



Hypertonic saline therapy in cystic fibrosis: do population shifts caused by the osmotic sensitivity of infecting bacteria explain the effectiveness of this treatment?

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Cystic fibrosis (CF) is caused by a defect in the CF transmembrane regulator that leads to depletion and dehydration of the airway surface liquid (ASL) of the lung epithelium, providing an environment that can be infected by bacteria leading to increased morbidity and mortality. *Pseudomonas aeruginosa* chronically infects more than 80% of CF patients and one hallmark of infection is the emergence of a mucoid phenotype associated with a worsening prognosis and more rapid decline in lung function. Hypertonic saline (HS) is a clinically proven treatment that improves mucociliary clearance through partial rehydration of the ASL of the lung. Strikingly, while HS therapy does not alter the prevalence of *P. aeruginosa* in the CF lung it does decrease the frequency of episodes of acute, severe illness known as infective exacerbations among CF patients. In this article, we propose a hypothesis whereby the positive clinical effects of HS treatment are explained by the osmotic sensitivity of the mucoid sub-population of *P. aeruginosa* in the CF lung leading to selection against this group in favor of the osmotically resistant non-mucoid variants.

Keywords: mucoid, *mucA*, alginate, infective exacerbation, *Pseudomonas aeruginosa*, metabolomics

INTRODUCTION

In this paper we will discuss the background to the use of hypertonic saline (HS) in the treatment of cystic fibrosis (CF) and propose a hypothesis whereby the positive effects of this approach can be explained by population shifts of *Pseudomonas aeruginosa*, the major chronic infecting bacterium of the CF lung.

CYSTIC FIBROSIS AND CHRONIC PSEUDOMONAS AERUGINOSA INFECTION

Cystic fibrosis is an inherited condition that results from mutations in the gene encoding the CF transmembrane regulator (CFTR), a cAMP-regulated chloride channel in epithelial tissues. These mutations lead to disruption of normal ion and water transport across the epithelium and so to thickened, dehydrated airway secretions resulting in impaired mucociliary clearance (Collins, 1992; Ratjen and Doring, 2003; Campodonico et al., 2008). There is strong evidence to support an airway surface liquid (ASL) depletion/dehydration theory and a model for ASL volume regulation and hence mucociliary clearance has been described for normal and CF epithelia (see **Figure 1**). This defect in the mucociliary escalator means that CF lungs are prone to chronic infection, which is the main cause of mortality and morbidity in CF patients (Dodge et al., 2007). While experimental data supports the polymicrobial nature of CF airway infections there is little evidence for serious lung damage occurring in CF in the absence of *P. aeruginosa* infection (Sibley et al., 2008; Tunney et al., 2008). *P. aeruginosa* chronically infects more than 80% of CF patients by adulthood leading to significantly

poorer lung function and increased risk of mortality than non-infected peers (Govan and Deretic, 1996; Govan et al., 2007; Murray et al., 2007). The high incidence of specific *P. aeruginosa* infection in CF is probably not explained solely by a defect in mucociliary clearance, as *P. aeruginosa* infection is less frequently encountered and usually occurs later in life in patients with primary ciliary dyskinesia (Noone et al., 2004; Barbato et al., 2009). Without aggressive treatment of initial *P. aeruginosa* infection most CF patients will develop chronic lung infection (Gibson et al., 2003).

THE MUCOID SWITCH

One hallmark of chronic *P. aeruginosa* CF infection is the emergence of an alginate over-producing, mucoid phenotype, which is associated with worsening of prognosis and an accelerated loss of lung function (Lyczak et al., 2002; Ramsey and Wozniak, 2005). The *algD* operon (PA3540–PA3551) encodes the alginate biosynthetic genes and is under the regulatory control of the alternative sigma factor AlgU (AlgT) (Martin et al., 1993; Hershberger et al., 1995). The *algU* gene is in an operon with the *mucABCD* genes, which play a role in regulating AlgU activity. In particular, the MucA protein acts as an anti-sigma factor specific for AlgU. MucA, in combination with MucB sequesters AlgU at the membrane surface, preventing it forming a RNA polymerase holoenzyme complex and so directing transcription of its target genes (Schurr et al., 1996; Boucher et al., 1997; Rowen and Deretic, 2000). The most common mechanism of mucoid switching by *P. aeruginosa* in the CF lung is through mutation of the *mucA* gene (the *mucA22* or *muc-22* mutation being most

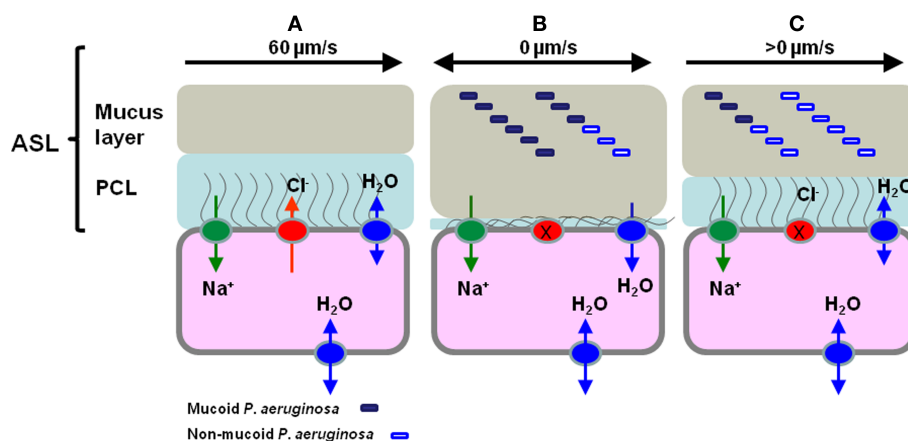


FIGURE 1 | The regulation of airway surface liquid (ASL) volume and *Pseudomonas aeruginosa* phenotype in normal, CF epithelia and CF epithelia following hypertonic saline treatment. (A) In normal epithelia CFTR (red sphere) plays a vital role in regulating the concentration of NaCl on airways surfaces, ensuring correct hydration of the ASL, which is comprised of the mucus and periciliary layers (PCL). **(B)** In the epithelia of the CF lung the PCL is depleted because of defective Cl⁻ secretion due to CFTR mutation and through increased adsorption of Na⁺ ions through ENaC (green sphere), as a consequence of ENaC being locked in the Na⁺ adsorptive mode. This results in continued absorption of water from the airway surface and resulting depletion of ASL, collapse of the PCL and sticky, dehydrated mucus in contact with epithelial cells (Matsui et al., 1998; Boucher, 2007; Livraghi and Randell, 2007).

The lack of PCL lubrication means that cilia cannot beat to move mucus out of the lung and this results in chronic bacterial infection indicated here by non-mucoid (open rectangle) and mucoid (filled rectangle) *P. aeruginosa*. **(C)** Following HS treatment it is proposed that ASL volume increases as it encourages transmembrane water movement and that this leads to a partial rehydration of the PCL and less sticky, partially rehydrated mucus and the possibility of some ciliary beating-mediated mucus clearance. Furthermore, we propose that the increased salinity of the ASL leads to a shift in the *P. aeruginosa* population as consequence of the osmotic sensitivity of the mucoid *P. aeruginosa* and that this population shift impacts on the frequency of infective exacerbations. Note that in this cartoon we do not attempt to distinguish planktonic and biofilm bacteria.

commonly encountered) that prevents the anti-sigma factor from sequestering AlgU, making it available to direct transcription of its target genes including the *algD* alginate biosynthetic operon. This mutation is a single base pair deletion that results in a truncated, dysfunctional MucA protein. AlgU-responsive promoter sequences are found for at least 35 genes and included many stress-induced genes (Firoved et al., 2002; Firoved and Deretic, 2003). The mutation also affects the production of a number of virulence factors (Whitchurch et al., 1996; Carterson et al., 2004; Wu et al., 2004). The selective advantage of mutation-driven alginate production in the CF lung is unclear, but it has been proposed to protect against phagocytosis (Schwarzmann and Boring, 1971) and reactive oxygen species (Mathee et al., 1999). Mucoidity is not an essential requirement for biofilm formation *in vitro*, as alginate is not a major component of non-mucoid *P. aeruginosa* biofilms (Ramsey and Wozniak, 2005), but it is likely that mucoid *P. aeruginosa* are part of the persistent biofilm communities in the CF lung (Hassett et al., 2009; Hoiby et al., 2010). Indeed, exposure to antibiotics triggers transient alginate production *in vitro* (Wood et al., 2006; Hoiby et al., 2010) and alginate-overproduction increases resistance to tobramycin in biofilm cultures (Hentzer et al., 2001). As more than 80% of adult CF patients have mucoid *P. aeruginosa*, mucoid biofilms may act as a reservoir for the infection of previously healthy parts of the lung through biofilm dispersal mechanisms that lead to the release of motile, non-mucoid bacteria (VanDevanter and Van Dalfsen, 2005; Kirov et al., 2007; Bjarnsholt et al., 2010), although this proposal has been challenged (Reid et al., 2006) and mucoid variants have been shown to be seeding dispersal proficient (Kirov et al., 2007). Treatments that would destroy or weaken biofilm structures may be of therapeutic benefit.

TREATMENT OF *P. AERUGINOSA* INFECTIONS

The severity of *P. aeruginosa* infection can be limited by early detection and aggressive antibiotic treatment before the bacteria convert to a mucoid phenotype, but it is extremely difficult to eradicate once established (Doring et al., 2000; Ratjen, 2001, 2006; Davies, 2006; Goss and Burns, 2007; Bendiak and Ratjen, 2009). When chronically infected, the patient will have repeated episodes of acute pulmonary exacerbations characterized by increased breathlessness, sputum production and feeling unwell and a reduction in lung function. Exacerbations are usually treated with a combination of systemic antibiotics (Davies, 2006; Bendiak and Ratjen, 2009; Foweraker, 2009); additionally, antibiotic therapy is supplemented by treatments designed to improve sputum clearance, such as chest physiotherapy and administration of nebulized recombinant deoxyribonuclease (rhDNase), although the later approach is relatively expensive leading to restricted use in many countries (Jones and Wallis, 2003).

HYPERTONIC SALINE TREATMENT

An alternative or supplementary therapy to improve mucociliary clearance either in the context of a long term maintenance therapy or during infective exacerbations is the use of hypertonic saline (HS). It is an inexpensive, straightforward treatment during which nebulized HS (3–7% NaCl), is inhaled as a fine mist through a mask or a mouthpiece; typically, 10 ml of saline is inhaled twice a day (Wark and McDonald, 2009).

In vitro studies first indicated that HS might be a useful as a potential mucotropic approach as it was found to reduce the viscoelasticity and increase the predicted cough clearability of sputum (King et al., 1997). A number of clinical studies followed that

supported the efficacy of nebulized HS in improving lung function in CF (Robinson et al., 1996, 1997, 1999). Two more recent clinical studies have shown clearly the short and long term benefits of nebulized HS. Elkins et al. (2006) reported a double blind, placebo-controlled, parallel group trial over 48 weeks of HS treatment in which 4 ml of HS (7%) was nebulized twice daily to CF patients with the control group receiving 0.9% saline. The rate of change in lung function was recorded as the forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1) and forced expiratory flow at 25–75% of FVC (FEV_{25-75}). While the individual measures did not show significant differences during the treatment period, a composite end point measurement (averaged FEV_1 and FVC across all post-randomization visits) was found to be significantly different between the two groups.

More strikingly, the HS treated group had significantly fewer pulmonary exacerbations and a significantly higher percentage of patients without exacerbations. This is particularly important given that frequency of exacerbations is strong predictor of morbidity and mortality in CF and so a treatment that reduces exacerbations is of major clinical relevance. Interestingly, however, HS treatment did not significantly alter the concentrations or prevalence of either *P. aeruginosa* or common co-infectors *Staphylococcus aureus* in sputum between treated and non-treated groups.

In a shorter but more intensive 2-week trial in which 5 ml of HS (7% NaCl) was administered four times daily a larger increase in pulmonary function was observed (Donaldson et al., 2006). No microbiological studies were done as part of this study.

A recent independent review of all controlled trials (12 in total) assessing HS compared to placebo or other mucolytic treatments concluded that HS does improve quality of life and reduces pulmonary exacerbations and that there was sufficient evidence to recommend using HS on CF patients (Wark and McDonald, 2009). There are side effects of HS therapy that include more frequent coughing, sore throat and chest tightness in some patients, as HS can irritate the airways, and these can lead to patient compliance problems (Elkins et al., 2006). Inhaled mannitol is an alternative non-ionic osmotic agent to HS to promote airway rehydration and to increase mucociliary clearance. It has shown some promising effects on lung function, but its effects on pulmonary exacerbations and lung microbiology has not been tested (Robinson et al., 1999; Jaques et al., 2008; Minasian et al., 2008; Daviskas et al., 2010).

HOW DOES HYPERTONIC SALINE TREATMENT WORK?

The exact mechanism by which HS treatment works is unclear, but several ways as to how it could improve mucus clearance have been proposed: by inducing osmotic flow of water into the mucus layer so improving mucus rheology, through its action directly on the mucus, breaking the ionic bonds leading to lower viscosity and elasticity; and/or by increasing the ion concentration in the mucus and a subsequent reduction of intra and inter-molecular mucus repulsion leading to more compact mucus that could be more effectively cleared (Robinson et al., 1997; Wark and McDonald, 2009).

Donaldson et al. (2006) proposed that HS produced a sustained increase in airway surface hydration and elevation in ASL volume that improves mucociliary clearance from the airways, so protecting relatively non-obstructed airways from exogenous insults that would slow mucus clearance and promote intrapulmonary

spread of bacterial infection. They postulated the involvement of airway aquaporin water channels (AQPs) in the modulation of ASL volume. However, experimental evidence has subsequently been reported against this explanation of the data, indicating the need to identify alternate mechanisms (Levin et al., 2006).

An alternative is that HS treatment acts directly on the infecting bacteria in the CF lung, among which *P. aeruginosa* is usually the most prominent. *P. aeruginosa* motility was shown to be sensitive to NaCl exposure (Havasi et al., 2008) and the study suggests that the clinical benefits attributed to HS treatment might be in part due to these effects on bacterial motility. Although not specifically discussed in the paper, it is reasonable to think that a reduction in flagellin [which serves as a pathogen associated molecular pattern (PAMP) and activates the innate immune response; Morris et al., 2009; Raoust et al., 2009; Campodonico et al., 2010] could lead to a reduction in inflammatory responses in the CF lung. The importance of motility during chronic infection of the CF lung remains uncertain, as *P. aeruginosa* is thought to populate the lung in biofilm-like communities (Moreau-Marquis et al., 2008; Davies and Bilton, 2009; Hassett et al., 2010) and following biofilm establishment the bacteria lose their flagella. However, motility might be involved in mobilization of *P. aeruginosa* to infect new sites in the lung using flagella-based swimming and swarming motility (VanDevanter and Van Dalfsen, 2005; Kirov et al., 2007).

AN ALTERNATIVE HYPOTHESIS TO EXPLAIN THE EFFECT OF HYPERTONIC SALINE TREATMENT ON INFECTIVE EXACERBATIONS

Based on recently published data we propose an alternative hypothesis whereby long term HS treatment leads to a shift in *P. aeruginosa* sub-populations in the CF lung, away from mucoid variants, that are so strongly associated with increased morbidity, to non-mucoid strains.

We recently applied a nuclear magnetic resonance (NMR)-based extracellular metabolomics approach called time resolved metabolic footprinting (TReF; Behrends et al., 2009) to investigate metabolic changes in mucoid *P. aeruginosa* *mucA22* mutants to determine the overall metabolic and physiological changes that are caused by the mucoid switch (Behrends et al., 2010). We found changes in the levels of various metabolites associated with osmotic tolerance, including glycine-betaine, trehalose, and glutamate in the mucoid *mucA22* mutant. Quantitative comparison of the endometabolome of wildtype and isogenic *mucA22* mutant cells showed differences in the accumulation of osmo-protectants, suggesting a switch in osmo-protectant preference from glycine-betaine to trehalose in the *mucA22* mutant. Subsequent physiological experiments showed that the *mucA22* mutant was significantly more sensitive to osmotic stress than the isogenic parental *P. aeruginosa* PA01 strain. The effect was tested over a concentration range of 0.2–0.8 M NaCl with mucoid *P. aeruginosa* showing increased sensitivity over the complete range (Behrends et al., 2010). The *mucA22* mutant was also more sensitive to the alternative osmotic stressor sucrose, showing it was not just an effect of NaCl.

Ambient NaCl concentrations in the CF lung are in the region of 0.15 M (Smith et al., 1996), so while the concentration that NaCl reaches in ASL or sputum following HS treatment is not known, it is likely that *P. aeruginosa* will encounter osmotic stress when NaCl at

a concentration of between 0.5 and 1.2 M is added during HS treatment. Strikingly, the *mucA22* mutation affected osmotic tolerance only in the stationary phase of growth in laboratory culture, where it had a growth inhibitory effect on the *mucA22* mutant independent of the presence of osmo-protectants in the medium (Behrends et al., 2010). This is in contrast to previously described osmotic stress responsive systems that have an effect on growth in exponential phase and can be rescued by addition of osmo-protectants like glycine-betaine (Aspedon et al., 2006). This is an intriguing observation as it links the effects of the switch to mucoidity to stationary phase physiology. Following initial colonization *P. aeruginosa* is likely to find itself mostly in a biofilm like structure in which a significant fraction of the cells will spend much of their time in a non-growing state, dividing infrequently and so physiologically resemble the stationary phase of the planktonic growth (Davies and Bilton, 2009; Bjarnsholt et al., 2010; Hassett et al., 2010).

While the mucoid shift in *P. aeruginosa* that occurs in most adult patients is well documented, it is rarely a complete takeover and the most common situation is the co-existence of mucoid, non-mucoid and other morphotypes in the CF lung population. We hypothesize that the increased osmotic sensitivity of mucoid *P. aeruginosa* could explain part of the beneficial effects of HS treatment on CF patients. We argue that prolonged HS treatment will select against osmotically sensitive mucoid strains and in favor of the greater osmotically resistant non-mucoid strains (Figure 1).

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