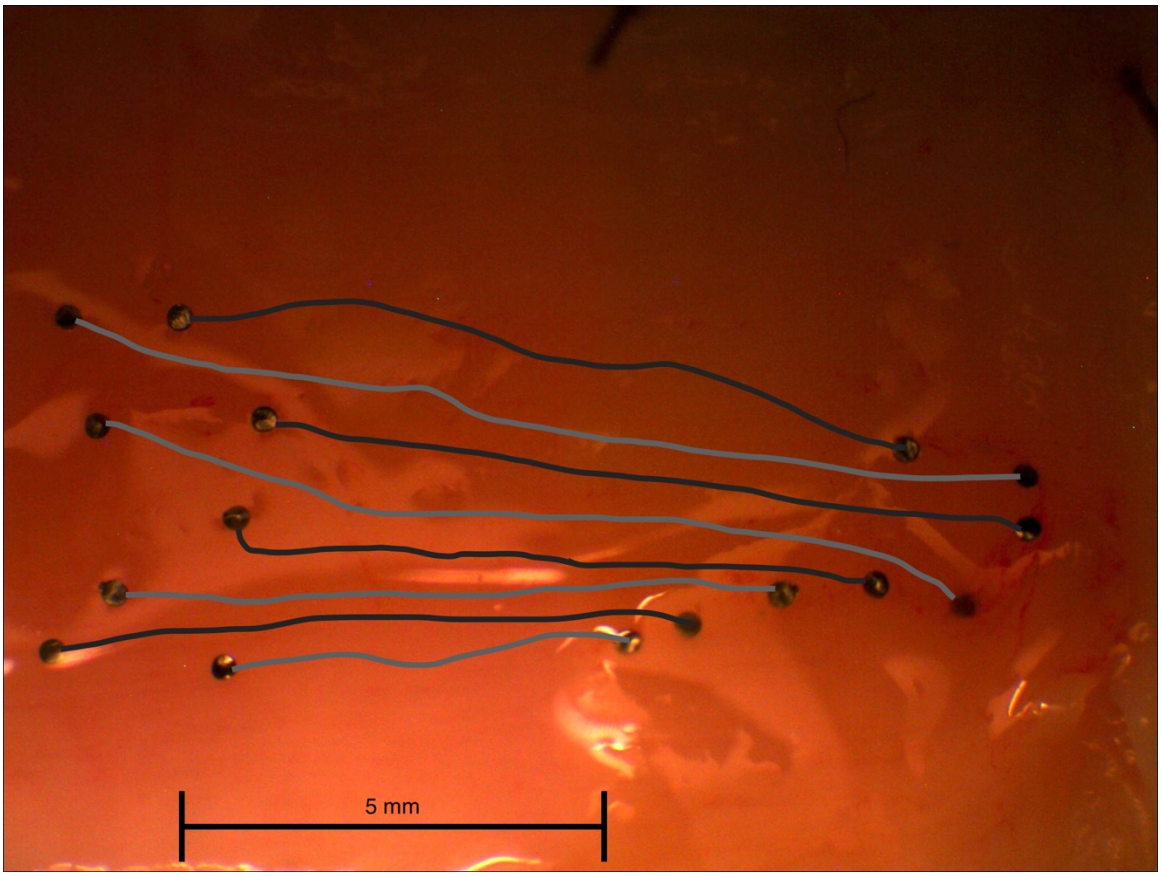


Figure 2-4. Particle movement traced over a 20-minute interval in ex vivo *swine trachea preparation following 90-seconds of HTS nebulization. This figure consists of the first and last images merged. Particles placed on the most distal (left) end of the trachea in an approximate 4x4 position travel toward the proximal (right) end of the trachea.*

2.3 Analysis of Mucociliary Clearance Rate

The efficacy of mucociliary clearance could be understood as how many particles are cleared and how fast the airway ciliary escalator is capable of removing them. Thus, a treatment for improving MCC would, ideally, increase both the number of particles cleared and the how fast they are removed. However, determining if a treatment improved clearance using our *ex vivo* preparation was more complicated than it initially seemed. For example, a preparation where a single tantalum disk moves 10 times faster after treatment would indicate that the treatment was very effective if one measures maximum MCC rate. However, if none of the other particles move at all, that would be a misguided conclusion according to our initial definition, which included the number of cleared particles to determine MCC efficacy. Similar problems arise when using other variables such as number of immobile or mobile particles, delay to start moving, average maximum particle speed, etc. (please see Appendix 1 for a detailed description of the development of the MCC assay and measurements). Thus, we decided to measure several variables that, together, provide evidence of MCC efficacy under different conditions.

2.3.1 Number of Particles that Travel 5 mm

The most informative parameter was the average number of particles that travelled a minimum of 5 mm. The distance of 5 mm was established because it was large enough to detect differences between treatment groups. Other distances were tested, including 1 mm, 1.5 mm, 2 mm, and 4 mm, but were less sensitive to differences between groups. Distances larger than 5 mm become gradually more problematic, as changes in particle movement cannot be definitely attributed to MCC; for example, the presence of tissue damage near the end of the tissue, or accumulation of mucus at the end of a preparation, could both artificially reduce movement. This parameter is effective at representing the capacity of a treatment to induce relatively high levels of MCC.

2.3.2 Average Maximum Particle Speed

Average maximum speed was also measured using the overall distance travelled in a 30-second interval, i.e., the time between two consecutive images. Taking the time-point in which the particle travelled the greatest distance allowed for maximum speed to be calculated, which could be then averaged between all particles and experiments. It should be noted that, in order to be included in calculations of speed, the particle was required to have travelled a minimum of 1 mm. This requirement was to prevent results from being skewed in treatment groups that have a higher proportion of particles that are immobile or minimally mobile. Further, this allows for the comparison of movement behaviour of particles between treatment groups when particles/mucus are reasonably mobile.

2.3.3 Number of Minimally Mobile Particles

The last parameter of interest was the number of particles that were minimally mobile, which was defined as travelling less than 1 mm. This measure helps to demonstrate a treatment's ability to induce movement. While the number of particles that travel 5mm shows how well a treatment can propagate movement over longer distances, this measure tells us how well a treatment can initiate movement. The two measures are complementary, but the induction of movement does not necessarily imply high levels of movement, as will be shown later on.

2.3.4 Maximum Distance Travelled in 20 Minutes in CFTR-/- Piglets

Measures of clearance in the CFTR-/- piglets varied slightly, likely due to the substantial differences in piglet health. With the CFTR-/- piglets, each trachea was divided into two for two experiments: HTS and HTS + TTX. Significant heterogeneity in the health of CF piglets necessitated normalizing each piglet to itself, and then performing paired *t*-tests. While speed calculations and the number of minimally mobile particles in the CFTR-/- piglets remained unchanged, the number of particles observed to travel 5 mm had to be modified to accommodate interindividual differences observed. Instead of determining how many particles travelled 5 mm, we decided to determine how far particles travelled as a percentage of the maximum distance in

that animal. We measured the maximum distance any particle travelled for a given piglet in 20 minutes, and then calculated percentage values for each other particle. For example, if the most mobile particle in a pig (i.e., examining all particles from both experiments performed in a single piglet) travelled 1 mm, then another particle from the same piglet that travelled 0.6 mm would be assigned a value of 60%. In doing so, it helped to control for the differences that existed between animals related to the health of the animal, and allowed for more effective comparisons between treatment groups. With the standard value of 5 mm, it was often the case that zero particles crossed that distance threshold in an animal, regardless of treatment type. In essence, we are still comparing the relative ability of the treatment groups to promote high levels of MCC, whereby a higher average percentage means that there was greater MCC, normalized to the pig's health. Normalizing each piglet to itself was not ideal, but it was a necessary decision to avoid massive variance due to small sample size and high heterogeneity in the piglets. This was not an issue for the wild-type control animals, and we did not consistently measure the same two treatment groups in each animal, so we opted to remain consistent with previously described wild-type animal analyses. Overall, both analyses measure the same thing, but are presented in slightly different ways.

2.4 Statistical Analyses

A variety of statistical analyses were performed. For wild-type juvenile swine experiments (Chapter 3), comparisons of particles travelling 5 mm, minimally mobile particles, and mean maximum particle speed were analyzed using one-way ANOVAs with Tukey's HSD post-hoc multiple comparisons. Additionally, Bartlett's test for homogeneity of variances was used and, in the event of violation of this assumption, Brown-Forsythe correction for unequal variances was applied. For the percentage of particles cleared over time, a mixed methods two-way ANOVA using Greenhouse-Geiser epsilon correction factor when Mauchly's sphericity test failed. Tukey's HSD post-hoc multiple comparisons were used to determine where significant differences occurred.

In CFTR-/- swine, paired *t*-tests were used. Each piglet had its trachea divided into two sections: one would receive HTS alone, and one would receive HTS following preincubation with TTX. This allowed each piglet act as its own control. 1-week- and 1-day-old wild-type control

piglets used unpaired *t*-tests, alongside F-tests for ensuring equal variances. If unequal variances were detected, Welch's correction was applied. The rationale behind using a different method of comparison was that, unlike in CFTR-/- swine, there was not high levels of heterogeneity between experiments, and thus normalizing each wild-type swine to itself was unnecessary. Additionally, incomplete experimental groups were used in these controls, and so each piglet did not necessarily receive the same two treatments of HTS and HTS + TTX. Rather than removing a number of experiments and only using the pigs that received these two treatment groups, it was decided to perform an unpaired *t*-test. For the purpose of determining differences between HTS and HTS + TTX in these wild-type controls, this method was sufficient.

2.5 Inclusion/Exclusion Criteria

Not all experiments were included in the data analysis for this thesis. The most basic, common reason for exclusion was puncturing of the mucosal layer during cartilage dissection. This would cause fluid to enter the tracheal lumen, where it could potentially influence particle movement later on. Experiments with less than three mobile particles were excluded, as it was a sign of unobservable epithelial damage. Preliminary experiments found that preparations with surface damage displaced substantially less particle movement. Additionally, areas of the tissue affected by clamping displayed little to no movement, further supporting this hypothesis. Damage to the luminal surface was difficult to observe directly, so these criteria were created to avoid attributing minimal movement to the treatment group in cases where the tissue's health may have been a confound. For this reason, it was decided that a reasonably healthy tissue would display movement of three or more particles. For the purpose of exclusion, an immobile particle was defined as any particle that completely lacked mobility, excluding any movement that could clearly be attributed to tissue movement (e.g., through smooth muscle contraction).

CHAPTER 3: HYPERTONIC SALINE NEBULIZATION TRIGGERS AN INCREASE IN MUCOCILIARY CLEARANCE MEDIATED BY THE NERVOUS SYSTEM

Inhaled nebulized hypertonic saline is a commonly used treatment in CF patients to restore hydration and improve lung clearance (Robinson et al., 1996). Currently, the exact mechanisms of HTS treatment effects on the airway are not completely understood (Tildy & Rogers, 2015). Gaining a better understanding of how HTS improves MCC (Robinson et al., 1996) could be hugely beneficial in creating new therapies or improving existing ones by extending their duration or enhancing the intensity.

It is well-understood that HTS functions, in some part, through the production of an osmotic gradient that draws fluid into the airway to increase the ASL height (Goralski et al., 2018), and thus MCC (Elkins et al., 2006; Tildy & Rogers, 2015). However, it has been shown that HTS stimulates airway sensory neurons to trigger ASL secretion by airway submucosal glands. It has been shown that there is an approximate 50% reduction in the effects of HTS treatment on ASL height *in vivo* when pigs are treated with neural blockers atropine and lidocaine (Luan et al., 2019). The same observation was made in *ex vivo* swine trachea preparations using neural blockers, including TTX (Luan et al., 2019). Thus, it was proposed that HTS triggers a neural response, whereby the nervous system is recruited to facilitate the production and secretion of fluid into the airways and improve hydration (Luan et al., 2019).

We hypothesized that the HTS-triggered increase in mucociliary clearance is also dependent on the stimulation of the nervous system. Our results show that inhibition of neuronal function does, indeed, reduce the HTS-triggered mucociliary clearance. Moreover, stimulation of airway sensory nerves with capsaicin or menthol stimulates clearance. Menthol acts primarily through TRPM8 receptors in the airways to initiate autonomic responses, such as mucosal secretions, which are mediated by C-fibres and Aδ-fibres (McKemy, 2007; Plevkova et al., 2012; Xing et al., 2008). Capsaicin acts primarily through TRPV1 receptors, which are predominant in sensory C-fibres (Bessac & Jordt, 2008; Lee & Gu, 2009).

3.1 Results

3.1.1 Assay Validation

The initial goal of the project was to validate the mucociliary clearance assay by confirming that it can detect changes in MCC between treatment groups. It is well-established that HTS causes a significant increase in lung function through improved MCC (Elkins et al., 2006; Robinson et al., 1996). To validate the methods, it was necessary to first replicate these findings, using our assay to show a significant increase in MCC in tracheas that received nebulized HTS treatment. The HTS treatment group showed 5.3 ± 0.6 (mean ± SEM) particles that cleared 5 mm, which was significantly larger than the control groups. The ITS treatment group, which acted as a nebulization control for HTS, had only 1.9 ± 0.7 particles crossing the 5mm distance threshold. Lastly, the No Treatment control group showed 2.0 ± 1.4 particles crossing 5mm (Fig. 3-1; n = 15 for HTS, n = 13 for ITS, n = 6 for No Treatment; $p < 0.05$, one-way ANOVA with Tukey's HSD post-hoc testing). These findings support the use of this assay for comparing MCC of different treatment groups. It was expected that HTS should cause an increase in MCC over tissues treated with ITS and those that lacked nebulization treatment, and therefore we can conclude that these data are comparable with existing clinical evidence of HTS efficacy (Elkins et al., 2006; Robinson et al., 1996)

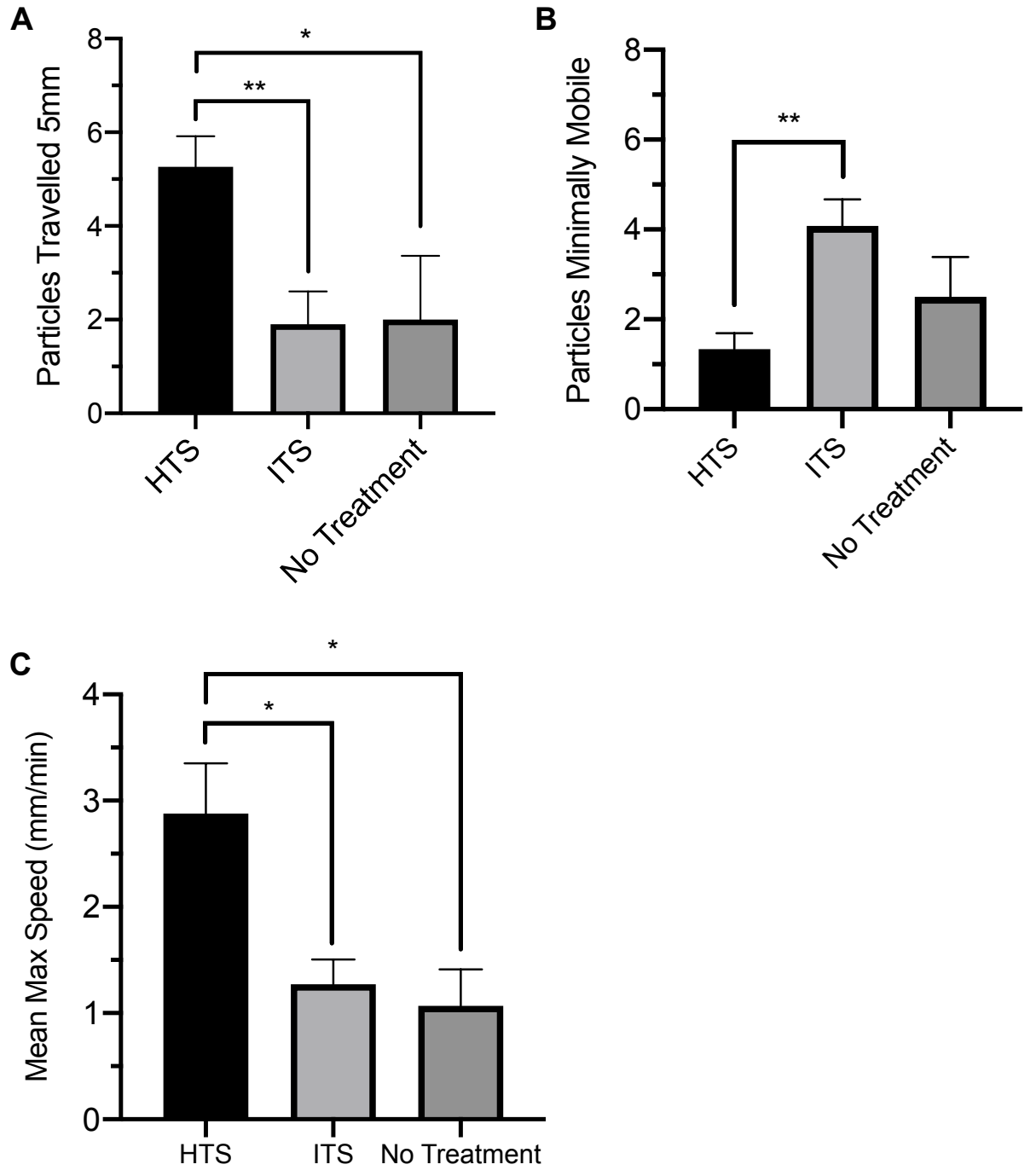


Figure 3-1. Mucociliary clearance in excised wild-type 5-week-old swine trachea following treatment with hypertonic saline, isotonic saline, or no treatment (HTS, n = 15 trachea sections from 15 swine; ITS, n = 13 trachea sections from 13 swine; No treatment, n = 6 from 5 swine). A) Average number of particles that travelled a minimum of 5 mm in the 20-minute imaging interval. HTS was shown to cause a significant increase in the mean particles travelled 5mm over ITS,

whereby q (31) = 4.710, p = 0.0062. HTS also caused a significant increase over No Treatment, whereby q (31) = 3.586, p = 0.0424. B) Average number of minimally mobile particles between the groups. HTS led to significantly fewer minimally mobile particles when compared with ITS, whereby q (31) = 5.544, p = 0.0013. No statistically significant difference was observed between HTS and No Treatment. C) Mean maximum particle speed. HTS was shown to have a significantly higher mean maximum particle speed when compared with ITS, p = 0.0183. HTS was also shown to have a significantly higher mean maximum particle speed when compared with No Treatment, whereby p = 0.0179. Data are presented as mean ± SEM. Means for A and B were analyzed with ordinary one-way ANOVA and Tukey's HSD multiple comparisons. Brown-Forysthe correction for unequal variances was applied to C with Dunnett's T3 multiple comparisons testing. Significance level was set at p = 0.05.

Similarly, preparations treated with HTS displayed 1.3 ± 0.4 particles travelling less than 1 mm (i.e., minimally mobile) compared to ITS-treated preparations, which showed 4.1 ± 0.6 particles minimally mobile (Fig. 3-1B, $p < 0.05$, one-way ANOVA with Tukey's HSD post-hoc testing). No other significant differences were observed, but the small n-value of the No Treatment group possibly accounts for the lack of statistically significant difference between HTS and No Treatment groups; further repetitions are necessary to better characterize this relationship. Lastly, examining Figure 3-1C for mean maximum particle speed, it is apparent that HTS causes a significant increase in mean maximum particle speed when compared with ITS and No Treatment (Fig. 3-1C, $p < 0.05$, one-way ANOVA with Brown-Forsythe Correction and Dunnett's T3 multiple comparisons). HTS showed a mean maximum particle speed of 2.9 ± 0.5 mm/min, while ITS and No Treatment showed 1.3 ± 0.2 mm/min and 1.1 ± 0.3 mm/min, respectively.

The results demonstrate that our mucociliary clearance assay is capable of measuring HTS-triggered increase in clearance and, thus, can be used to study other treatments, including alternative airway rehydration treatments.

3.1.2 Blocking Neuronal Function Inhibits the Effect of HTS Nebulization on MCC

Treatment with nebulized HTS triggers neurogenic ASL secretion (Luan et al., 2019). This effect can be blocked by treatment with the sodium channel blocker tetrodotoxin (TTX), which functions by inhibiting action potentials in some neurons (Scholz et al., 1998). Thus, we tested the effect of TTX on HTS-triggered mucociliary clearance.

While HTS treatment triggered 5.3 ± 0.6 particles to travel 5 mm, preincubation with TTX significantly reduced the number of cleared particles to 1.6 ± 0.6, similar to the results of ITS treatment (Fig. 3.2A; n = 15 for HTS, n = 17 for HTS + TTX, n = 13 for ITS; $p < 0.05$, one-way ANOVA and Tukey's HSD post-hoc multiple comparisons). Consistent with this result is the finding that HTS also had significantly fewer particles that were minimally mobile when compared with HTS + TTX (Fig. 3.2B; $p < 0.05$, one-way ANOVA with Tukey's HSD post-hoc multiple comparisons). HTS alone saw 1.3 ± 0.4 minimally mobile particles, whereas preincubation with TTX prior to HTS nebulization raised that value to 4.5 ± 0.7. The number of minimally mobile particles in the ITS group was similar to that of HTS + TTX. Lastly, no statistically significant differences in mean maximum particle speed were observed in tissues treated with TTX, suggesting that the speed of mobile particles is similar, but less particles are mobile overall when TTX is applied.

These findings provide evidence that the neurogenic effect of HTS treatment contributes to the stimulation of MCC by increasing the number of cleared particles, but has no effect on the mucociliary clearance speed.

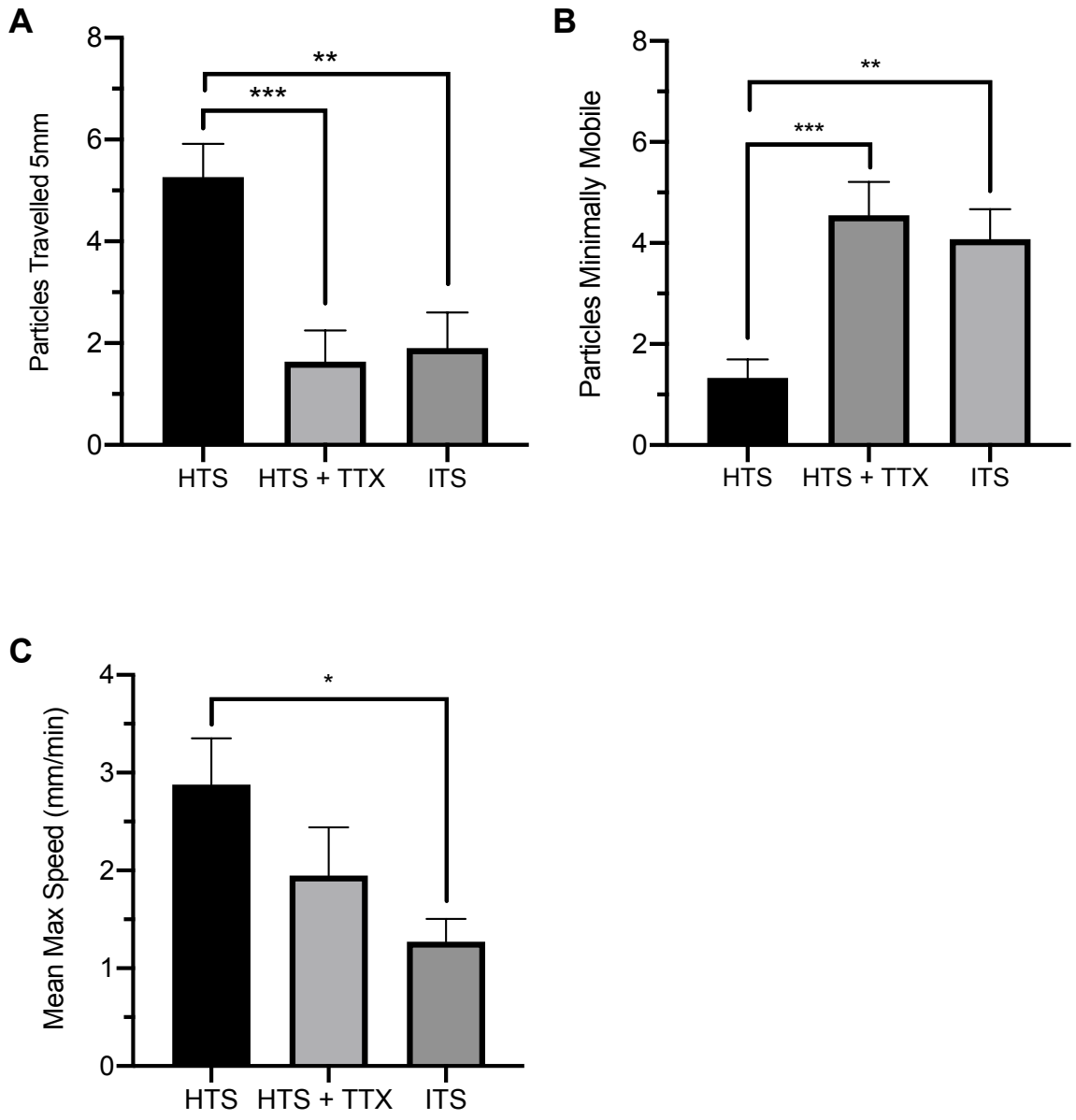


Figure 3-2. Mucociliary clearance in excised wild-type 5-week-old swine trachea following treatment with hypertonic saline, isotonic saline, or a combination of HTS and TTX (HTS, n = 15 trachea sections from 15 swine; ITS, n = 13 trachea sections from 13 swine; HTS + TTX, n = 17 from 17 swine). A) Average number of particles that travelled a minimum of 5 mm. HTS caused a significant increase in the mean particles travelled 5 mm versus the HTS + TTX treatment group, whereby q (42) = 5.738, p = 0.0006. HTS was shown to cause an increase over ITS as well, whereby q (42) = 4.970, p = 0.003. B) Number of minimally mobile particles. HTS caused a significant reduction compared to ITS. HTS also caused a significant reduction in minimally

mobile particles when compared with HTS + TTX, whereby q (42) = 5.870, p = 0.0005. C) Mean maximum particle speed, and found HTS to be significantly greater than ITS, as described earlier. No significant difference in maximum particle speed was observed between HTS + TTX and ITS or HTS. Data are presented as mean ± SEM. Means for A and B were analyzed with ordinary one-way ANOVA and Tukey's HSD multiple comparisons. Brown-Forysthe correction for unequal variances was applied to the ANOVA for C, with Dunnett's T3 multiple comparisons testing. Significance level was set at p = 0.05.

3.1.3 Sensory Agonists Increase Mucociliary Clearance

To further test the potential role of the nervous system in improving mucociliary clearance, we tested the effect of adding the sensory agonists capsaicin and menthol. Capsaicin has been shown to increase HTS-triggered airway ASL production (Luan et a., 2019). Addition of capsaicin to ITS treatment increased the number of particles cleared from 1.9 ± 0.7 in ITS to 5.6 ± 1.4 for ITS + Capsaicin-treated preparations (Fig. 3-3A; n = 15 for HTS, n = 13 for ITS, n = 5 for ITS + Capsaicin, n = 7 for HTS + Capsaicin; $p < 0.05$; one-way ANOVA and Tukey's HSD post-hoc multiple comparisons). Interestingly, capsaicin had no significant effect on HTS-treated preparations, where the number of particles cleared was 5.3 ± 0.9 for HTS + Capsaicin, and 5.6 ± 0.6 for HTS alone ($p > 0.05$, one-way ANOVA and Tukey's HSD post-hoc multiple comparisons).

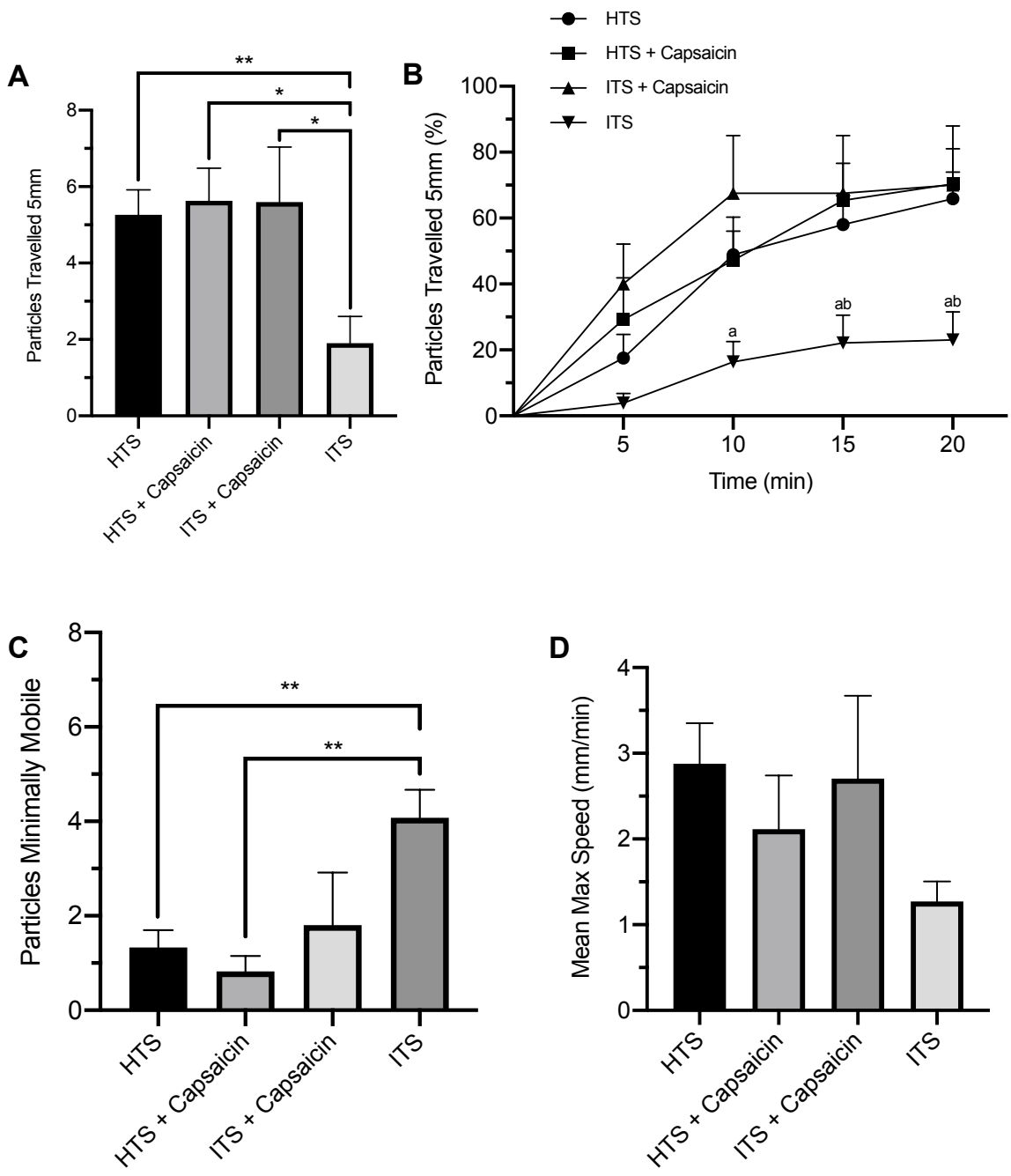


Figure 3-3. Mucociliary clearance in excised wild-type 5-week-old swine trachea following treatment with HTS, ITS, a combination of HTS and capsaicin, or a combination of ITS and capsaicin (HTS, n = 15 trachea sections from 15 swine; ITS, n = 13 trachea sections from 13 swine; HTS + Capsaicin, n = 7 from 7 swine; ITS + Capsaicin, n = 5 from 5 swine). A) Average number of particles that travelled a minimum of 5 mm. HTS was shown to cause a significant increase in the mean particles travelled 5 mm over the ITS treatment group. HTS + Capsaicin

caused a significant increase over ITS, whereby q (36) = 4.393, p = 0.0185. ITS + Capsaicin caused a significant increase over ITS as well, whereby q (36) = 3.881, p = 0.0445. No statistically significant differences were observed between HTS, HTS + Capsaicin, or ITS + Capsaicin treatment groups. B) Percentage of particles that have travelled 5 mm as a function of time. 'a' denotes a statistically significant difference from HTS for a given time point. 'b' denotes a statistically significant difference from HTS + Capsaicin for a given time point. Only HTS was greater than ITS at minute 5, while both HTS + Capsaicin and HTS were statistically significantly greater than ITS at minute 15 and 20, p < 0.05. C) Average number of minimally mobile particles. HTS was shown to have significantly less than ITS. HTS + Capsaicin also showed a significant reduction in minimally mobile particles when compared with ITS, whereby q (36) = 5.575, p = 0.0019. No additional significant differences were observed. D) Mean maximum particle speed. No statistically significant differences were observed. Data are presented as mean ± SEM. Means in A, C, and D were analyzed with ordinary one-way ANOVA and Tukey's HSD multiple comparisons post-hoc testing. Means in B were compared using mixed methods two-way ANOVA with Greenhouse-Geiser correction factor where necessary, and Tukey's HSD post-hoc testing. Significance level was set at p = 0.05.

Similarly, HTS + Capsaicin showed 0.8 ± 0.3 minimally mobile particles, which was significantly less than the 4.1 ± 0.6 minimally mobile particles observed in ITS (Fig. 3-3A; $p < 0.05$, one-way ANOVA and Tukey's HSD post-hoc multiple comparisons). ITS + Capsaicin, which had 1.8 ± 1.1 minimally mobile particles, was not significantly different from ITS (Fig. 3-3C; $p > 0.05$, one-way ANOVA and Tukey's HSD multiple comparisons). The small sample size of ITS + Capsaicin will necessitate additional experiments. Lastly, no significant differences in mean maximum particle speed were observed when tissues were treated with capsaicin (Fig. 3-3D; $p > 0.05$, one-way ANOVA).

Despite there being no difference between HTS and HTS + Capsaicin in the measurements described above, it was still possible that there may be a difference with respect to the time course of the response to these treatments. Thus, in order to study the time course of the effect of the treatment we analyzed the cumulative percentage of particles that have travelled a distance of 5 mm as a function of time (Fig. 3-3B). At minute 10, minute 15, and minute 20, a statistically

significant difference can be observed between HTS and ITS (Fig 3-3B, $p < 0.05$, mixed methods two-way ANOVA with Greenhouse-Geiser correction and Tukey's HSD post-hoc testing). At minute 15 and minute 20, HTS + Capsaicin showed greater clearance compared to ITS (Fig 3-3B, $p < 0.05$, mixed methods two-way ANOVA with Greenhouse-Geiser correction and Tukey's HSD post-hoc testing). Despite there being an absolute difference in clearance between ITS + Capsaicin and ITS alone (Fig. 3-3A), no significant difference was observed in this analysis. It can be observed that ITS + Capsaicin is highly similar to both HTS and HTS + Capsaicin groups, and therefore additional experiments are likely necessary to better represent this relationship.

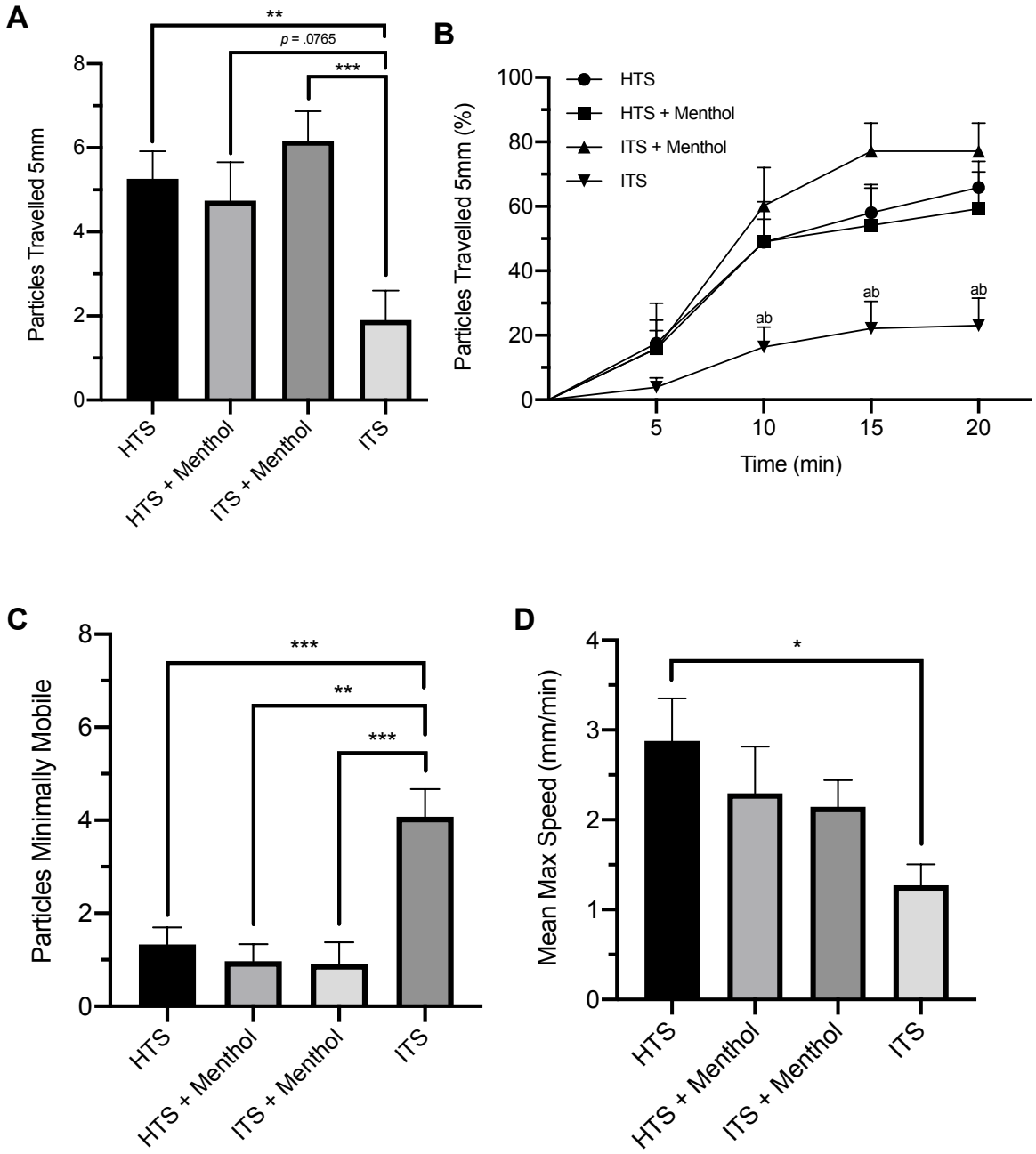


Figure 3-4. Mucociliary clearance in wild-type 5-week-old excised swine trachea following treatment with HTS, ITS, a combination of HTS and menthol, or a combination of ITS and menthol (HTS, n = 15 trachea sections from 15 swine; ITS, n = 13 trachea sections from 13 swine; HTS + Menthol, n = 7 from 7 swine; ITS + Menthol, n =10 from 10 swine). A) Average number of particles that travelled a minimum of 5 mm in the 20-minute imaging interval. HTS was shown to cause a significant increase in the mean particles travelled 5 mm over the ITS treatment group, whereby

q (41) = 5.154, p = 0.004. ITS + Menthol caused a significant increase over ITS, whereby q (41) = 5.890, p = 0.0009. HTS + Menthol did not cause a statistically significant difference over the ITS treatment group. B) Percentage of particles that have travelled 5 mm as a function of time. 'a' denotes a statistically significant difference from HTS for a given time point. 'b' denotes a statistically significant difference from ITS + Menthol for a given time point. ITS + Menthol and HTS were statistically significantly greater than ITS at minute 10, 15, and 20, p < 0.05. No other significant differences were observed. C) Number of minimally mobile particles. HTS + Menthol showed significantly less minimally mobile particles compared with ITS, whereby q (41) = 5.798, p = 0.0011. ITS + Menthol showed a significant reduction compared with ITS, whereby q (41) = 6.581, p = 0.0002. No significant differences were observed between HTS, HTS + Menthol, and ITS + Menthol. D) compares mean maximum particle speed. HTS shows significantly greater maximum particle speed than ITS. No additional significant differences were observed. Data are presented as mean ± SEM. Means in A, C, and D were analyzed with ordinary one-way ANOVA and Tukey's HSD multiple comparisons post-hoc testing. Means in B were compared using mixed methods two-way ANOVA with Greenhouse-Geiser correction factor where necessary, and Tukey's HSD post-hoc testing. Significance level was set at p = 0.05.

Menthol was the second sensory agonist used to test the involvement of the nervous system in MCC (Fig. 3-4). Menthol has been shown to stimulate airway secretions (Chiyotani et al., 1994), and appears to have a positive effect on mucus clearance in non-CF obstructive lung disease (Hasani et al., 2003), making it a solid candidate for testing. ITS + Menthol caused 6.2 ± 0.7 particles to travel 5 mm, which was significantly more than the 1.9 ± 0.7 particles in ITS (Fig. 3-4A; n = 15 for HTS, n = 13 for ITS, n = 10 for ITS + Menthol, n = 7 for HTS + Menthol; $p < 0.05$, one-way ANOVA with Tukey's HSD multiple comparisons). No significant differences were observed between HTS, HTS + Menthol, or ITS + Menthol groups, and they appear approximately equivalent in their number of particles that travel 5 mm. HTS + Menthol was not significantly greater than ITS (Fig. 3-4A; $p > 0.05$, one-way ANOVA with Tukey's HSD multiple comparisons). That said, the HTS + Menthol group had the smallest n-value in this comparison and the sample size should be increased.

When comparing the cumulative percentage of particles that have travelled a distance of 5 mm as a function of time, no statistically significant difference was observed at 5 minutes (Fig. 3-4B, $p > 0.05$, mixed methods two-way ANOVA with Greenhouse-Geiser correction). At 10, 15, and 20 minutes, ITS + Menthol and HTS both showed statistically higher percentages than ITS (Fig. 3-4B, $p < 0.05$, mixed methods two-way ANOVA with Greenhouse-Geiser correction and Tukey's HSD multiple comparisons). Similar to 3-4A, no significant difference was observed between HTS + Menthol and ITS. HTS + Menthol appears to be approximately equivalent to the HTS treatment group, and so additional experiments may help to clarify this relationship.

Comparing the average number of minimally mobile particles (Fig. 3-4C), a few significant differences were observed. As described earlier, HTS had significantly less minimally mobile particles when compared with ITS (Fig. 3-4C; $p < 0.05$, one-way ANOVA with Tukey's HSD multiple comparisons). ITS + Menthol showed 0.91 ± 0.5 minimally mobile particles, a significant reduction when compared to ITS (Fig. 3-4C; $p < 0.05$, one-way ANOVA with Tukey's HSD multiple comparisons). Interestingly, despite not causing a significant increase in particles travelling 5 mm (Fig. 3-4A), HTS + Menthol, which had 1.0 ± 0.4 minimally mobile particles, did cause a significant reduction in minimally mobile particles when compared with ITS (Fig. 3-4C; $p < 0.05$, one-way ANOVA with Tukey's HSD multiple comparisons). No statistically significant differences were observed between HTS, HTS + Menthol, and ITS + Menthol. Mean maximum particle speed was not shown to be different between either HTS + Menthol or ITS + Menthol when compared with ITS or HTS (Fig. 3-4D; $p > 0.05$, one-way ANOVA). These findings highlight menthol as a strong potentiator of MCC, as evidenced by the ability of ITS + Menthol to outperform ITS alone.

3.2 Discussion

The key finding in our research is that HTS-mediated increases in MCC involve the nervous system. HTS nebulization caused a significant increase in both particles that travel large (5 mm) distances and a significant reduction in minimally mobile particles (Fig. 3-1) when compared with an ITS-treated preparation. The effect of HTS nebulization on MCC were remarkably similar to those reported on the effect of the treatment on the changes in airway surface

liquid layer height reported in previous studies from our lab (Luan et al., 2019). First, No Treatment and ITS treatment groups were approximately equal in the change in ASL at all time-points, including the end time-point of 18 minutes. Similarly, we see close values for the number of particles travelled 5 mm (Fig. 3-1A). When comparing the change in ASL between treatment groups at the end-time (18 minutes), the study showed that HTS-treated tracheas had close to double the change in ASL height compared to ITS and No Treatment. Comparatively, the MCC experiments described above show that no treatment and ITS treatment had between one-third and one-half of the clearance seen in HTS-treated preparations (Fig. 3-1A). With respect to minimally mobile particles (Fig. 3-1B), we can also see that there are fewer particles remaining stationary or minimally mobile in the HTS-treated tissues. Finally, we saw that there was an increase in the mean maximum particle speed in HTS tissues, when compared with ITS or No Treatment. Overall, we can conclude that, when compared with ITS or No Treatment, HTS experiments demonstrate more mobile particles, a greater number of particles travelling long distances, and higher velocities of clearance. These experiments allowed us to achieve our first objective, whereby we sought to confirm that our assay was sensitive to changes in MCC that occur in response to treatments. From here, the next step was determining what role, if any, the nervous system plays in HTS-triggered MCC.

The experiments comparing the use of HTS + TTX to HTS alone address the main question of this thesis: is the HTS-mediated increase in MCC dependent on the nervous system? After finding that HTS-mediated increases in ASL height are reduced by roughly 50% in the presence of neural blockers (Luan et al., 2019), the next logical step was to determine whether this translated to any effect in the more clinically relevant parameter of MCC. It was expected that there would be a reduction in MCC in response to HTS when the tissue was incubated using TTX, a voltage-gated Na^+ channel blocker (Scholz et al., 1998). Initially, it was expected that HTS + TTX would have less clearance when compared with HTS alone, but still exhibit greater clearance than ITS. This is because of *in vivo* and *ex vivo* ASL height experiments, where HTS combined with neural blockers showed a 50% reduction in HTS-mediated increases in ASL height, indicating that some level of HTS response was still present with neural blockers since the osmotic effect of HTS would not be affected by neural blockers (Luan et al., 2019). Figure 3-2A showed that TTX significantly blocked HTS-induced increase in MCC, as there was no significant difference between HTS +

TTX and ITS. It is apparent that this degree of neural block eliminated the effects of HTS on particle clearance, according to this parameter of MCC. HTS + TTX also had a significantly greater number of minimally mobile particles when compared with HTS. Therefore, not only is the nervous system important for causing mucus and the associated particles to travel long distances, but it is also important in initiating particle movement.

Despite these differences, there was no apparent change in particle speed when HTS was preceded by TTX incubation. This finding may indicate that mobile particles behave similarly, but the true difference between treatments is in the number of particles being cleared. Overall, the experiments performed using TTX allowed us to support our hypothesis that HTS-triggered MCC is dependent on the nervous system.

Thus, blocking the neurogenic contribution to HTS treatment inhibits ~50% of treatment-induced airway fluid secretion (Luan et al., 2019), but completely blocks the HTS-induced increase in MCC. These results suggest that the importance of the nervous system plays a larger role in HTS treatment than previously thought. One of the limitations of our experimental approach is that, since our *ex vivo* preparations are missing all innervation from the central nervous system, the HTS treatment must only recruit the airway intrinsic neural network for its effects. Experiments *in vivo* could help to determine if this trend is still observed with the involvement of the central nervous system. Regardless, it is clear that the nervous system can be harnessed to promote mucus clearance, and the next step was to investigate whether sensory agonists could be used to potentiate the effects of either HTS or ITS on MCC.

3.2.1 Sensory Mechanism

Live swine treated with ITS and capsaicin have been shown to cause a statistically significant increase in ASL height when compared with ITS alone, indicating that capsaicin triggers ASL production (Luan et al., 2019). The same study found that adding capsaicin to HTS treatment did not potentiate the effects of HTS, therefore suggesting that they both may be acting on C-fibres (Luan et al., 2019). This finding prompted experiments examining how capsaicin may affect MCC through nebulization with either HTS or ITS (Fig. 3-3). We hypothesized that adding

capsaicin to HTS may cause a minor improvement in treatment efficacy, and that the addition of capsaicin to ITS treatment will cause a substantial increase in clearance when compared to ITS alone, while not achieving levels of clearance observed in HTS or HTS + Capsaicin. ITS + Capsaicin caused much greater clearance than that produced by ITS alone. Unexpectedly, ITS + Capsaicin appears approximately as effective as HTS and HTS + Capsaicin treatment groups (Fig. 3-3A). Since ITS + capsaicin is promoting airway hydration only through the neural mediated sensory pathways, with no contribution from an osmotic effect (Luan et al., 2019; Szallasi, 2001), we must conclude that nervous system stimulation produces the same increase in MCC as HTS nebulization.

Similar results were obtained when preparations were treated with menthol. Menthol has been shown to stimulate transepithelial Cl^- secretion in the airways, and has been suggested to influence MCC (Chiyotani et al., 1994). Furthermore, aromatic compounds, such as menthol, are able to promote mucus clearance in obstructive lung disease (Hasani et al., 2003). Therefore, we decided to investigate how menthol might influence MCC when combined with HTS or ITS (Fig. 3-4). We hypothesized that HTS + Menthol would be equivalent or better than HTS alone in promoting MCC, while ITS + Menthol would be less effective than HTS, but more effective than ITS.

Our results indicated that ITS + Menthol was, as expected, much more effective than ITS alone, while HTS + Menthol did not appear to cause any statistically significant improvements. It is surprising that HTS + Menthol does not appear to cause a significant increase in particles cleared 5 mm when compared with the ITS group. However, it is noteworthy that the n-value is relatively small for the HTS + Menthol group (n = 7). The *p*-value is relatively small, despite being not significant, and so further experiments will need to be performed to confirm the validity of this finding. It is possible, however, that the combination of menthol and HTS promotes excessive fluid secretion, to the point that it abrogates movement. The excised trachea preparation, unlike *in vivo*, is only ~1-1.5 inches in length, and has an end-point where fluid and mucus accumulate over time; the accumulation of fluid and mucus may artificially inhibit movement. It is not unreasonable to suggest that, in the event of excess fluid, it causes some degree of additional movement inhibition when compared other experiments. Despite this finding, we did find that HTS + Menthol caused

a significant decrease in the number of minimally mobile particles (Fig. 3-4B), with values similar to those of ITS + Menthol and HTS. What this indicates is that HTS + Menthol is roughly equal to HTS and ITS + Menthol in its capacity to induce particle movement, which is, in itself, an important indicator of treatment efficacy. Interestingly, ITS + Menthol was able to reproduce the effects of HTS, and to a greater level of significance. Similar to our results with capsaicin, the effect of ITS + Menthol indicate that stimulation of the sensory neurons is sufficient to replicate the effect of HTS, highlighting the importance of the nervous system in MCC. It is also important to consider, however, that menthol may also stimulate ciliary beat frequency (Neher et al., 2008). This makes it difficult to determine the origin of menthol's MCC-promoting effects, but it is likely a combination of fluid secretions and heightened ciliary beat frequency. Regardless of the mechanism, these findings could have exciting implications for treatment of CF-related airway disease.

Taken together, our results indicate that the nervous system plays a major role in regulating MCC and airway hydration, and thus it may be an ideal target to produce novel or improved therapies. Modulators of the nervous system may be used to increase the duration and intensity of HTS-trigger rehydration of the airway and to improve MCC, while reducing the negative side effects.

CHAPTER 4: THE CONTRIBUTION OF THE NERVOUS SYSTEM TO HTS-TRIGGERED INCREASE IN MUCOCILIARY CLEARANCE IN CYSTIC FIBROSIS SWINE

Hypertonic saline nebulization is regularly used in patients with cystic fibrosis to improve the rheological properties of the airway fluid and increase mucociliary clearance. Thus, we decided to study whether the HTS-triggered increase in mucociliary clearance is dependent on the nervous system in CF models, as we have described in wild-type swine (Chapter 3). We investigated the relationship between HTS and the nervous system in two CF models: transgenic CFTR knockout (CFTR-/-) swine, as well as wild-type swine trachea preparations treated with the CFTR blocker, CFTRinh-172. While the use of CFTR blockers to model CF provides a simple method to study the contribution of CFTR to CF phenotype, the CFTR-/- swine model is a much more powerful model. CFTR-/- swine have demonstrated similar disease progression to that of human CF patients, including lung pathogenesis. Moreover, CFTR -/- pigs possess a reduced ability to protect against pathogens, as well as a higher incidence of lung infections, comparable to what is observed in humans (Stoltz et al., 2010). Lastly, CFTR -/- swine exhibit airway mucus buildup, bacterial infection, inflammation, and obstruction within the first few months of life (Ostedgaard et al., 2011; Stoltz et al., 2010).

While it is clear that HTS-triggered increases in MCC is dependent on the nervous system in wild-type swine (see Chapter 3 above), it is not immediately clear that the same would be true in CF airways. The lack of functional CFTR may affect neural function (since CFTR is expressed in sensory neurons), fluid secretion, and cilia movement. Thus, this chapter of the dissertation will focus on experiments performed in the two aforementioned models of CF, as well as their controls.

The initial step of this series of experiments was to test the effect of blocking CFTR in 5-week-old wild-type animals using the blocker CFTRinh-172, allowing for direct comparisons with the results presented in Chapter 3. The second step was to perform experiments using CFTR-/- piglets.

4.1 Results

4.1.1 Blocking CFTR in Wild-Type Swine Does Not Affect HTS-Triggered MCC

CFTRinh-172 is a molecule used to pharmacologically inhibit the CFTR channel (Caci et al., 2008), without affecting non-CFTR chloride channels (Ma et al., 2002). The rationale behind using this drug is that it provided us with a relatively inexpensive method for modeling CF conditions in wild-type pigs. We hypothesized that HTS nebulization will increase MCC in CFTRinh-172-treated preparations. Further, we expected that this increase would be mediated, at least in part, by the nervous system. Thus, we incubated preparations with CFTRinh-172 and treatment them with HTS, HTS + TTX, or ITS.

The HTS treatment group with no CFTRinh172 caused 5.3 ± 0.6 particles to travel 5 mm, compared to 4.6 ± 1.2 particles in HTS + CFTRinh-172 preparations, a difference which was not significant (Fig. 4-1A; n = 15 for HTS, n = 8 for HTS + CFTRinh172, n = 10 for HTS + TTX + CFTRinh172, n = 7 for ITS + CFTRinh172; $p > 0.05$, one-way ANOVA and Tukey's HSD multiple comparisons). HTS + TTX + CFTRinh-172 showed 2.9 ± 0.7 particles traveling 5 mm, but this was not significantly different from the other treatment groups. Lastly, ITS + CFTRinh-172 had 2.0 ± 0.6 particles travelling 5 mm, which was significantly less than HTS alone (Fig. 4-1A, $p < 0.05$, one-way ANOVA and Tukey's HSD multiple comparisons).

HTS treated preparations had a statistically significant reduction in minimally mobile particles when compared with ITS + CFTRinh-172 (Fig. 4-1B; $p < 0.05$, one-way ANOVA and Tukey's HSD multiple comparisons), but no other statistically significant differences were observed. Lastly, no statistically significant differences were observed in mean maximum particle speed between these groups (Fig. 4-1C, $p > 0.05$, one-way ANOVA). Further experiments are planned to increase the sample sizes between the groups.

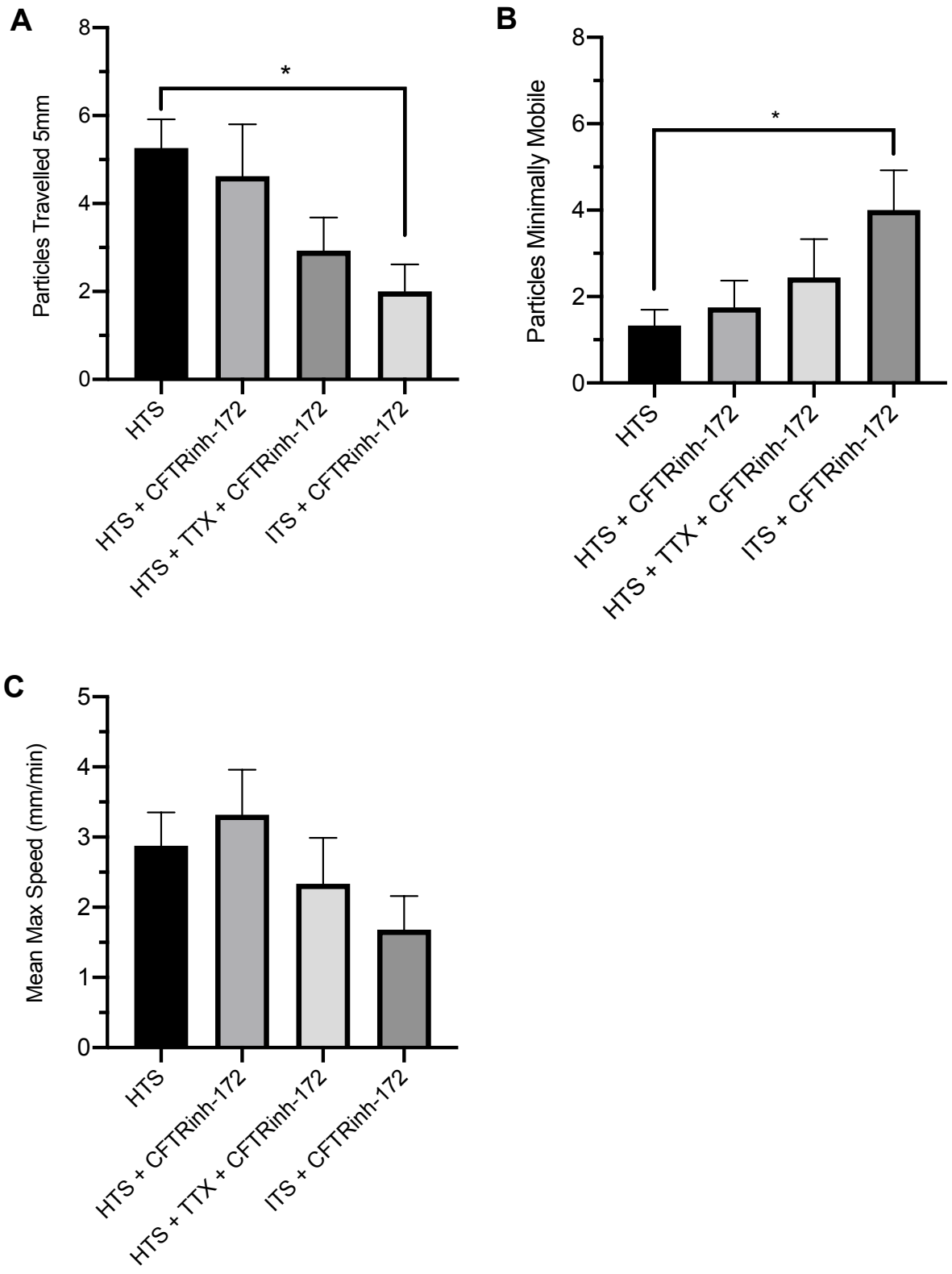


Figure 4-1. MCC in excised wild-type 5-week-old swine trachea preparations incubated with CFTRinh-172 prior to nebulization (HTS, n = 15 from 15 swine; HTS + CFTRinh-172, n = 8 from

7 swine, HTS + TTX + CFTRinh-172, n = 10 from 10 swine; ITS + CFTRinh-172, n = 7 from 7 swine). A) Average number of particles that travelled a minimum of 5 mm in the 20-minute imaging interval. HTS showed a statistically significant increase in particles travelling 5 mm over ITS + CFTRinh-172, whereby q (36) = 3.975, p = 0.0380. No other statistically significant differences were observed. B) Minimally mobile particles between the treatment groups. HTS showed a statistically significant reduction in minimally mobile particles when compared with ITS + CFTRinh-172, whereby q (35) = 4.104, p = 0.0309. No other statistically significant differences were observed. C) Mean maximum speed of each treatment group. No statistically significant differences in maximum speed were observed between treatment groups. Results are reported as mean ± SEM. Statistics for all figures were calculating using a simple one-way ANOVA and Tukey's HSD post-hoc multiple comparisons where necessary. For all analyses, the assumption of homogeneity of variance was met, as determined by Bartlett's test. Significance level was set at p < 0.05.

4.1.2 Blocking Neuronal Function Improves the Effects of HTS Nebulization on MCC in CFTR-/- Piglets

The trachea of 1- to 7-day-old male CFTR-/- swine were dissected and used to study the effect of HTS on MCC. Each CFTR-/- trachea was divided into two sections, with one portion receiving HTS alone, and the other receiving HTS + TTX treatment. The two experimental groups were then compared in each piglet using a paired Student's *t*-test. As explained in the method section, we determined a maximum distance travelled for each pig, and each particle from both trachea sections from that pig were then compared, as a percentage, to that maximum distance. This modified parameter still measures the ability of a treatment to cause MCC over relatively long distances, but normalizes each pig to itself, helping to account for substantial heterogeneity observed between pigs in health status and their response to treatment. A higher average percentage indicates greater levels of MCC.

The results show that the average percentage of maximal distance travelled in HTS-treated preparations was 17.7% ± 6.3%, while in preparations treated with HTS + TTX the particles travelled significantly farther, at 46.4% ± 8.7% (Fig 4-2A; n = 4 pigs; $p < 0.05$, paired *t*-test).

Therefore, it appears that the partial neural block induced by TTX actually improves HTS-mediated MCC in CFTR-/- piglets. Disaggregating the data into the response by each piglet shows that all preparations show the same trend, but with piglet 5 and piglet 6 having statistically significantly differences (Fig. 4-2B; $p < 0.05$, multiple paired *t*-tests). No statistically significant difference was observed in piglet 2 or piglet 7.

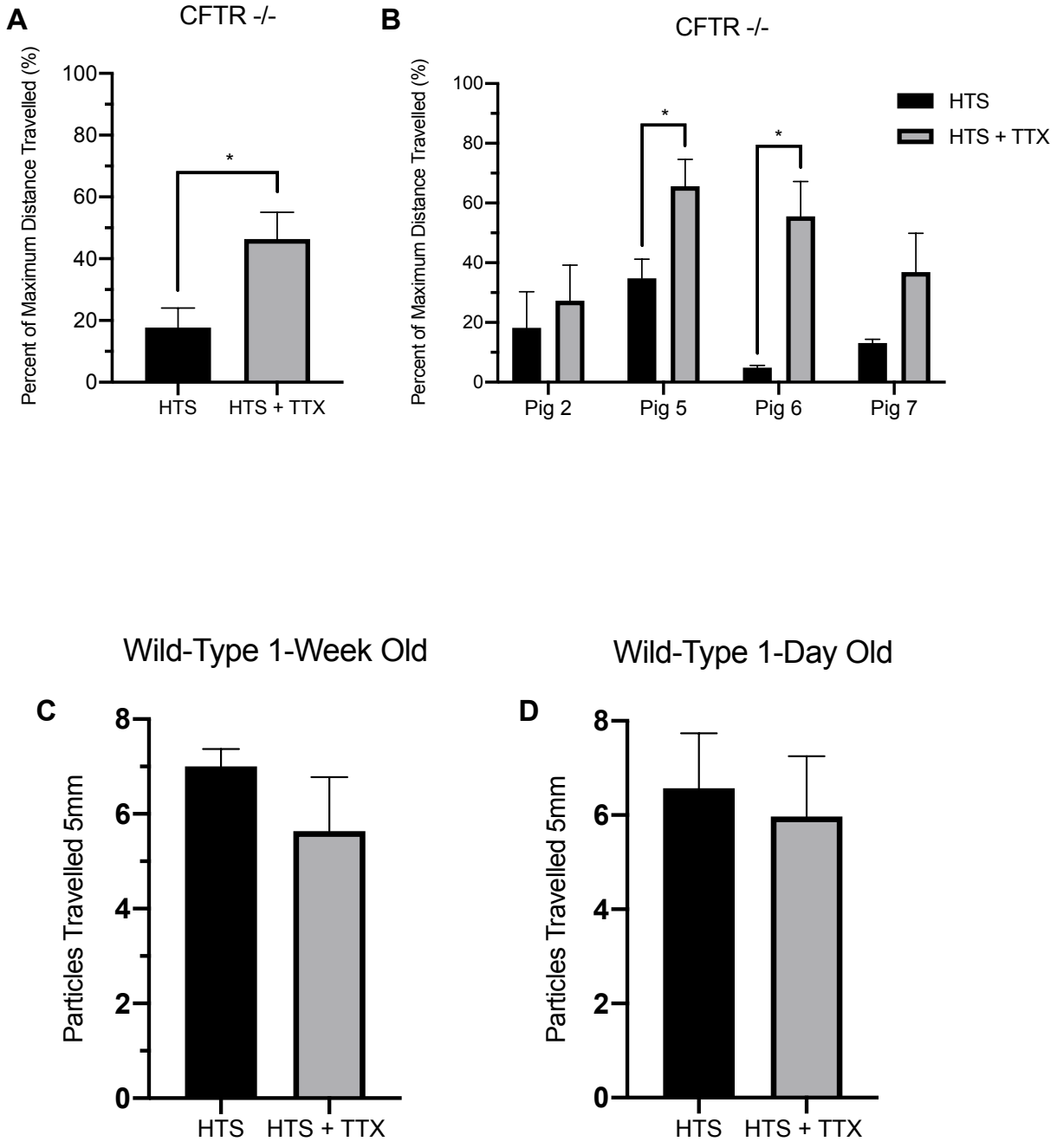


Figure 4-2. Mucociliary clearance in ex vivo swine trachea preparations following treatment with HTS or HTS + TTX. A) Average percentage of maximum distance travelled in CFTR -/- piglets (HTS, n = 4 trachea sections from 4 swine; HTS + TTX, n = 4 trachea sections from 4 swine). It was found that there is a statistically significant increase in percentage of maximum clearance in HTS + TTX when compared with HTS, whereby t (3) = 3.313, p = 0.0453. Statistics were calculated using a paired t-test. B) Average percentage of maximum distance travelled for each

individual piglet. Pig 5 and pig 6 showed a statistically significantly higher clearance in the HTS + TTX treatment groups. Statistics were calculated using multiple paired t-tests. C) and D) Average number of particles travelling 5 mm in 1-week-old and 1-day-old controls, respectively. In C, HTS (n = 13 trachea sections, from 13 swine) showed no statistically significant difference from HTS + TTX (n = 7 trachea sections from 7 swine). In D, HTS (n = 5 trachea sections from 5 swine) showed no statistically significant difference from HTS + TTX (n = 5 from 5 swine). Statistics were calculated using an unpaired t-test, with the addition of Welch's correction for unequal variances in C only. Results are reported as mean ± SEM. Significance level was set at p = 0.05.

Comparing the average number of particles travelling at least 5 mm between HTS and HTS + TTX in 1-week-old wild-type controls, HTS showed 7.0 ± 0.4 particles travelling 5 mm, while HTS + TTX showed 5.6 ± 1.1 particles. No statistically significant difference was observed between the HTS and HTS + TTX treatment groups (Fig. 4-2C; n = 13 for HTS, n = 7 for HTS+TTX; $p > 0.05$, Welch's unpaired *t*-test). Similarly, we compared these treatments in 1-day-old piglets (Fig. 4-2D). HTS showed a 6.6 ± 1.2 particles travelling 5mm, while HTS + TTX showed 6.0 ± 1.3 particles. No statistically significant difference was observed between the two groups (Fig. 4-2D; n = 5 for HTS, n= 5 for HTS + TTX; $p > 0.05$, unpaired *t*-test). Therefore, it appears that there is no statistically significant difference in the number of particles travelling 5 mm between the HTS and HTS + TTX treatment groups in wild-type young swine.

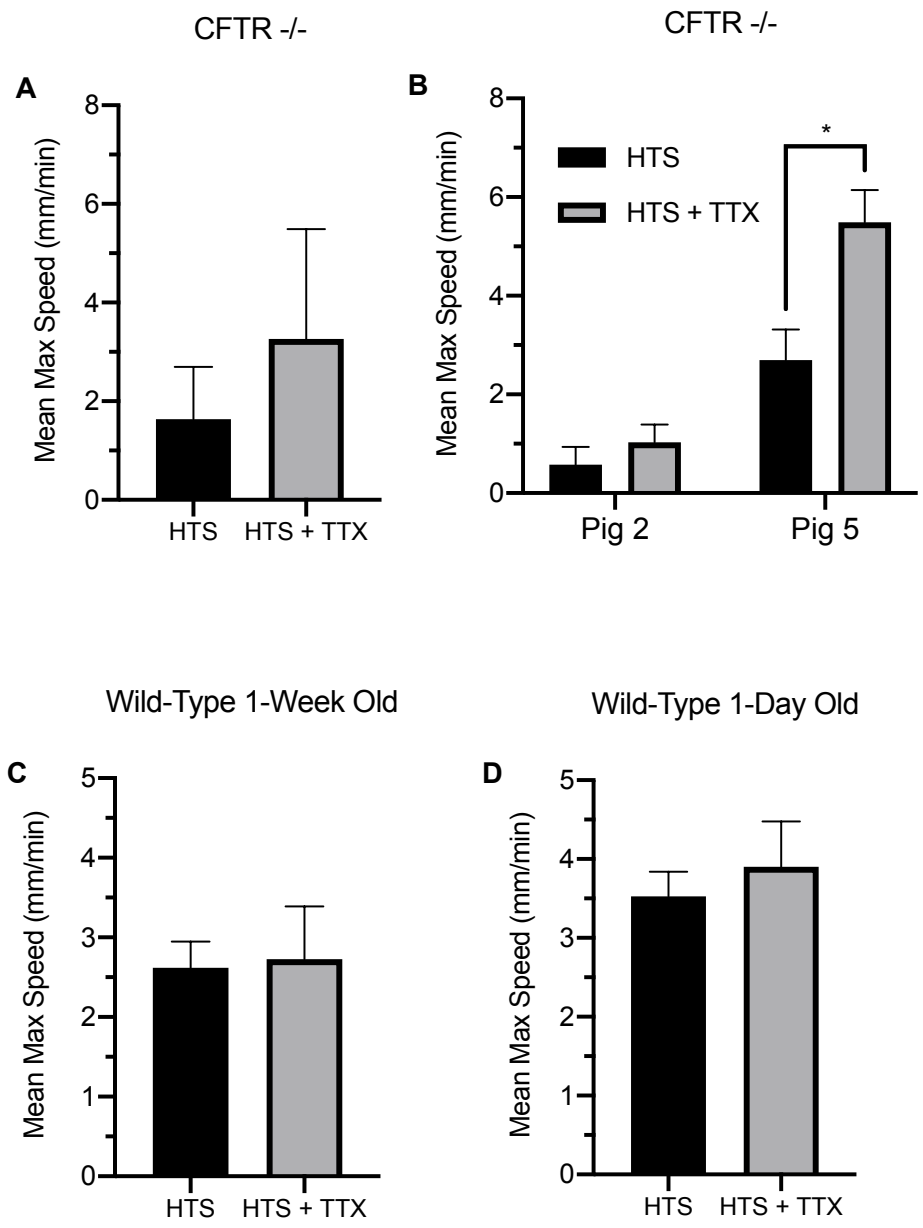


Figure 4-3. Mean maximum particle speed in ex vivo *swine trachea preparations following nebulization treatment. A) CFTR-/- piglets treated with either HTS (n = 2 trachea sections from 2 swine) or HTS + TTX (n = 2 from 2 swine). There was no statistically significant difference observed between HTS and HTS + TTX. Statistics were performed using a paired t-test. B) break down of the paired t-test from A into individual comparisons. In pig 5, HTS + TTX appears to have a statistically significantly higher mean maximum particle speed when compared with HTS, whereby t (14) = 3.099, p = 0.0156. C) and D) compare HTS with HTS + TTX in wild-type 1-week-old and 1-day-old piglets, respectively. No statistically significant differences were observed*

between HTS (n = 13 from 13 swine) and HTS + TTX (n = 7 from 7 swine) in 1-week-old swine. No statistically significant differences were observed between HTS (n = 5 from 5 swine) and HTS + TTX (n = 5 from 5 swine) in 1-day-old piglets. Statistics for C and D were calculated using an unpaired t-test, as well as an F-test to ensure equal variances. Results for each figure are reported as mean ± SEM. Significance level was set at p = 0.05.

Mean maximum particle speed was calculated and compared between HTS and HTS + TTX treatment groups in CFTR-/- swine, as well as wild-type 1-week- and 1-day-old controls. Only two CFTR-/- swine met the criteria to be included in this analysis. In CFTR-/- swine, there was no apparent effect on neural block on mean maximum particle speed (Fig. 4-3A; $p > 0.05$, paired *t*-test). HTS (n = 2) had a mean maximum particle speed of 1.6 ± 1.1 mm/min, while HTS + TTX (n = 2) was shown to have 3.3 ± 2.2 mm/min. Figure 4-3B breaks down the comparison to show individual paired *t*-tests performed for each pig. Here, it can be seen that in pig 5, HTS + TTX group had a statistically significant increase in mean maximum particle speed when compared with HTS (Fig. 4-3B; $p < 0.05$, paired *t*-test). The small sample size of this analysis makes it difficult to draw any conclusions, but I will defer to the lack of statistically significant difference observed in 4-3A. Figure 4-3C and 4-3D compare the mean maximum particle speed of wild-type controls, using 1-week- and 1-day-old pigs respectively. No statistically significant difference was observed in either comparison (Fig. 4-3C and Fig. 4-3D; $p > 0.05$, unpaired *t*-test), as the mean maximum particle speed between HTS and HTS + TTX appear approximately equal.

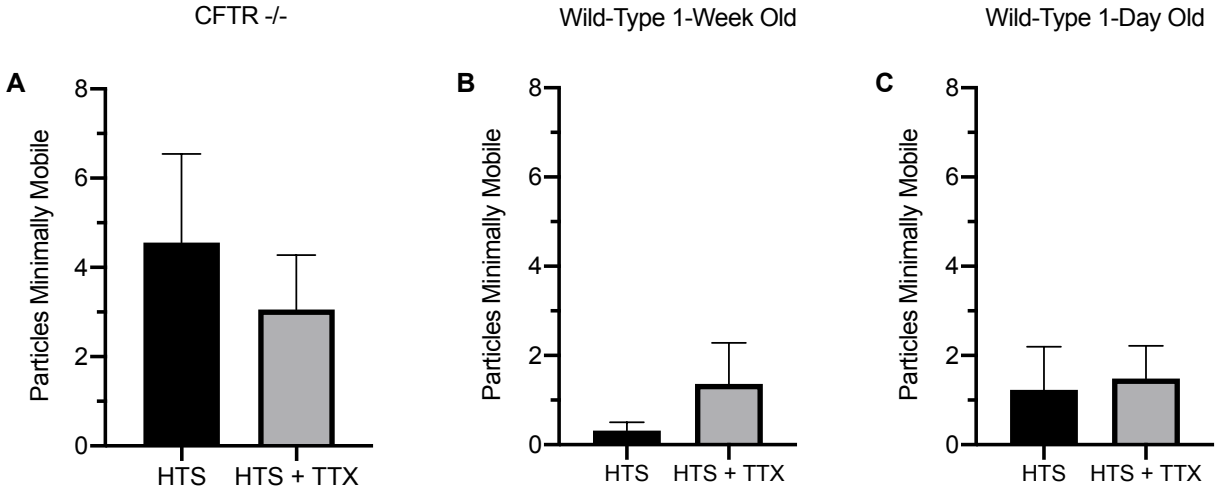


Figure 4-4. Mean number of tantalum particles minimally mobile during imaging of ex vivo swine trachea preparations. A) compares HTS (n = 4 trachea preparations from 4 swine) and HTS + TTX (n = 4 from 4 swine) treatment groups in CFTR-/- pigs. No statistically significant difference was observed between HTS and HTS + TTX groups. Statistics were calculated using a paired t-test. B) and C) show control experiments comparing HTS and HTS + TTX groups in wild-type 1-week-old and 1-day-old swine, respectively. No statistically significant difference was observed between HTS (n = 13 from 13 swine) or HTS + TTX (n = 7 from 7 swine) in B. Statistics for B were calculated using an unpaired t-test with Welch's correction for unequal variances. No statistically significant difference was observed between HTS (n = 5 from 5 swine) and HTS + TTX (n = 5 from 5 swine) in C. Statistics for C were calculated using an ordinary unpaired t-test, with an F-test to ensure equality of variances. Results are reported as mean ± SEM. Significance level was set at p = .05.

Figure 4-4A compares HTS and HTS + TTX treatment groups in CFTR-/- swine. HTS was shown to have 4.6 ± 2.0 particles that were minimally mobile, while HTS + TTX showed 3.1 ± 1.2 minimally mobile particles. This was not a statistically significant difference (Fig. 4-4A; n = 4 for HTS and HTS+TTX; $p > 0.05$, paired *t*-test). In wild-type 1-week-old controls, HTS had 0.3 ± 0.2 particles minimally mobile, while HTS + TTX showed 1.4 ± 0.9 particles, which was not a statistically significant difference (Fig. 4-4B; n = 13 for HTS, n = 7 for HTS + TTX; $p > 0.05$, unpaired *t*-test with Welch's correction for unequal variances). Lastly, in wild-type 1-day-old piglets, the number of minimally mobile particles was not statistically significant between HTS and HTS + TTX-treated preparations (Fig. 4-4C; n = 5 for HTS and HTS + TTX; $p > 0.05$, unpaired *t*-test). Therefore, no statistically significant differences were observed in either wild-type or CFTR-/- piglets.

4.3 Discussion

The most salient result was the surprising effect of TTX on CFTR-/- swine preparations. While blocking the nervous system reduces the HTS-triggered ASL production (Luan et al. 2019), and completely blocks HTS-induced increases in MCC in wild-type swine (Chapter 3), CFTR-/- preparations actually displayed an increase in HTS-induced increase in MCC after blocking the nervous system. More work would be needed to fully understand the reasons behind improved MCC after blocking the nervous system in CF; however, there are some indications of how this may work in the literature. One study has shown that impaired mucociliary transport in CF airways may be a result of CF submucosal glands secreting mucus strands that fail to detach from the gland ducts, thereby inhibiting MCC (Hoegger et al., 2014). HTS nebulization may act on the CF airway by improving the hydration of the airway and rheological properties of the mucus, which results in improved lung function (Donaldson et al., 2006). However, if mucus strands secreted by submucosal glands fail to detach from the submucosal glands, then it is possible that neural stimulation of submucosal gland secretions via HTS may actually have a counterproductive effect on MCC through this pathway. Thus, blocking the neurogenic response inhibits glandular secretion and leads to a decrease in mucus tethering to the glands. By partially blocking the neural component of this interaction via TTX, there would be less neural stimulation of submucosal gland secretions via these sensory networks. Instead, HTS would be, in the presence of TTX, relying

more heavily on an osmotically driven transepithelial secretion of fluid. While overall changes in ASL height may be reduced when compared to HTS alone, it would also potentially result in a reduction in tethered mucus strands (Hoegger et al., 2014) that fail to detach. This may also explain the finding that there is no significant difference in the number of minimally mobile particles between HTS and HTS + TTX (Fig. 4-4A). Impaired mucus detachment from the submucosal glands (Hoegger et al., 2014) may not influence the ability of mucus and particles to travel small distances. The relatively small distance of 1 mm may be achieved reasonably well in both HTS and HTS + TTX treatment conditions (i.e., even when there are more tethered mucus strands), but the real difference resides in their respective abilities to promote MCC over longer distances. These unforeseen findings may suggest that the neural component of HTS treatment in CF conditions could actually be preventing HTS from achieving its full potential in promoting MCC. Additional studies using varied methods will be necessary to further characterize this relationship.

The results obtained from wild-type swine treated with CFTRinh-172 were fundamentally different from those observed in CFTR-/- swine. Our data seems to indicate that there are no significant differences in the overall clearance, or speed of clearance, between tissues treated with just HTS, and those treated with CFTRinh-172 or CFTRinh-172 and TTX. These results are different from those in 5-week-old animals (Chapter 3), where TTX reduces MCC. It was surprising to see that there was no difference between HTS and HTS + TTX in 1- and 7-day-old animals. It is possible that, at this young age in healthy animals, MCC is already occurring optimally, such that addition of HTS has no significant effect on further improving MCC, and thus there would be no effect of TTX on reducing MCC. However, this result may also be an artifact of the experimental set up. It is possible that the methods employed for incubation with CFTRinh-172 may not have allowed it to properly penetrate the mucosa, thereby preventing access to the CFTR channel. Moreover, the sample size for the wild-type preparations incubated with CFTRinh172 may be too small. Therefore, to ensure the accurate characterization of this relationship, additional experiments have been planned. Regardless of the reason behind the lack of significant difference in these wild-type control groups, the result does allow us to conclude that the trends observed in CFTR-/- piglets are likely not a product of their age. Overall, these findings were unexpected, and are quite different than the results found in the CFTR-/- animals and those in Chapter 3.

CHAPTER 5: GENERAL CONCLUSION

The nervous system does, in fact, play a role in HTS-mediated changes in MCC. In wild-type swine, blocking the nervous system via preincubation with TTX essentially removed the effects that HTS has on mucociliary clearance. While we expected there to be some reduction in MCC in response to TTX, we did not expect HTS to lose, essentially, the entirety of its effects. This finding suggests that, despite HTS causing a significant increase in ASL height when the nervous system is blocked in comparison to ITS (Luan et al., 2019), there may be complete absence of the treatment's effects on MCC without the neural component.

Our CFTR-/- results showed that blocking the neurogenic response to HTS actually improved HTS-induced increases in MCC. This finding was entirely unexpected, refuting all of our prior hypotheses that blocking the nervous system would attenuate the response to HTS. While the reasoning for this behaviour remains somewhat uncertain, one possible explanation for this finding is that HTS stimulates submucosal gland secretions which, in CF airways, may fail to detach from the glands (Hoegger et al., 2014). By failing to detach, the mucus strands may enable clearance of particles over short distances, followed by failure to continue travelling. This is supported by the finding that, despite HTS + TTX showing a higher average percentage of maximal movement when compared to HTS alone (Fig. 4-2A), they demonstrated similar numbers of minimally mobile particles (Fig. 4-3A). This could be interpreted as both treatments having approximately the same propensity to induce movement, but preincubation with TTX allows for the particles, and thus the mucus, to travel longer distances. By blocking the nervous system, we suggest that there is only the osmotic fluid secretion, and a reduction in the prevalence of mucus strands that inhibit more distant movement.

5.1 Significance for the Development of New Treatments

Currently, in the year 2020, there is ongoing interest in developing an airway rehydration therapy superior to HTS, such as ENaC blockers to inhibit airway surface liquid reabsorption (clinical trials for Parion PS-G201; Boehringer Ingelheim BI1265162; AstraZeneca AZD5643; Novartis QBW276). There is also interest in increasing tolerability and effectiveness by replacing

HTS with mannitol or hypertonic xylitol (Singh et al., 2020) (clinical trial registration NCT00928135), or adding hyaluronic acid to HTS formulation to increase "pleasantness" (Máiz et al., 2018). However, there are no treatments under development that exploit the contribution of the nervous system to MCC.

Our results could have interesting implications for HTS treatment of CF lung disease as well as non-CF bronchiectasis. We show that the nervous system is a major contributor to improved MCC after HTS nebulization in non-CF tissues. Addition of capsaicin or menthol to HTS formulation does not seem to further increase the rate of MCC, according to our results; however, it may have a large benefit on the duration of the treatment. Luan et al. (2019) showed that adding capsaicin or menthol to the HTS formulation increased the duration of the nebulized treatment. HTS alone produced an increase in hydration for 90 min, while adding capsaicin or menthol extended the effect to 4 hours. Thus, even though we did not measure an increase in MCC in the timeframe of our experiments (~20 min), it is possible that adding capsaicin or menthol may result in an increased MCC for a longer period of time after nebulization.

Addition of sensory agonists may have negative effects on the patients by inducing cough, though this may assist in the clearance of mucus. It is important to consider that increased coughing may make it a less tolerable treatment for certain patients. We also saw that menthol clearly has a beneficial effect on MCC when combined with ITS, and may be equivalent to HTS alone when combined with HTS. While some patients struggle to tolerate HTS due to its emergency reflex responses, such as coughing, epiglottal closure, and airway constriction (Wine, 2007), menthol has actually been shown to increase cough reflex threshold (Wise et al., 2012). ITS appears to be harmless (Driessen et al., 2013), and if it does not cause these unwanted effects, it is possible that using ITS to deliver nebulized menthol could be a valid alternative to HTS treatment for CF and non-CF related lung disease. Additionally, while HTS causes unwanted neurogenic inflammation (Umeno et al., 1990; Wine, 2007), menthol has been shown to have counter-irritant effects, thereby reducing neurogenic inflammation (Andersen et al., 2016). Through its counter-irritant and cough-suppressant effects, the presence of menthol may make HTS more tolerable in certain patients, thereby helping patient adherence and improving lung health in the process. The highly tolerable nature of menthol and the potential for its effects on airway secretions (Chiyotani et al., 1994) and

MCC make it a strong candidate for improving upon HTS as an airway rehydration treatment in CF and the treatment of non-CF bronchiectasis. Thus, further work needs to be done to formulate a combination of HTS and sensory agonists that may be well-tolerated by live animals, as well as humans, for future research and drug development. Lastly, because we observed the neurogenic response to HTS to be inhibitory of movement in CFTR-/- swine, it would be necessary to perform experiments using sensory agonists in a CF model before any suggestions can be made about its efficacy in treating CF-related lung disease.

Overall, the nervous system is clearly a powerful entity in regulating MCC, and plays a substantial role in this commonly used treatment for CF-related lung disease. By understanding how HTS functions to improve lung health and promote MCC, we may find methods of harnessing the nervous system in such a way that enhances the effects of existing treatments, or replaces them altogether. While this study is limited to making inferences about CF and non-CF swine, it provides valuable insight that will direct future inquiries into this treatment strategy, as well as the nervous system as it relates to CF. A great deal remains to be discovered about this complex system, but it is clear that an enhanced understanding of nervous system's role in mediating mucociliary clearance in the context of HTS certainly has the potential to improve the health of many patients with CF.

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APPENDIX: DEVELOPMENT OF THE MUCOCILIARY CLEARANCE ASSAY

CF respiratory pathogenesis largely stems from impaired MCC resulting from impaired ion transport (Donaldson et al., 2007). Modulating rheological properties of mucus and promoting clearance has been a common theme in treatment (Robinson & Bye, 2002), including HTS. Experiments that model MCC have great potential in understanding the impaired clearance in CF patients, as well as the effects of treatments. While techniques exist to measure MCC, there has been limited success in detecting a difference between CF and non-CF patients (Robinson et al., 2000; Robinson & Bye, 2002). Recently, *ex vivo* techniques have been developed, whereby radiodense tantalum microdisks were placed on sections of trachea and monitored over time (Hoegger et al., 2014). Using these *ex vivo* MCC techniques, it was concluded that impaired mucociliary transport (MCT) was, as expected, a primary defect in CF patients. They went on to suggest that the loss of CFTR channels in submucosal glands may be the reason for failed mucus detachment from these glands, thus causing impaired MCT (Hoegger et al., 2014). With other studies reporting evidence that airway surface dehydration may initiate CF respiratory pathogenesis (Boucher, 2007), our lab sought to develop a MCC assay that could be used to study airway rehydration treatments, such as HTS, and their effects on MCC.

Beginning in September of 2018, the initial goal of the project was to develop an assay that could effectively show MCC, while being sensitive enough to also detect changes in MCC between treatment groups. Previous iterations prior to my tenure in the lab had limited success, but their work contributed substantially to the success had with my experiments. The assay we developed is partially based off an assay used by the Hoegger lab (Hoegger et al., 2014). They developed a technique whereby mucociliary transport (MCT) was tracked using radiodense 350 μm diameter tantalum microdisks, with X-ray computed tomography used to measure movement of the disks. We adopted the use of tantalum for the particles, first beginning with tantalum powder. The benefit of the powder was that there were particles substantially smaller than 350 μm, representing a more physiological context for particle clearance; it is rare that particles of that size are cleared via MCC, with most inhaled particles and pathogens being significantly smaller (Thomas, 2013). Preliminary experiments prior to my work in the lab demonstrated limited clearance of the powder. One possible explanation for this is that the powder frequently aggregated, creating clumps of material

much larger than the 350 μm disks employed by Hoegger. This led to us adopting the use of microdisks. Using a micro-hole puncher we produced tantalum microdisks with a diameter of 250 μm, and a thickness of 25 μm. While still larger than most inhaled particles, it was a major step in creating a functional assay and is a definite advantage over other existing *ex vivo* MCC assays. This was the first of the key differences that made our assay preferable to the experimental questions we wanted to answer.

A second major difference between our methods and those described by Hoegger is that they used saline to submerge the luminal surface of the trachea during imaging (Hoegger et al., 2014). For our objectives and goals, this method was problematic. Foremost, the use of saline to submerge the trachea during imaging would wash out the effects of any treatment applied to the airway and it would make it impossible to nebulize the lumen of the airway. HTS has previously been shown to cause an increase in the height of the ASL (Luan et al., 2019); therefore, the addition of fluid to the surface after nebulization would essentially negate the benefits of HTS, and equalize fluid heights between treatment groups. We were interested in determining if this increase in ASL height could be responsible for the improved clearance caused by HTS and, to do so, we needed an assay that kept the luminal surface of the trachea relatively clear of exogenous fluid after the initial wash with phosphate buffer solution (PBS). Though PBS itself could have an effect on clearance, we observed minimal residual fluid by the time imaging was occurring ~25 minutes later. Additionally, the PBS wash occurred prior to nebulization, creating a standardized starting point that provides a clearer picture of the effects of a treatment on MCC. The goal of preventing fluid from entering the lumen created a problem for treating the tissue with drugs such as CFTRinh-172 and TTX. With the lumen clamped off to prevent fluid entry, and the cartilage protecting the outer-surface of the mucosa, we realized the need to dissect the cartilage. Our assay is unique in that it involves removal of a portion of the cartilage to allow for pharmacological agents to reach the mucosal layer from the outside. This allowed us to overcome the challenge of treating the tissue with neural blockers and CFTR blockers.

Cleaning the Trachea

One of the major challenges that had to be overcome during the development of this technique was the cleaning of the lumen of the trachea prior to experimentation. There was considerable heterogeneity between different animals, as well as separate preparations of the same animal, in the amount of blood, fluid, and other unwanted matter in the trachea. Having inconsistent baseline fluid levels, or the presence of material or fluid that could potentially abrogate particle movement or irritate the trachea to cause fluid secretions, was a confounding effect that had to be eliminated. The initial method used to clean the tissue was sterile medical gauze bandages. This was somewhat effective in removing blood and other fluid that entered the airways during the euthanasia of the animal; however, it was soon observed that particles displayed minimal movement, likely as a result of epithelial damage. The delicate nature of the epithelium and its cilia meant that tactile force without fine control would cause damage that could not be standardized between experiments. The more cleaning a trachea needed, the more damage that would result from cleaning. This led to the use of sterile Q-tips to give greater control in cleaning. The Q-tip was used to spot-clean areas with high prevalence of blood or other matter. This method was more effective than gauze in cleaning the preparation, but once again, compared to tissues that lacked cleaning, these preparations did not exhibit as much clearance. We concluded that this was likely the result of damage to the epithelium from excessive force. The realization was that the trachea had to be cleaned without physically touching the surface with a cleaning object. The use of PBS was suggested, whereby trachea would be repeatedly dunked in PBS to remove blood, foam, and other contaminants. This method was found to be effective in cleaning the airway without causing damage to the epithelium.

Future Directions with the Assay

My work in developing the assay was from September-November 2018, but previous students had spent significant time (~2 years) working on the assay and making contributions that allowed me to achieve success with it. Their work was just as important in getting the assay to its current functional state. I expect my work and ideas will similarly help further improve and iterate upon this technique, with the following plans in progress:

The use of labelled bacteria, such as GDP-labelled *Pseudomonas aeruginosa*, will hopefully be used in future iterations of this assay. As mentioned, the tantalum disks used are relatively large compared to the actual size of inhaled particles and pathogens (Barbara H. Iglewski, 1996). In CF, chronic respiratory tract infections, including those of *Pseudomonas aeruginosa* (Speert et al., 2002), are relevant to health outcomes; therefore, improving this assay to measure movement of bacteria could provide valuable information about how treatments may improve clearance of these pathogens. Some have suggested that there could be differential movement of microscopic particles, including pathogens, compared to more macroscopic particles, such as the microdisks we currently employ, but this has yet to be explored. The use of fluorescent microscope-based imaging and fluorescing bacteria could provide valuable information regarding the potential differential clearance of small versus large particles. It has been suggested that the mucus strands play a role in adhering to and dragging macroscopic particles, with the underlying ASL being more involved in the transport of pathogens and other microscopic particles.