REVIEW

Inhaled hypertonic saline for cystic fibrosis: Reviewing the potential evidence for modulation of neutrophil signalling and function

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Abstract

Cystic fibrosis (CF) is a multisystem disorder with significantly shortened life expectancy. The major cause of mortality and morbidity is lung disease with increasing pulmonary exacerbations and decline in lung function predicting significantly poorer outcomes. The pathogenesis of lung disease in CF is characterised in part by decreased airway surface liquid volume and subsequent failure of normal mucociliary clearance. This leads to accumulation of viscous mucus in the CF airway, providing an ideal environment for bacterial pathogens to grow and colonise, propagating airway inflammation in CF. The use of nebulised hypertonic saline (HTS) treatments has been shown to improve mucus clearance in CF and impact positively upon exacerbations, quality of life, and lung function. Several mechanisms of HTS likely improve outcome, resulting in clinically relevant enhancement in disease parameters related to increase in mucociliary clearance. There is increasing evidence to suggest that HTS is also beneficial through its anti-inflammatory properties and its ability to reduce bacterial activity and biofilm formation. This review will first describe the use of HTS in treatment of CF focusing on its efficacy and tolerability. The emphasis will then change to the potential benefits of aerosolized HTS for the attenuation of receptor mediated neutrophil functions, including down-regulation of oxidative burst activity, adhesion molecule expression, and the suppression of neutrophil degranulation of proteolytic enzymes.

Key words: Cystic fibrosis; Hypertonic saline; Mucociliary clearance; Neutrophils and inflammation

Core tip: The pathogenesis of lung disease in cystic

fibrosis (CF) is characterised by decreased airway surface liquid volume and subsequent failure of normal mucociliary clearance. Therapies acting against airway mucus in CF include aerosolized hypertonic saline (HTS). It has been shown that HTS aids mucociliary clearance by restoring the liquid layer lining the airways. However, recent studies are beginning to broaden our view on the beneficial effects of HTS, which now extend to include anti-inflammatory properties. This review aims to discuss the therapeutic benefits of HTS and to identify the potential benefits of aerosolized HTS for attenuation of neutrophil function.

INTRODUCTION TO CYSTIC FIBROSIS AND THE ROLE OF NEUTROPHILS IN DEVELOPING AIRWAYS DISEASE

Cystic fibrosis (CF) is a complex genetic disease leading to increased risk of chronic lung disease resulting in terminal respiratory failure[1,2]. CF is an autosomal recessive disorder caused by mutations in the CF transmembrane conductance regulator (CFTR) chloride channel. Over 1900 CFTR mutations leading to defective chloride transport have been identified to date[3] and result in misfolding of the CFTR protein. Reported mutations can be categorised into different classes depending on whether the mutation alters CFTR processing (Classes I, II and V) or results in dysregulated chloride secretion (Classes III, IV, VI) (Figure 1). The most common mutation is deletion of phenylalanine at position 508 (ΔF508) which occurs in approximately 70% of patients with CF, and 90% of CF suffers carry one copy[4]. Defects in CFTR protein function not only impact upon cAMP-dependent chloride secretion but also result in increased epithelial sodium channel (ENaC)-mediated ion absorption in the superficial airway epithelium[5,6]. CFTR absence or malfunction causes increased water re-absorption across airway epithelial cells leading to dehydration of the airway surface liquid (ASL) layer, persistent mucus hypersecretion and airflow obstruction[7]. Dehydration of the ASL layer and mucus accumulation has been implicated in exacerbated airway inflammation[8] and decline in lung function predicts significantly poorer outcomes[9]. Therapeutic interventions to improve mucus clearance is a necessary treatment in CF[10]. Hypertonic saline (HTS) at concentrations of 3% or higher is widely used to improve mucociliary clearance,

as this increases the tonicity of the ASL creating an osmotic gradient drawing water into the airway hence improving ASL and facilitating removal of airway secretions (Figure 2). Furthermore, HTS improves lung function and quality of life and significantly decreases the frequency of exacerbations[11,12] and is generally well tolerated.

When considering the different immune cells present in the CF lung it has been documented that neutrophils account for approximately 60%-70% of immune cells in CF airway samples[13]. Key studies have demonstrated that infiltration of neutrophils into the airways occurs early in the course of CF lung disease and that neu-trophil-released granule proteins, particularly neutrophil elastase (NE), play a crucial pathological role[14,15]. The expression of functional CFTR on the plasma membrane of neutrophils has been the topic of great debate[16-19] with studies specifying intrinsic abnormalities due to a lack of CFTR function[20,21]. Reports on reprogrammed cell activity secondary to chronic bacterial infection and inflammation have also been documented[22]. Moreover, persistent mTOR and CREB pathway activation in CF airway neutrophils[23] and augmented cell surface nutrient transporter expression are consistent with metabolic adaptation[24].

Regardless of the cause of impaired neutrophil activity however, the fundamental consequence is neutrophil mediated tissue proteolysis. Excessive neutrophil recruitment to the lung, results in prolific NE degrading protease activity and inflammation that can eventually become chronic and lead to tissue destruction. The recognition that aerosolized HTS may moderate neutrophil cytotoxicity and may function to restrain an exuberant inflammatory response in CF, provides a possible strategy for mitigating inappropriate neutrophil activity. This review will initially describe the use of HTS in treatment of CF and then extend the focus of HTS beyond mucociliary, to the potential benefits of aerosolized hyperosmolar therapy for the modulation of neutrophil activity within the confines of the CF airways. Our review of the literature was carried out using the MEDLINE database (from 1976 to the year 2014), Google Scholar and The Cochrane Library databases using several appropriate generic terms.

CLINICAL EFFICACY OF NEBULISED HTS IN CF

The use of HTS treatments has been shown to improve mucus clearance in CF and impact upon exacerbations, quality of life and improve lung function[12]. Early studies demonstrated an acute dose-response relationship between inhaled saline concentration and mucociliary clearance[25], with short-term HTS administration improving mucociliary clearance and lung function with acceptable tolerability[26]. In 2006, the National Hypertonic Saline in Cystic Fibrosis Study Group provided the first evidence for the long-term efficacy
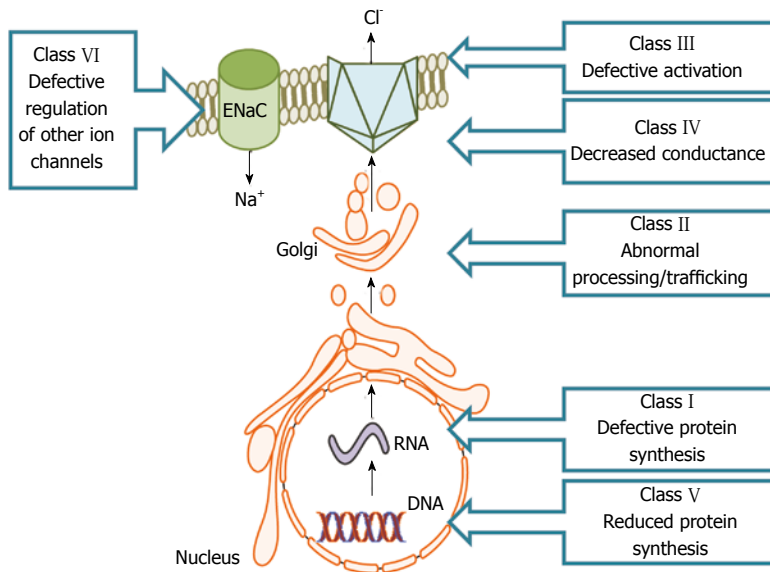
Figure 1 Classification of cystic fibrosis transmembrane conductance regulator mutations. CFTR mutations are classified into six groups according to their effect on CFTR function. Class I mutations affect biosynthesis, while class II mutations affect protein processing. Milder mutations such as class II to Class V impair CFTR channel function. CFTR: Cystic fibrosis transmembrane conductance regulator.

of HTS in individuals with CF. The study randomised 164 patients with CF to receive HTS (7%) or isotonic (0.9%) saline for 48 wk. Using forced vital capacity and forced expiratory volume in 1 s (FEV1) to assess the rate of change of lung function, no significant difference was observed between the two groups, but there was a statistically significant difference in the absolute change in lung function. More importantly, this study demonstrated an impressive reduction in the frequency of exacerbations in the HTS group, with fewer days missed from work or school. Furthermore, significant improvements in quality of life were observed, particularly with regard to mental health on quality of life questionnaires after long-term HTS therapy[12].

A further study by Donaldson *et al*[26] showed that repeated use of 7% HTS generated both acute and sustained improvements in mucociliary clearance while improving FEV1 following four-times-daily treatment for 14 d, when compared to HTS given in conjunction with the ENaC inhibitor amiloride, however this study lacked a 0.9% saline control group, and as a result the effect of HTS could only be compared to patient baselines. Robinson *et al*[27], in a study employing radioaerosol technique, examined the acute effect of a single administration by aerosolization of 7% HTS, amiloride, or a combination of HTS and amiloride, or a 0.9% saline control. Results demonstrated that treatment with HTS alone significantly increased mucociliary clearance compared to treatment with HTS/amiloride combined, and both of these therapies were in turn significantly more effective than isotonic saline or amiloride alone.

The efficacy of HTS in improving mucociliary clearance may also be related to the volume administered as studies of 4 mL or 5 mL aerosolized HTS[12,26] recorded smaller improvements in lung function compared to a 10 mL volume[11,28]. In 2011, Dmello *et al*[29] used a multivariate logistic regression analysis to assess 340 CF exacerbations, 99 of them involving treatment with HTS. The results confirmed the beneficial effect of HTS with regard to reduction of pulmonary exacerbation

frequency, even in those with "severe" CF lung disease, categorised as those with an FEV1 below 40%. A further study, on the use of HTS during hospitalization for adult exacerbations of CF showed that nebulized treatment accelerated the recovery of FEV1 to base-line[30]. However, there is conflicting evidence on the effectiveness of HTS upon lung function and FEV1 and a Cochrane review summarising all clinical trials of HTS in CF demonstrated a significant but minimal increase in FEV1 with a mean change of 4.15% after 4 wk, however at 48 wk this was not significant and was reduced to 2.31%[31].

While spirometry, primarily FEV1, represents the measure of lung function used in the majority of HTS studies to date, the use of lung clearance index (LCI), a measure of ventilation inhomogeneity derived from the multiple breath washout test, is increasingly being employed for the early detection of CF respiratory disease[32]. LCI has been shown to be a better predictor of later lung function abnormalities than FEV1[33] and also correlates well with structural changes[34,35]. LCI has been shown to detect treatment responses to HTS in children with CF aged 6-18 years who have normal baseline spirometry[36]. It should be noted that while these studies when analysed together formed the basis for HTS use in the majority of CF centres in Europe and North America, the data for the most part only apply to adults, with a relative lack of evidence for use in children. Studies of HTS use in the CF child population have shown satisfactory safety and tolerability profiles[37-39], but it is still unclear as to whether or not HTS treatment confers a clinical benefit upon this group. This may be in part due to the fact that younger individuals typically have less-advanced lung disease, nonetheless it is still well tolerated even in very young children aged between 12 and 30 mo[38]. Although there is good evidence to suggest that HTS is of benefit regarding the enhancement of mucociliary clearance in adults, one study of HTS in CF children aged between 7-14 years published by Laube *et al*[40] demonstrated only
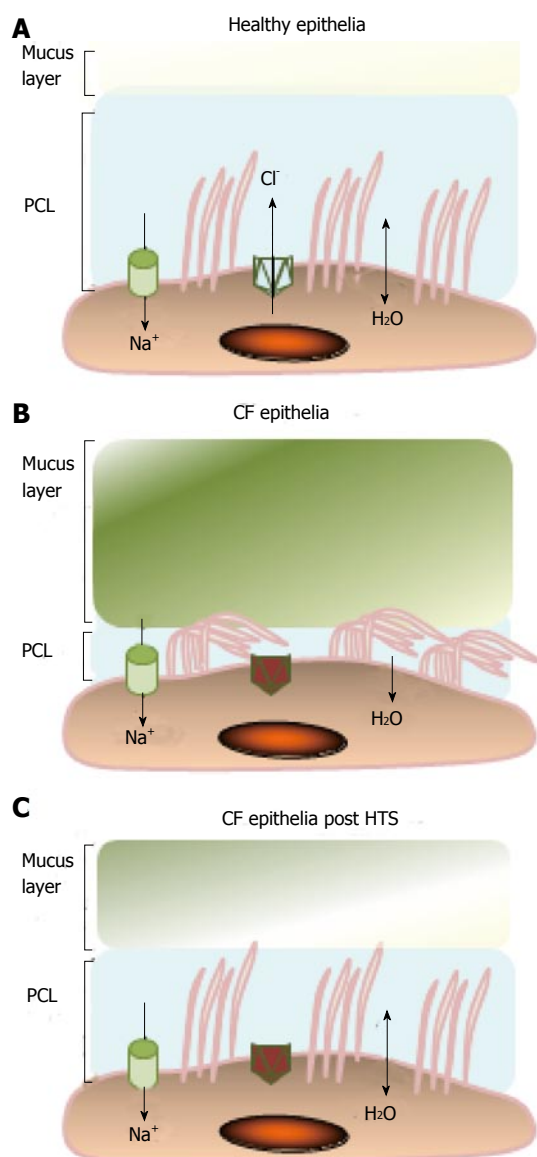
Figure 2 Effects of hypertonic saline on the airway surface liquid in cystic fibrosis. A: In healthy airway epithelia, CFTR plays a vital role in regulating hydration of the airway surface liquid (ASL) constructed of the periciliary layer (PCL) and the mucus layer; B: Due to defective CFTR in individuals with CF, Cl⁻ secretion is impaired and Na⁺ absorption through ENaC is increased resulting in dehydration of the ASL and accumulation of thick mucus causing reduced PCL height; C: Treatment with hypertonic saline assists osmosis of water into the ASL and thus rehydrates the mucus and partially restores the PCL allowing for easier clearance of mucus. CFTR: Cystic fibrosis transmembrane conductance regulator.

negligible acute clearance effects, however, it should be noted, that this was a single-dose study. A recent trial, from the North Carolina group at Chapel Hill, of HTS in CF children with normal lung function has shown some interesting results. This trial compares 6% HTS to 0.12% saline, with both arms of the study receiving 4 mL three times daily for four weeks. While mucociliary clearance was largely unaltered at 2 h after the initial dose, a significant acceleration of mucociliary clearance lasting greater than 12 h following the final dose was observed[41]. This sustained effect suggests that single-dose studies may not be ideal predictors of mucociliary

clearance in these individuals. A further study, by Amin et al[36] using LCI to evaluate ventilation heterogeneity in individuals aged between 6 and 18 years with CF with normal spirometry, demonstrated a significant improvement in ventilation after four weeks of HTS treatment. Moreover, recent evidence has demonstrated that HTS is also beneficial through its ability to reduce *Pseudomonas aeruginosa* activity[42] and also to disrupt biofilm formation[43].

TOLERABILITY OF HTS IN CYSTIC FIBROSIS

Although an acute dose-response relationship between inhaled saline concentration and mucociliary clearance exists, data showing better or worse clinical efficacy with concentrations other than 7% are lacking. In this regard most clinical trials show that both 3% and 7% HTS are more effective than placebo[31], however one clinical trial in a paediatric population demonstrated a superior effect with 3% HTS. In this study, the 3% group had significantly higher FEV1 on day 14 and day 28 compared to the group receiving 7%[44], however this study was not extended beyond 28 d, so it is unclear whether there is a truly superior dose, and the majority of trials have employed 7% HTS. Moreover, the percentage of HTS administered not only has implications for clinical efficacy, but also for patient adherence, since as doses increase (from 3% to 7%), so do nebulisation times, taste and tolerability, all important factors for compliance[45]. A 1997 study by Robinson et al[25] showing increasing levels of sputum clearance with increasing concentrations of saline also noted that factors such as cough and oropharyngeal irritation increased in tandem with sputum clearance, and were highly disconcerting at concentrations approaching 12%, setting the ceiling of tolerability for the study. Tolerability is often a key determinant of the dose selected for an individual patient, with pre-treatment with bronchodilators aimed at facilitating a higher concentration. Roughly 5% of CF patients undergoing treatment with HTS will experience bronchospasm severe enough to restrict use[8]. A commonly-used starting point for HTS is 7%, with bronchodilator pre-treatment, and with the willingness to down-titrate should patient comfort be sufficiently compromised. Administration of 7% HTS in conjunction with 0.1% hyaluronic acid *via* the aerosolised route has been shown to significantly improve tolerability and pleasantness when compared with 7% HTS alone[46].

EFFECT OF HTS ON LEVELS OF AIRWAY INFLAMMATORY MEDIATORS INVOLVED IN NEUTROPHIL RECRUITMENT AND ACTIVATION

Circulating neutrophils are initially found in a resting

state, and become primed upon exposure to chemo-tactic stimuli comprising pathogenic molecules such as *N*-formyl peptides, cytokines including tumour necrosis factor-α (TNF-α) and chemokines including interleukin (IL)-8[47]. The release of cytokines and chemokines including IL-8 and granulocyte macrophage colony stimulating factor (GM-CSF) by CF epithelial cells functions to signal to circulating immune cells resulting in increased numbers of neutrophils and macrophages localized to the airways[48,49]. IL-8 binds to the chemokine (C-X-C motif) receptor 1 (CXCR1) and CXCR2 on the plasma membrane of neutrophils resulting in cell adhesion[50] and migration[51]. In turn, synthesis and release of TNF-α and IL-1β by recruited macrophages, and NE induced secretion of IL-8 and IL-6 by upper airway epithelial cells, perpetuate the cycle of inflammation[52,53]. In addition, NE activity in BAL fluid is associated with early airways disease in children with CF[54] and both NE and TNF-α up-regulate leukotriene B$_4$ (LTB$_4$) production by macrophages[55,56], the latter a potent lipid inflammatory mediator. It has also been documented that CF lung epithelial cells release IL-8 in the absence of pathogens suggesting a persistent pro-inflammatory state[13,14]. Moreover, upon bacterial challenge studies have shown that the level of IL-8 released in response to infection is significantly increased in CF airway epithelial cells compared to CFTR sufficient cells and this has in part been explained by the plasma membrane surface expression of asialoganglioside 1 and toll-like receptor 4[57,58].

Observations of increased cell migration and neutrophil-dominated chronic airway inflammation at an early age in children with CF[59], supports the need for potential therapies that may target airway inflammatory mediators of neutrophil priming and migration. In this regard the ability of HTS to act as an anti-inflammatory, or alternatively pro-inflammatory agent, was studied by Chan *et al*[60]. IB3-1 bronchial epithelial cells containing the DF508/W1282X CF mutation were exposed to increasing concentrations of HTS *ex vivo* and secreted IL-8 levels were quantified. Results revealed that CFTR mutated bronchial epithelial cells produced an exaggerated level of both basal and NaCl-induced IL-8 production, indicating that HTS was acting as a pro-inflammatory stimuli[60]. However, the highest concentration of HTS employed in this study was 125 mmol/L, which is in contrast to the therapeutic concentration of HTS used *in vivo* (513 mmol/L; 3%). Nevertheless, this effect of HTS was echoed by studies that demonstrated that hyperosmolar solutions stimulated cytokine production by bronchial epithelial cells *via* p38 mitogen-activated protein kinases activation[61] and in CF bronchial gland cells *via* the NF-κB pathway[62]. Similarly, a study carried out by Shapiro *et al*[63] demonstrated that human peripheral blood mononuclear cells exposed to increasing concentrations of NaCl in combination with bacterial lipopolysaccharide or IL-1 exhibited increased protein expression of IL-8, IL-1β and TNF-α.

HTS continues to be used as a therapy available for the treatment of patients with CF[64] and in contrast to the HTS-induced increased expression of IL-8 in *in vitro* studies, a number of *in vivo* studies have measured IL-8 levels following HTS treatment. These included a long term controlled trial of inhaled HTS in patients with CF, compared to inhaled isotonic saline, with no significant difference in sputum IL-8 levels found between the groups[12]. Two further studies also investigated IL-8 levels in CF sputum post HTS (3% and 7%) nebulisation, with results showing no significant alteration in IL-8 levels[65,66]. Moreover, an investigation designed to assess the effect of 7% HTS on airway inflammation in CF, with outcome measurements including altered IL-8, myeloperoxidase (MPO) and NE levels, revealed no increase in free IL-8 and the study did not support the capacity of HTS to promote inflammation in CF[67]. Furthermore, in human pulmonary microvascular endothelial cells the ability of increasing concentrations of HTS (ranging from 140 mmol/L to 170 mmol/L NaCl) to significantly reduce TNF-α-induced IL-8 release was established[68]. However, the concentration of HTS utilised was far below that used therapeutically. More recently, the functionality of HTS in reducing levels of IL-8 bound to glycosaminoglycans (GAGs) within the CF airways was observed. Within the CF airways, the dehydrated thick mucus contains raised levels of anionic GAGs formed on the surface of bronchial epithelial cell[69], the most abundant including heparan sulphate (HS) and chondroitin sulphate (CS)[70,71]. Of major importance, increased quantities of GAGs have been found in airway samples from individuals with CF[72]. The immobilization of IL-8 by GAGs plays a major role in the establishment of gradients of the chemokine that contribute to the recruitment of neutrophils during inflammatory exacer-bations[73]. The use of an IL-8 decoy (PA401) with enhanced GAG binding ability[74], or the removal of HS and CS lead to a significant reduction in the detection of this chemokine[75]. Moreover, disruption of this interaction with increasing ionic concentrations (7% HTS) displaces IL-8 from GAGs, subjecting the former to clearance by proteolytic activity by NE[76] (Figure 3). Although only a small number of patients were recruited to this latter study, and the effect of HTS on other immunomodulatory mediators in the CF airways was not evaluated, results are in line with the ability of aerosolized HTS in an animal model of acute lung injury to reduce levels of the murine analogue of IL-8, cytokine-induced neutrophil chemoattractant-1, by 44%[77].

ABILITY OF HTS TO IMPACT UPON NEUTROPHIL ADHESION AND MIGRATION

Pro-inflammatory stimuli, either individually or in com-bination, can stimulate the neutrophil to change mor-
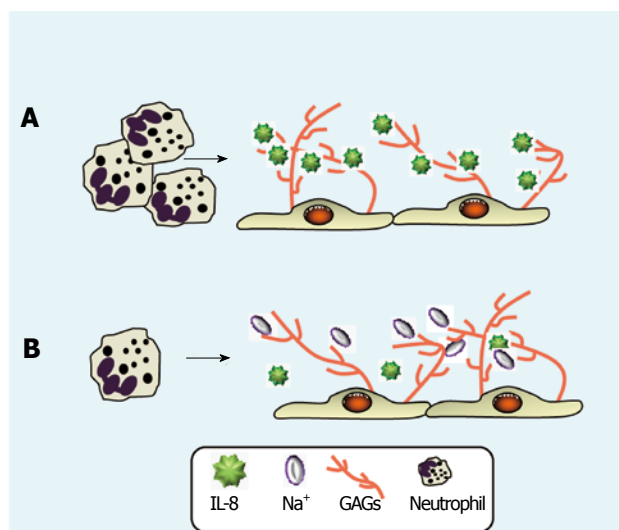
Figure 3 Hypertonic saline reduces levels of interleukin-8 in cystic fibrosis airway samples thereby reducing neutrophil migration. A: The chemokine IL-8 is a key mediator of inflammation in patients with CF and increases neutrophil migration to the airways. GAGs possess the ability to influence the chemokine profile of the CF lung by binding IL-8 and protecting it from proteolytic degradation; B: HTS functions to disrupt IL-8: GAG complexes, rendering the chemokine susceptible to proteolytic degradation. Clinical application of HTS may serve to decrease the inflammatory burden in the CF lung *in vivo*. CF: Cystic fibrosis; GAGs: Glycosaminoglycans; HTS: Hypertonic saline; IL-8: Interleukin-8.

phology and migrate to the airways, the latter being a multistep process. Initially, after a chemotatic signal is received, the neutrophil reversibly binds to the vascular endothelium through the interactions between P-selectin and E-selectin found on the epithelium, with L-selectin expressed on the neutrophil surface. Rolling of neutrophils involves interaction between these selectins and glycoproteins such as P-selectin glycoprotein ligand (PSGL1) which is expressed by the endothelium and leukocytes. This mediated rolling of the cell allows new bonds to form before breaking of older bonds and shedding of L-selectin[78]. This slow rolling then allows for tighter bonds to form between β2 integrins expressed on the neutrophil surface including CD11b/CD18 and the corresponding ligands, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2. Once neutrophils have adhered to the endothelial wall, tight junctions between endothelial cells become loose and allow transmigration. Neutrophils then follow a gradient of immobilised chemoattractants and travel to the airways along collagen and elastin fibres[78] and movement through the extracellular matrix is facilitated by release of proteolytic enzymes including metalloproteases and NE[79,80].

The capacity of HTS to reduce neutrophil migration as a result of lowering levels of the potent neutrophil chemoattractant IL-8 has been investigated. In this regard, the consequence of disruption of interactions between IL-8 and GAGs within the CF lung was addressed by assessing the chemotactic potency of sputum *ex vivo* following nebulized HTS

treatment, with results demonstrating a reduction in the neutrophil chemotactic index[76]. Although IL-8 is a major chemotactic factor in CF, it is not the only chemoattractant found in the CF airways. Thus this latter study should be extended to evaluate the effect of HTS on additional chemoattractants including levels of formyl peptides, C5a[81], and the more recently described chemotactic peptide, proline-glycine-proline[82]. Nevertheless, in agreement with these latter findings, Aitken *et al*[65] showed that the percentage of neutrophils in liquefied sputum samples significantly decreased post HTS (3%) nebulization. Moreover, recent data indicates that HTS can inhibit platelet activating factor (PAF) stimulated cell adhesion. In this regard, exposure of neutrophils to PAF characteristically leads to increased CD11b/CD18 surface expression, and adhesion of PAF activated neutrophils was significantly inhibited by pretreatment with HTS, indicating that HTS may influence functional changes in neutrophils[68]. This concept is further supported by a study demonstrating that HTS considerably reduced neutrophil chemotaxis in response to zymosan-activated serum[83]. Moreover, HTS treatment decreased the number of neutrophils migrating to the airways in a rat model[84], and has been shown to reduce neutrophil adhesion and rolling in a murine model[85]. Although this latter study did not evaluate the neutrophil plasma membrane surface expression of either L-selection or CD11b, diminished levels of both adhesion molecules in response to HTS had previously been documented[86,87]. Moreover, while the use of animal models provides in-depth information on the efficacy of HTS usage, they are not representative of human disease and in particular the use of murine models in the study of CF is limited, as CF mice fail to develop spontaneous lung disease or chronic bacterial infection[88].

IMPACT OF HTS ON NEUTROPHIL CELLULAR PROCESSES INCLUDING NADPH OXIDASE ACTIVITY AND DEGRANULATION

The process of neutrophil mediated bacterial clearance can be divided into two main procedures, those that are oxygen independent, and those that are oxygen dependent. These two cell processes are tightly regulated, and upon dysregulation, can result in release of reactive oxygen species and proteolytic enzymes to the surrounding lung tissues, as occurs in CF. Reactive oxygen species are produced by reduction of consumed oxygen. This reaction is catalysed by the NADPH oxidase, an enzyme complex that consists of two membrane proteins, p22^{phox} and gp91^{phox}, that constitute the heterodimeric flavoprotein cytochrome b$_{558}$, and four cytosolic components p67^{phox}, p47^{phox}, p40^{phox} and p21^{rac} (Figure 4). In the resting neutrophil the majority of membrane-associated flavoprotein
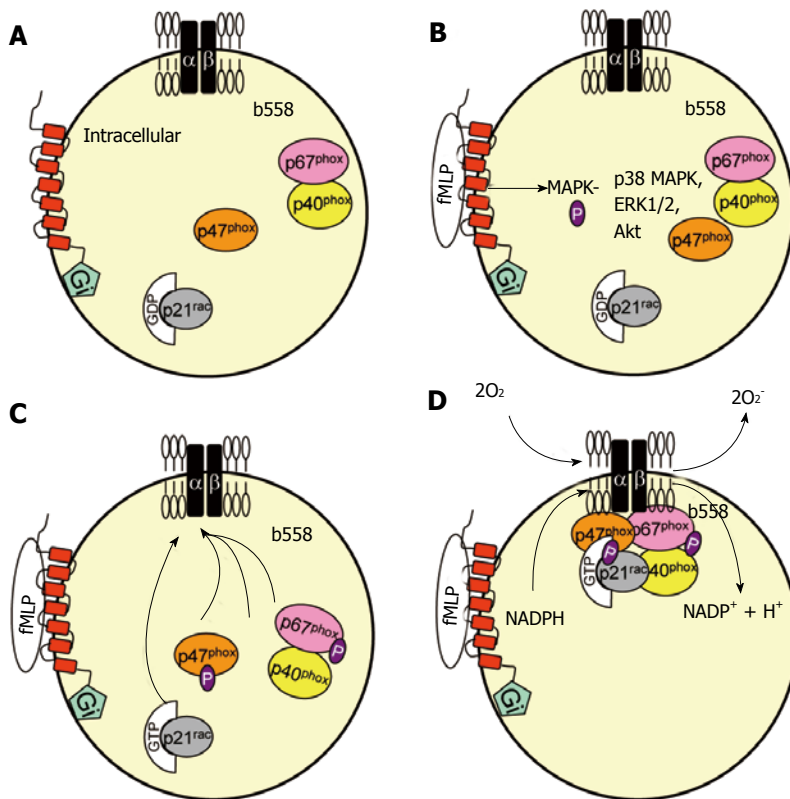
Figure 4 Schematic illustration of the NADPH oxidase of resting and formyl peptides activated cells. The neutrophil NADPH oxidase generates superoxide (O_2^-) and secondary oxygen-derived toxic products in response to bacteria or a variety of soluble stimuli (fMLP). A: The enzyme is dormant in resting neutrophils. The active site of this enzyme is located in an integral membrane cytochrome, b_{558}, which consists of the two subunits $gp91^{phox}$ and $p21^{phox}$ (subunits); B: Stimulation of the neutrophil by fMLP induces activation and phosphorylation (P) of a number of kinases including Akt; C: $P21^{rac}$ is converted into the active GTP-bound form and the phosphorylation of the cytosolic components ($p67^{phox}$, $p47^{phox}$ and $p40^{phox}$) occurs; D: These subunits then translocate to the membrane where they interact with cytochrome b_{558} to initiate reactive oxygen species production. fMLP: Formyl peptides.

cytochrome b_{558} is localised to secondary granules and the plasma membrane, whereas components $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ are localised within the cytosol together with GDP-bound $p21^{rac}$. Upon priming of the neutrophil with proinflammatory stimuli including fMLP or TNF-, partial assembly of the NADPH oxidase occurs involving phosphorylation of $p67^{phox}$ and $p47^{phox}$ followed by translocation to the flavocytochrome. The NADPH oxidase complex becomes fully assembled upon recruitment of GTP-bound $p21^{rac}$. Upon assembly, the active oxidase reduces NADPH and electrons are transferred *via* the flavocytochrome across the membrane to oxygen creating superoxide (O_2^-), which dismutates to hydrogen peroxide (H_2O_2). H_2O_2 generated during the oxidative burst has limited bactericidal properties and the best-defined function of H_2O_2 in the antimicrobial activities of neutrophils comes from the function of H_2O_2 as a substrate for MPO in the presence of halides [chloride (Cl^-)], resulting in the formation of hypochlorous acid (HOCl). HOCl is the most bactericidal oxidant known to be produced by neutrophils and as Dakin's solution, was extensively used in medicine in the treatment of topical wounds until antibiotics became available. Conversely, neutrophil-derived reactive oxygen species have been implicated in activation of NF-κB, release of pro-inflammatory mediators, inhibition of

apoptosis and recurring DNA damage[89].

The second mechanism contributing to bacterial killing is mediated by enzymes stored in granules (Figure 5). Essential serine proteases stored in primary granules include NE, cathepsin G and proteinase 3. Each azurophilic granule contains 5.33 mmol/L NE, corresponding to approximately 67000 molecules per granule. NE, protease 3 and cathepsin G, are found in similar amounts and distribution in neutrophils[90], however much of the research has focused on NE as it is the main mediator of proteolysis (Figure 6). As NE up-regulates expression of *other proteases* it has been suggested that neutralization of NE activity is central to reducing the overall protease burden within the airways[91]. In addition, NE plays a central role in activation of matrix metalloproteinases (MMPs) which are synthesized in an inactive zymogen precursor form[92]. For example, MMP-9 which exists as a pro-form, exhibits a molecular mass of 92 kDa which is cleaved by NE into an active molecule that is 72-kDa in size[93]. In turn, increased levels of active MMP-9 can lead to the increased production of chemotactic peptides[94] and extensive airway remodelling and inflammation[95,96]. Thus, of the serine proteases, NE is the most harmful in the lung[97] and it has been proposed as a target for therapeutic intervention in
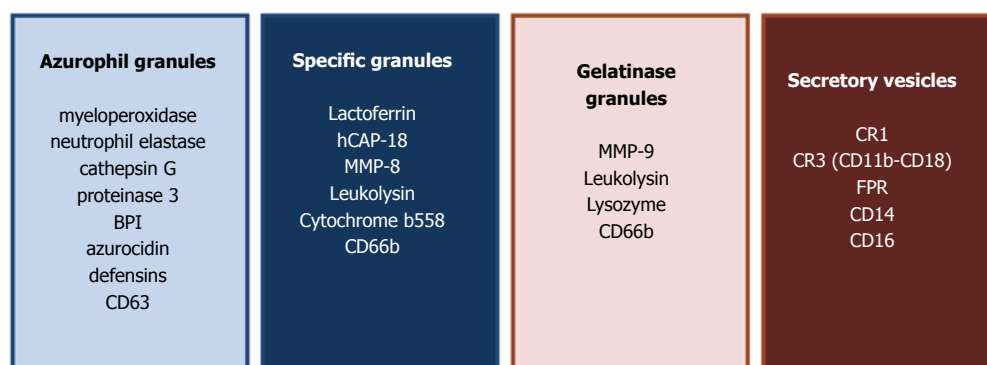
Figure 5 Components of neutrophil granules. The second mechanism of bacterial killing is mediated by enzymes stored in granules. Essential serine proteases stored in primary granules include NE, cathepsin G and proteinase 3. Other bactericidal proteins of primary granules include defensins, azurocidin and bactericidal permeability-increasing (BPI) protein, which mutually function to destabilise bacterial membranes. Additional antibacterial proteins stored in secondary or specific granules include lactoferrin, the 18-kDa human cathelicidin antimicrobial protein (hCAP18) and lysozyme. Lactoferrin, an iron binding protein displays antimicrobial properties by limiting iron availability and exhibits direct antmicrobial and antifungal properties independent of iron-binding. LL-37, the CX-terminal peptide of hCAP-18, disrupts the integrity of bacterial membranes and can neutralise bacterial endotoxins. The gelatinase or tertiary granules contain mainly gelatinase (MMP-9) whose main function is to degrade type V collagen in the extracellular matrix to aid neutrophil migration. In addition to these three granule types, neutrophils also contain secretory vesicles that contain a reservoir of essential receptors and integrins. All are degranulated to the outside of the cell or into the phagocytic vacuole.
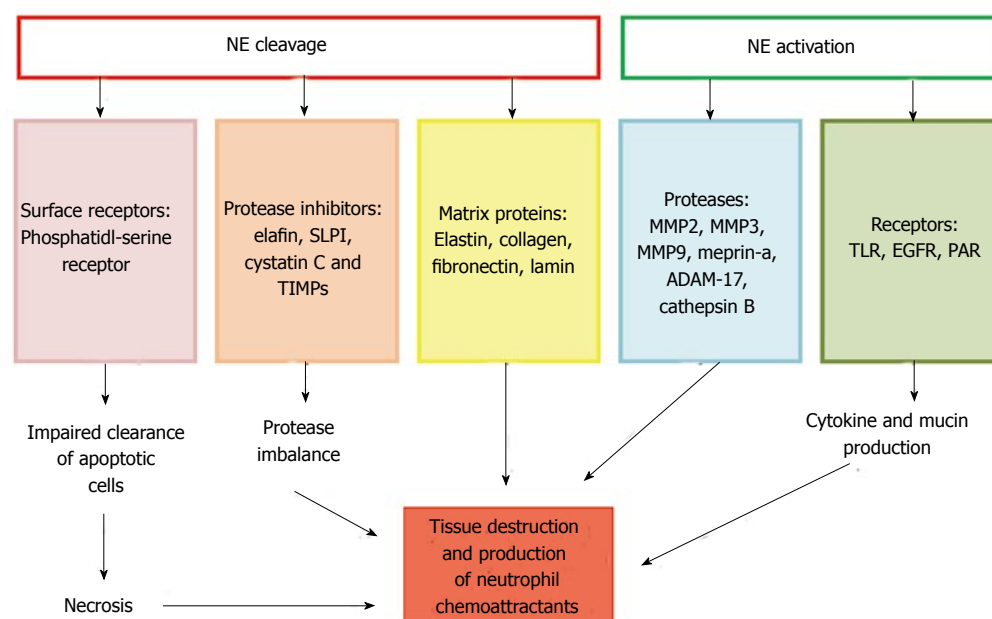
Figure 6 Potential effects of active neutrophil elastase in cystic fibrosis. Excessive NE activity can lead to proteolysis causing protease-antiprotease imbalance by cleaving protease inhibitors. Cleavage of matrix proteins and surface receptors leads to tissue damage and prolonged immune response, respectively. NE can further activate pro-inflammatory molecules (MMPs and ADAM-17) and receptors. These cumulative effects exacerbate tissue destruction and hyper-inflammation. NE: Neutrophil elastase; CF: Cystic fibrosis; MMPs: Matrix metalloproteinases.

CF[98,99]. Unopposed NE proteolytic action can degrade molecules important in control of inflammation including receptors[100], particularly those required for clearance of apoptotic neutrophils[101] or bacterial phagocytosis[102,103]. NE can also inactivate and degrade antiproteases including elafin[104], alpha-1 antitrypsin and secretory leukocyte inhibitor[105]. As a consequence of the proteases/antiprotease imbalance, lung tissue is irreversibly damaged, dramatically reducing lung function and ultimately causing respiratory failure[106]. In short, HTS therapies that may modulate exuberant oxidase and degranulation activity may be used as

powerful anti-inflammatories within the setting of CF airways disease.

A number of *in vitro* investigations have documented that sodium chloride slows neutrophil activity[107] and a study evaluating the effect of HTS on the mechanisms of activation of the NADPH oxidase revealed that stimulated translocation of p67^{phox} to the neutrophil membrane in response to PAF was prevented. Moreover, in *in vitro* cell-free oxidase assays, the membrane content of p67^{phox} post PAF activation was increased in support of oxidase activity, whereas control unstimulated and HTS-PAF activated membranes contained equiva-

lent p67[phox] protein content[108]. Although these results support the potential of HTS to modulate oxidase activity, the concentration of HTS utilised was 180 mmol/L, which is below therapeutic HTS and therefore higher concentrations of HTS should be investigated to determine the effect on p67[phox] membrane translocation. Nevertheless, the inhibitory effect of HTS on a second stimuli involving fMLP-induced NADPH oxidase was also confirmed. The described inhibition occurred in a dose-dependent manner with results indicating that transient increases in osmolality caused prolonged suppression of neutrophil O_2^- production to the outside of the cell, as measured by cytochrome c reduction[83]. The mechanism of inhibition was explored and shown to involve blockade of mitogen activated protein kinase (MAPK) ERK 1/2 and p38 signalling[83]. Moreover, H_2O_2 production to the outside of the cell post fMLP activation was equally reduced by two concentrations of HTS ([Na^+] = 180 mmol/L and 200 mmol/L)[109]. In contrast however, and of major importance, intracellular formation of reactive oxygen species upon bacterial phagocytosis was potentiated with increasing osmolar strength[110]. Despite osmotic down-regulation of p38 and ERK-1, this later study demonstrated enhanced intracellular O_2^- generation in response to bacterial challenge suggesting that HTS may attenuate tissue injury by compromising neutrophil cytotoxic capacity, and additionally appears to enhance the response to bacteria. This may be a further beneficial role of HTS when aerosolized clinically in CF[110].

With respect to the ability of HTS to modulate the degranulation process, Junger *et al*[83] demonstrated that neutrophils exposed to > 50 mmol/L HTS alone released increased levels of MPO and NE, however, when cells were exposed simultaneously to inflammatory levels of fMLP and increasing concentrations of HTS, as would be expected in the CF airways, the fMLP-stimulated primary granule release of MPO[20] and NE was inhibited in a dose dependent manner. Moreover, HTS induced changes in the actin cytoskeleton have been reported[111] and linked to the hypertonic inhibition of neutrophil degranulation. HTS instigated a twofold increase in F-actin formation and abrogated the mobilization of all granule types suggesting cytoskeletal remodelling as a key component in the neutrophil-suppressive anti-inflammatory effects of HTS[112]. As neutrophils in individuals with CF demonstrate enhanced secretion of NE and MPO, the potential of aerosolized HTS to prevent primary granule release would have tremendous clinical implications. Furthermore, despite the fact that there is abundant extracellular neutrophil released hCAP-18/LL-37 in the lungs of individuals with CF, the lung fluid from patients exhibits poor antimicrobial activity. A recent study has demonstrated that the antimicrobial activity of endogenous hCAP-18/LL-37 in CF BAL fluid is rendered inactive by binding GAGs but is liberated following nebulized HTS[113]. The effect of HTS on levels of additional antimicrobial peptides and proteins within the CF airways was not evaluated but this study does suggest that a strategy whereby nebulized HTS augments antimicrobial activity may provide optimization of the innate antimicrobial activity in the setting of CF.

CONCLUSION

HTS treatment is associated with an improvement in lung function and marked benefits with respect to exacerbations[26,114,115]. Significant inflammation in the airways manifests from a very young age in CF most likely due to a combination of intrinsic innate immune dysregulation and infection. The obvious most effective treatment remains correction of CFTR dysfunction at a very early age, thereby curtailing development of airway inflammation. However, in the absence of CFTR ion channel modulators for each individual's genotype it will remain important to modulate or suppress the inflammatory reactions of the disease. Although in children with CF, the use of inhaled HTS did not reduce the rate of pulmonary exacerbations[116], the described studies in the present review demonstrate dramatic *in vitro* effects of HTS on neutrophil function, limiting cellular processes that govern airway inflammation including cell adhesion, reactive oxygen species production and protease release. These reports support the concept that HTS may have beneficial anti-inflammatory effects other than simply increasing mucociliary clearance and thus further investigations of the potential mechanisms of this currently available therapy in CF is crucially required.

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