

## Hypertonic saline alters ion transport across the human airway epithelium

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**ABSTRACT:** Aerosolized hypertonic saline is currently being investigated as a new agent for the treatment of impaired mucociliary clearance which occurs in many respiratory diseases. Mannitol aerosols, in particular dry powder inhalers, have been proposed as an alternative treatment to saline, offering the same osmotic load with other benefits. However, the effects of these hypertonic aerosols on airway epithelial ion transport processes have not been tested in human subjects *in vivo*. This report examines the effect of these solutions on airway ion transport using the nasal potential difference (PD) technique.

Seven healthy nonsmoking adult volunteers were studied. On different days, a dose-response curve was constructed for the saline added to Krebs N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (HEPES) diluent. The reversibility of this saline effect was measured, and the response to additional saline (500 mM) and mannitol (1 M) compared.

Hypertonic saline decreased nasal PD in a dose-related manner, with mean (SEM) decreases in PD (less negative) of 6.6 (1.5), 7.6 (1.6), 10.0 (2.0), 13.1 (2.9) and 14.8 (3.2) mV (n=4) for addition of 150 mM, 250 mM, 500 mM, 1,200 mM and 2,000 mM NaCl to the Krebs HEPES diluent, respectively. The effect of hypertonic saline was fully reversible with washout for 3 min (presaline 15.9 (0.5) mV, postwashout 15.8 (1.1) mV, (n=4)). The hypertonic saline response was rapid in onset, sustained for at least 4 min, and decreased PD from 13.7 (1.7) mV to 5.1 (1.3) mV (n=7, p<0.001). In contrast, addition of mannitol to the perfusate did not significantly alter nasal PD, with a nonsignificant trend towards an increase (more negative) in the PD, (premannitol 13.9 (1.6) mV, postmannitol 15.3 (2.0) mV, n=7).

As the osmotic stimulus of the 1 M mannitol is similar to that of the 500 mM sodium chloride, the divergent nasal potential difference responses suggest that the response to the saline was specific to the sodium chloride itself and not the simultaneous change in osmolarity. This demonstrates that the human airway epithelium *in vivo* can respond to topical hypertonic saline independent of the altered osmolarity.

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The human airways are covered by a thin layer of fluid, the airway surface liquid (ASL), which protects the underlying epithelial cells from drying, and traps inhaled airborne particles and bacteria. The ASL is thought to comprise a watery periciliary or sol layer, over which floats the gel or mucous layer. Though the exact composition and volume of these 2 layers is currently debated, the ASL is thought to be largely regulated by the ion transport processes across the airway epithelium, namely sodium absorption and chloride secretion. Optimal functioning of the ASL is required to maximise mucociliary clearance (MCC), preventing accumulation of mucous and inhaled particles. Retained mucous and particles are a site for bacterial infection, which can then lead to airflow limitation, chronic cough and airway damage.

As impaired MCC is thought to be involved in the pathogenesis of a number of respiratory disorders, treatments have been devised to improve MCC through

physical removal of the retained secretions or the use of pharmacological agents. Chest physiotherapy, an example of physical removal of secretions, is extremely effective, but expensive and time consuming in the long term. For this reason a number of pharmacological therapies have been developed to increase MCC.

Recently, interest has focussed on the use of nebulised therapy, in particular hypertonic saline and mannitol, as potential new treatments to increase MCC. Topical application of hypertonic saline *via* nebuliser has been shown to increase MCC in patients with cystic fibrosis (CF) [1] and a 2 week clinical trial has demonstrated that 10 mL of 6% saline improved symptoms and lung function [2]. Similarly inhaled mannitol has been shown to increase MCC in subjects with CF [3] and idiopathic bronchiectasis [4]. However, the mechanism of action of both hypertonic saline and mannitol remains speculative. Whilst it has been hypothesised that the aerosols provide an osmotic

load to the ASL, no studies have examined the effects of hypertonic saline and mannitol on airway epithelial ion transport in human subjects.

The nasal potential difference (PD) technique, which involves passage of a small exploring electrode along the floor of the nose, allows *in vivo* measurement of ion transport across the human airway epithelium, bypassing the difficulties of *in vitro* techniques. The nasal PD technique has previously been used to study the effect of topical antibiotics on human airway epithelial ion transport [5], and to investigate potential new aerosol therapies for CF [6]. In the current study, the effects of topical application of hypertonic saline and mannitol on nasal PD have been investigated, demonstrating that saline, but not mannitol, rapidly and reversibly decreases nasal PD in normal human subjects.

### Methods

Nasal PD was measured using previously described methods [5, 7, 8]. Briefly, the exploring electrode consisted of a double lumen silicone rubber tube with the openings of both lumens at the same site, 3 mm from the tip. One lumen was filled with an equal mixture of saline and electrocardiography electrode cream, connected to a high impedance voltmeter *via* a silver/silver chloride electrode. The second lumen was perfused with the different solutions as outlined below, using a peristaltic pump which provided a continuous flow of 4 mL·min<sup>-1</sup> throughout the perfusion period. The reference electrode consisted of a second silver/silver chloride electrode placed over an area of abraded skin on the forearm, again connected to the voltmeter. Prior to recordings, the offset of the electrodes was measured and appropriate corrections made to recorded values. In all cases, the tests started with an initial period of stabilization using standard Krebs N-[2-hydroxyethyl]piperazine-N-[2-ethanesulphonic acid] (HEPES) solution. Following stabilization of the nasal PD, the diluent was changed to the various hypertonic solutions as indicated below, with different hypertonic responses measured on different days, in random order.

Perfusion commenced with Krebs HEPES solution of composition (mM): Na<sup>+</sup> 140, K<sup>+</sup> 6, Ca<sup>2+</sup> 2, Mg<sup>2+</sup> 1, Cl<sup>-</sup> 152, glucose 10 and HEPES 10, titrated to pH 7.4. Hypertonic solutions were produced volumetrically, so that the total concentrations of Na<sup>+</sup> and Cl<sup>-</sup> in the Krebs+500 mM saline were 640 mM and 652 mM, respectively. In the dose response testing, the solutions were perfused sequentially as indicated. Due to the 3 mL dead space of the perfusion system, the new perfusate reached the catheter tip approximately 45 s following solution change. Fresh stock solutions were prepared daily and diluted as required. All solutions were perfused at room temperature (21–23°C).

### Subjects

Non-smoking control subjects (4 female and 3 male, aged 18–40 yrs) with no history of respiratory disease were recruited from the hospital staff for testing. No

subjects had undergone nasal surgery, and all tests were performed at least 4 weeks following an upper respiratory tract infection. The study was approved by the hospital ethics committee and all subjects gave written informed consent.

### Statistical analysis

The two-tailed paired t-test was used for comparison and the null hypothesis was rejected at  $p < 0.05$ . For discussion purposes, increases and decreases refer to the absolute magnitude of the PD, which was lumen negative.

### Results

Initial studies measured the response of the nasal epithelium to different concentrations of added NaCl. Following stabilisation of the PD in the Krebs HEPES diluent, the nose was perfused for 3 minutes with each of the following solutions: Krebs+150, Krebs+250, Krebs+500, Krebs+1,200 and Krebs+2,000 mM NaCl. This was associated with significant ( $p < 0.05$ ) decreases in the mean (SEM) nasal PD of 6.6 (1.5), 7.6 (1.6), 10.0 (2.0), 13.1 (2.9) and 14.8 (3.2) mV ( $n=4$ ) (fig. 1). As the higher concentrations of saline present in Krebs+1,200 and Krebs+2,000 mM NaCl also resulted in subjective discomfort in the nose during the testing, a solution of Krebs+500 mM NaCl was selected for further investigation. This gave approximately 70% of the maximal response without causing any discomfort. All subsequent studies of hypertonic saline in this paper were then performed with Krebs+500 mM NaCl.

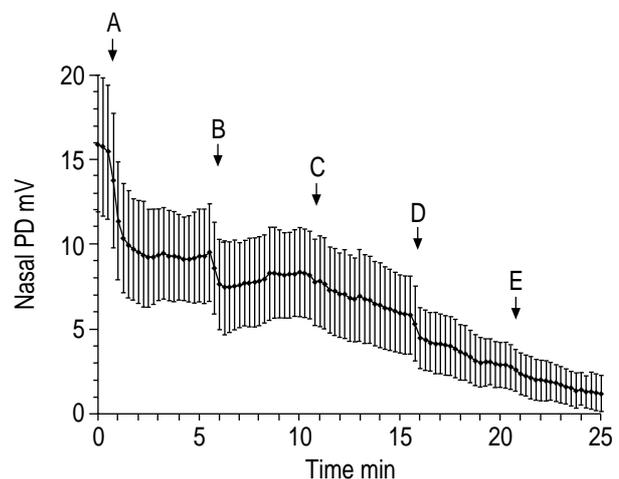


Fig. 1. – The effect of additional saline on the nasal potential difference (PD) in four normal subjects. Each new solution reached the nose approximately 45 s after the perfusate was changed, reflecting the dead space of the system. The solutions tested are indicated in the bar at the top of the graph. Data are expressed as mean  $\pm$  SEM. The PD is expressed in absolute terms, and was lumen negative. Arrows indicate Krebs+NaCl solutions of the following concentrations; A: 150 mM NaCl; B: 250 mM; C: 500 mM; D: 1.2 M; E: 2.0 M.

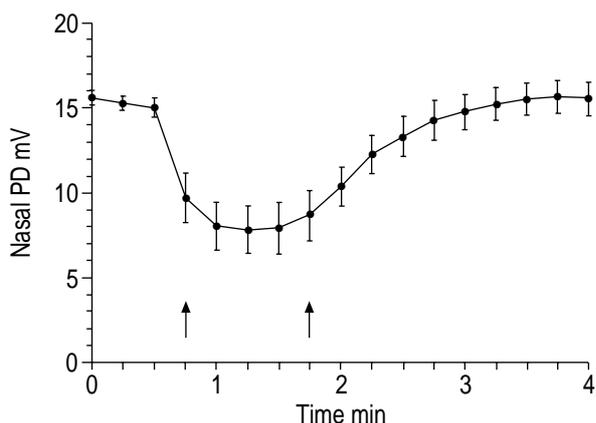


Fig. 2. – The effect of additional saline (500 mM) added to the Krebs HEPES N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid], on the nasal potential difference (PD) in four normal subjects. The new solution reached the nose approximately 45 s after the perfusate was changed (indicated by arrows), reflecting the dead space of the system. After 1 min of perfusion with the hypertonic saline solution, the perfusate returned to Krebs HEPES diluent to assess the effect of washout. Data are expressed as mean  $\pm$  SEM. The PD is expressed in absolute terms, and was lumen negative.

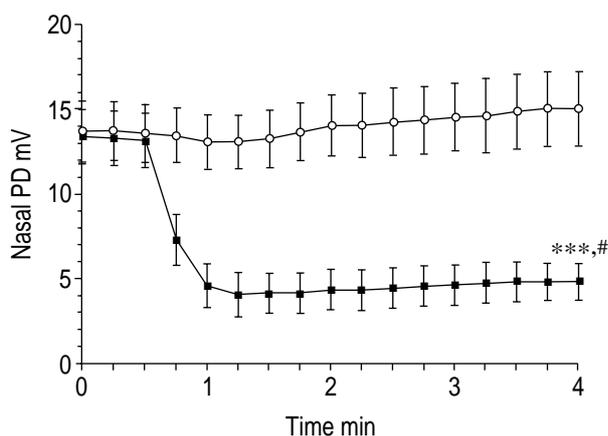


Fig. 3. – The effect of additional saline (500 mM) or mannitol (1 M) added to the Krebs HEPES N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid], on the nasal potential difference (PD) in 7 normal subjects. The new solution reached the nose approximately 45 s after the perfusate was changed, reflecting the dead space of the system. Data are expressed as mean  $\pm$  SEM. The PD is expressed in absolute terms, and was lumen negative. ■: saline; ○: mannitol. \*\*\*:  $p < 0.001$  versus baseline presaline; #:  $p < 0.0001$  versus mannitol.

To determine whether this response may be related to epithelial toxicity, the reversibility of the hypertonic saline response was measured. As shown in fig. 2, following 1 min of perfusion with Krebs+500 mM NaCl, washout for 3 min reversed the response (presaline 15.9 (0.5) mV, postwashout 15.8 (1.1) mV, (n=4)).

In seven subjects the change to Krebs+500 mM NaCl was associated with a significant decrease in nasal PD from 13.7 (1.7) mV to 5.1 (1.3) mV ( $p < 0.001$ ). This response was rapid in onset and sustained for at least 4 min. In contrast, the addition of mannitol (1,000 mM) to the perfusate did not significantly alter

nasal PD, with a trend to an increase in the PD, (premannitol 13.9 (1.6) mV, postmannitol 15.3 (2.0) mV, n=7,  $p=0.14$ ), as shown in figure 3.

### Discussion

This study has demonstrated that topical application of hypertonic saline, Krebs+500 mM NaCl, corresponding to a stimulus of ~3% saline, decreases nasal potential difference. The response to the hypertonic saline was rapid, reversible and dose related, suggesting a direct ion transport effect rather than nonspecific epithelial toxicity. In contrast, application of a similar osmotic load through hypertonic mannitol did not decrease the nasal PD, with a nonsignificant increase (more negative). This suggests that the ionic composition of the saline, and not the osmotic load, was involved in the saline response. As amiloride pretreatment can block the saline response in normal subjects (unpublished data), passive diffusion of Cl<sup>-</sup> ions down the paracellular pathway would not explain the changes in nasal PD. Therefore it is hypothesized that the saline directly alters ion transport processes across the normal human airway epithelium. This is the first demonstration in human subjects *in vivo* that increasing the NaCl concentration on the surface alters airway epithelial ion transport, independent of any effect of osmolarity.

Previous studies measuring the effect of hypertonic solutions on epithelial cells *in vitro* have found varying results. Early studies in canine trachea, a predominantly Cl<sup>-</sup> secreting epithelium, have shown that additional luminal NaCl (75 mM) decreased short-circuit current (*I*<sub>sc</sub>) and increased tissue conductance [9]. The decrease in *I*<sub>sc</sub> predominantly reflected an increase in mucosal to serosal Cl<sup>-</sup> movement, which was proportional to the increased Cl<sup>-</sup> concentration on the mucosal surface. Interestingly, addition of (150 mM) mannitol to the luminal surface decreased Na<sup>+</sup> absorption across the canine trachea without significant changes in either Cl<sup>-</sup> movement or tissue conductance. MAN *et al.* [10] demonstrated that addition of 100 mM mannitol to the luminal fluid decreased PD across the canine trachea by 3.9 mV, with a similar decrease in *I*<sub>sc</sub>. Interestingly there was a small increase in tissue resistance, opposite to the increased conductance found by YANKASKAS *et al.* [9].

In monolayers of cultured human nasal polyp cells, WILLUMSEN *et al.* [11] measured the effects of raised luminal and serosal osmolality. Addition of 150 mM mannitol to the luminal surface was associated with decreases (less negative) in both apical (*V*<sub>a</sub>) and basolateral (*V*<sub>b</sub>) membrane potentials, with small but variable effects on the transepithelial membrane potential (*V*<sub>t</sub>) in different monolayers. Addition of 75 mM NaCl to the luminal solution also decreased both *V*<sub>a</sub> and *V*<sub>b</sub>, but did not alter *V*<sub>t</sub> [11]. The nonsignificant increase in nasal PD found in the current study corresponds with the variable effects on *V*<sub>t</sub> found in the nasal polyp monolayers; *V*<sub>a</sub> and *V*<sub>b</sub> cannot be assessed using the nasal PD technique. Similarly, the changes in epithelial cell volume noted in the nasal polyp cultures cannot be measured by the current technique. Further studies will be necessary to deter-

mine whether luminal mannitol does alter ion transport *in vivo*, with similar but opposite effects resulting in no significant overall response.

However, the responses to luminal saline appear to differ in the two studies with a significant decrease in nasal PD in the current study contrasting with the lack of change in  $V_t$  found by WILLUMSEN *et al.* [11]. This may simply reflect the different doses used in the respective studies – the only dose reported in the WILLUMSEN *et al.* study was 75 mM, whilst the smallest dose used in the current study was 150 mM. Another possible explanation for the differences between the two studies is the use of nasal polyp cells, which are known to exhibit ion transport processes which differ from those found in normal airway epithelial cells [12]. The nasal PD also avoids the difficulties of cell culture techniques, in particular the submersion culture process and the hormones and antibiotics required to maintain the cells in a viable state. Finally, another alternative may be the presence of hormonal factors which may control ion transport *in vivo*, but are not maintained when the cells are grown into monolayers *in vitro*. Irrespective of the basis for the differences between the *in vivo* and the *in vitro* studies, the current report provides important new information about the ion transport responses of the human airway epithelium *in vivo*.

The nasal PD response to increasing concentrations of NaCl in the current study corresponds with the dose-related effects of saline on mucociliary clearance. ROBINSON *et al.* [1] found that aerosols of 0.9%, 3%, 7% and 12% saline were associated with isotope clearance at 90 min of 13%, 20%, 24% and 26%, respectively. Whilst the MCC response may be related to the nasal PD effects demonstrated in the current study, there are a number of other possibilities, including increased ciliary beat frequency *in vivo* [13] and direct effects on sputum [14]. Furthermore, the divergent effects of hypertonic mannitol and saline on nasal PD do not correspond with the similar increases in MCC in a variety of clinical situations [3, 4, 15].

Hyperosmolarity is also a reliable stimulus to provoke bronchoconstriction in susceptible subjects. Hypertonic saline solutions have long been used in the laboratory as an indirect challenge agent, with more recent studies by ANDERSON *et al.* [16] studying the effects of inhaled mannitol. Whilst for the majority of subjects saline and mannitol produced qualitatively similar results, some subjects did show 10-fold higher sensitivity to one agent. Furthermore, ESCHENBACHER *et al.* [17] compared the effects of different solutions with the same osmolarity on the airways of asthmatic subjects. They demonstrated that 4% saline solution was significantly more potent at inducing bronchoconstriction than 18.3% dextrose in 1% saline, though both solutions had the same osmolarity. Similarly MAGYAR *et al.* [18] has reported that 1.3 M KCl was more potent than 1.7 M NaCl, (both solutions prepared at 10% weight/volume). This suggests that as well as the osmotic stimulus, the ionic composition may also be involved in bronchoconstrictor responses.

It is well recognized that both the osmolarity and ionic composition of an aerosol are important in the induction of cough. In an elegant series of experiments,

ESCHENBACHER *et al.* showed that hypertonicity, irrespective of ionic composition, induced both bronchoconstriction and cough [17]. Isosmotic saline solution induced neither bronchoconstriction or cough, but isosmotic dextrose, with a low Cl<sup>-</sup> concentration, induced cough in the absence of bronchoconstriction [17]. This suggests that hypertonicity and ionic composition can, in certain situations, be stimuli for different responses. The relationship between this and the nasal PD responses in the current study will require further investigation.

Finally, the differing nasal PD response to hypertonic saline and mannitol may have important implications for the development of new treatments for lung diseases. Clinical trials will be necessary to investigate each hypertonic solution individually, as the results from hypertonic saline and mannitol are not always interchangeable.

In conclusion, this study has demonstrated that topical application of hypertonic saline induces a rapid and reversible decrease in nasal potential difference *in vivo*, most likely reflecting altered ion transport across the human airway epithelium. The nonsignificant increase in nasal potential difference seen with hypertonic mannitol suggests that the two solutions exert different effects on the epithelium, dependent on their composition. Further studies are necessary to investigate the mechanisms involved in these responses.

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