Format for ANSWERING REVIEWERS

Date: 09/01/2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name:15421-review.doc).

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

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Name of Journal: World Journal of Critical Care Medicine

ESPS Manuscript NO: 15421

We thank the Editor and the Reviewers for their very comprehensive and constructive reviews of this manuscript

Reviewer 1:

Comment 1: This is a very well written narrative review. I think it highlights the potential role for hypertonic saline nebulisation for the management of inflammation and infection in the CF cohort.

Response 1: We thank the Reviewer for the positive and encouraging comments regarding our study.

Comment 2: My only major concern is the absence of information for the reader on:

a. the search strategy or
b. evaluation of the quality of the evidence included in the review.

Response 2:

In response to point a: 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. This information can now be found on page 4, 2nd paragraph, of the revised manuscript.

In response to point b: we have evaluated the quality of the evidence included in the review. This new information can be found in the revised manuscript and reads as follows:

Page 8: “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 days, so it is unclear whether there is a truly superior dose, and the majority of trials have employed 7% HTS”.

Page 10: “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 125mM, which is in contrast to the therapeutic concentration of HTS used in vivo (513mM; 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)”.

Page 10-11: “Furthermore, in human pulmonary microvascular endothelial cells the ability of increasing concentrations of HTS (ranging from 140mM to 170mM 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”.

Page 11: “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)”.

Page 12: “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)”.

Page 13: “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)”.

Page 15: “Although these results support the potential of HTS to modulate oxidase activity, the concentration of HTS utilised was 180mmol/L, which is below therapeutic HTS and therefore higher concentrations of HTS should

be investigated to determine the effect on p67phox membrane translocation".

Page 17: "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".

Reviewer 2:

Comment 1: The authors present a fascinating, expert update on the literature regarding the effects of hypertonic saline on neutrophil-related pulmonary inflammation and immune regulation in cystic fibrosis.
Response 1: We thank the Reviewer for their positive comments regarding our study.

Comment 2: With the exception of scattered and minor misspellings and grammatical errors, this is an exceptional review.
Response 2: The revised manuscript has been fully checked and all minor misspellings and grammatical errors have been corrected throughout.

Reviewer 3: No comments made.

Reviewer 4:

Comment 1: This review first describes the use of HTS in treatment of CF focusing on its efficacy and tolerability. The paper is well-written, and well-organized.
Response 1: We thank the Reviewer for their encouraging comments regarding our study.

Comment 2: I have only two minor comments. 1. Which concentrations of Na is the best and which HTS treatment is associated with an improvement in lung function?
Response 2: In response to the Reviewers request concerning which concentration of Na is the best we have now added the following text on page 8:

"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 days, so it is unclear whether there is a truly superior dose, and the majority of trials

have employed 7% HTS".

Comment 3: 2. Please discuss the effect of HTS on the lung function in detail.

Response 3: As per the request of Reviewer 4 we have extended the section on the clinical efficacy of nebulised HTS in CF. This new text commences on page 5 and reads as follows:

"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 of HTS in individuals with CF. The study randomised 164 patients with CF to receive HTS (7%) or isotonic (0.9%) saline for 48 weeks. Using forced vital capacity (FVC) and forced expiratory volume in 1 second (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. 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 days, when compared to HTS given in conjunction with the ENaC inhibitor amiloride (26), 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, 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 (27). 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 4ml or 5ml aerosolized HTS (12, 26) recorded smaller improvements in lung function compared to a 10ml volume (11, 28). In 2011, Dmello et al. 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% (29). 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 baseline (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 weeks, however at 48 weeks 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 (MBW) 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 months (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. demonstrated only negligible acute clearance effects (40), 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 4ml three times daily for four weeks. While mucociliary clearance was largely unaltered at 2 hours after the initial dose, a significant acceleration of mucociliary clearance lasting greater than 12 hours 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. 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 (36). 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)".

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