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Hypertonic Aerosols Hydrate Airways Longer and Reduce Acidification Risk with Nonpermeating Cation and Permeating Anion Salts

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Abstract

Background: Hyperosmolar aerosols appear to promote or suppress upper airway dysfunction caused by dehydration in a composition-dependent manner. We sought to explore this composition dependence experimentally, in an interventional human clinical study, and theoretically, by numerical analysis of upper airway ion and water transport.
Methods: In a double-blinded, placebo-controlled clinical study, phonation threshold pressure (PTP) was measured prenasal and postnasal inhalation of hypertonic aerosols of NaCl, KCl, $CaCl_2$, and $MgCl_2$ in seven human subjects. Numerical analysis of water and solute exchanges in the upper airways following deposition of these same aerosols was performed using a mathematical model previously described in the literature.
Results: PTP decreased by 9%–22% relative to baseline ($p < 0.05$) for all salts within the first 30 minutes postadministration, indicating effective laryngeal hydration. Only $MgCl_2$ reduced PTP beyond 90 minutes (21% below baseline at 2 hours postadministration). By numerical analysis, we determined that, while airway water volume up to 15 minutes postdeposition is dictated by osmolarity, after 30 minutes, divalent cation salts, such as $MgCl_2$, better retain airway surface liquid (ASL) volume by slow paracellular clearance of the divalent cation. Fall of CFTR chloride flux with rise in ASL height, a promoter of airway acidification, appears to be a signature of permeating cation (NaCl) and nonpermeating anion (mannitol) aerosol deposition. For hypertonic aerosols that lack permeating cation and include permeating anion ($CaCl_2$ and $MgCl_2$), this acid-trigger signature does not exist.
Conclusions: Nonpermeating cation and permeating anion hypertonic aerosols appear to hydrate upper airways longer and, rather than provoke, may reduce laryngeal dysfunction such as cough and bronchoconstriction.

Keywords: bronchoconstriction, cough, hyperosmolar aerosol, hypertonic salts, laryngeal hydration, phonation

Introduction

Dehydration of the larynx occurs in the process of humidifying inhaled air.[1] Proximity to the environment and near-turbulent air formed within the larynx and trachea during normal tidal inhalation[2]—and with phonation or exercise on exhalation[3]—render evaporative rates higher than elsewhere in the respiratory system.[4] Given increasing prevalence of dry (often indoor) air, whole-body dehydration, overbreathing, and mouth breathing, which negates the humidifying

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function of the nose,[5] laryngeal dehydration appears an increasing contemporary threat to respiratory health and wellness.

Inflammation and elevated ATP accompany dehydration of the larynx and trachea,[6] promoting upper airway dysfunction.[6] Cilia beat more slowly in dehydrated airways,[7] such that particles inhaled and deposited do not clear as quickly as they do in well-hydrated airways. This increases their tendency to remain in the respiratory system, penetrate epithelial cells, and enter the systemic circulation.[8] Dehydrated upper airways further fail to humidify inhaled air; hence, dehydration can extend into the central and lower airways,[6,9] promoting inflammation throughout the respiratory tract. For these and other reasons, dehydration of the airways worsens symptoms and incidence of asthma,[10] chronic obstructive pulmonary disease (COPD),[11] influenza,[12] and COVID-19,[13] among other lung illnesses.

Vocal quality is lost with dehydration of the larynx,[14] making phonation a potential diagnostic for laryngeal hydration state. Prolonged vocal exercise, as in singing, can lead to vocal fold pathology in a dehydrated state.[15] Acidification of the larynx is a common challenge among singers,[16] who suffer more commonly from gastroesophageal reflux disease (GERD) than the general population.[17] Athletes face similar laryngeal challenges,[18] given the very high rates of air flow that occur during strenuous exercise.[19] Upper respiratory tract infections, cough, and exercise-induced bronchoconstriction are all frequent among athletes[20]; indeed, upper respiratory illness is the most common noninjury reason to report to a sports clinic, representing 30%–65% of all reports.[21]

Strategies to rehydrate the upper airways—from drinking copious quantities of water,[22] to breathing humid air,[23] or delivering isotonic and hypertonic salt and excipient aerosols to laryngeal tissue[24]—are frequently assessed by their ability to alter phonation quality, and particularly to reduce phonation threshold pressure (PTP),[22–24] the minimal subglottal pressure required to trigger vibration of the vocal folds on exhalation.[25,26]

Hydrating the larynx, through the ingestion of water, has been shown to reduce PTP by $\sim$10%,[27] while duration of the effect, and time of onset of hydration relative to time of water ingestion, remains unclear.[28] Theoretical analyses of PTP[29] reveal a linear relationship between PTP and dissipation of mechanical energy across the mucus and into the vocal fold tissue.[29,30] Hydration reduces vocal fold tissue viscosity,[31] lowering mechanical energy dissipation, while topical hydration of airway surface liquid (ASL) also reduces dissipation by lessening viscous stress within the mucus and periciliary layer (PCL) of the ASL.

The science and practice of upper airway hydration have advanced in recent years with the growing awareness of respiratory health consequences of dry airways.[32,33] The discovery that endogenous divalent salt cations (calcium and magnesium) clear more slowly than endogenous monovalent cations (sodium and potassium), reducing respiratory droplet generation for up to 6 hours postadministration of 8–15 μm mass median aerodynamic diameter (MMAD) aerosols, has made nasal divalent salt inhalation a practiced hygienic rite in the United States,[34,35] with potential for chronic cough treatment.[6]

Hypertonic aerosols of sodium chloride[36] and nonchloride osmolytes, such as mannitol,[37] are, however, recognized to promote cough in hypersensitive airways,[37] not relieve it, and therefore the question arises whether there might be compositional distinctions in the inflammatory and cough-provoking consequences of osmosis-induced rehydration of the upper airways, that is, whether certain hydrating hypertonic aerosols might promote inflammation and cough,[38] while other compositions might be anti-inflammatory and antitussive.

We explored these questions in a double-blinded placebo-controlled study of PTP at New York University. We analyzed our clinical results by the generalization of a recent mathematical model[39,40] that accounts for the principal ion transport pathways in the upper airways, expanding the model to include calcium and magnesium transport. We particularly explored hydration and CFTR flux kinetics as relates to tendency for acidification following deposition of aerosols of identical hyperosmolarity, possessing (1) a permeating cation and permeating anion (e.g., NaCl), (2) neither permeating cation nor permeating anion (e.g., mannitol), or (3) a nonpermeating cation and permeating anion (e.g., $MgCl_2$). The results of our studies are described here.

Methods

Materials

We prepared hypertonic solutions of NaCl, KCl, $CaCl_2$, and $MgCl_2$ as follows: 5% (weight/volume) hypertonic solutions of sodium chloride (Fisher Bioreagents), potassium chloride (Fisher Bioreagents), calcium chloride (Fisher Chemical), and magnesium chloride (Spectrum Chemical) were prepared by dissolving 5 g of the salts in 100 mL of high purity water (Thermo Scientific). Similarly, an isotonic solution of sodium chloride was prepared by dissolving 0.9 g of sodium chloride (Fisher Bioreagents) in 100 mL of high-purity water (Thermo Scientific).

The hypertonic and isotonic solutions were delivered as aerosols using a pump-spray device, widely used in pharmaceutical and cosmetic aerosol products in the United States and European Union, manufactured by AeroPump (Hochheim am Main, Germany) and supplied by Ursatec (Tholey, Germany). The hypertonic salts were delivered with mesh diameter 9 μm leading to MMAD size of 13 μm, while the isotonic saline was delivered with mesh diameter 13 μm leading to MMAD of 20 μm (Supplementary Data). The reason for choosing the former droplet size for the hypertonic aerosol was to achieve laryngeal and tracheal deposition with minimal exposure in the airways beyond the first carina. We chose the larger droplet size for the placebo to avoid penetration of the aerosol into the larynx, and thereby a potential active effect.

PTP was measured using the Aeroview System version 1.7.2 (Glottal Enterprises). The Aeroview System measures subglottic pressure through a plastic mask (Supplementary Data) placed over the nose and mouth, with a removable plastic tube assessing exhalation air pressure fitted into the corner of the mouth, and in the process of the phonation task described further below. The plastic mask surface was cleaned following each use with an alcohol wipe, the removable face seal cleaned with soap and water, and each participant received their own plastic tube mouthpiece, which was washed with soap and water between uses by the participant.

Clinical study design

Seven subjects successfully completed the clinical study at New York University (IRB approval No. IRB-FY2022-6445) over a period of 4 months from February through May 2023. PTP was evaluated for 2-hour postinhalation with subjects ''at rest,'' that is, they did not sing, speak other than in normal conversation, or otherwise physically exercise. At the start of the 2-hour protocol, each subject received one of the hypertonic salt aerosols or the placebo control.

Participants were recruited through e-mail and social media and invited to take part in a screening survey to determine eligibility to participate. Thirty-three participants volunteered and answered the screening questionnaire. Ten participants were identified and passed the inclusion criteria. Eight out of the 10 participants identified successfully concluded the entire data collection process. Table 1 summarizes inclusion and exclusion criteria for the human subject recruitment. We excluded one subject who had a deviated septum.[41]

The process for recruitment, vocal range identification, training, baseline PTP measurement, and PTP measurement before and after intervention is described in the Supplementary Data. For each reported PTP measurement at a given condition, three measurements were made and the average of these three measurements was reported with the standard deviation value (Supplementary Data). Intrasubject PTP variation tended to be greatest from day to day (see PTP Baseline data in Supplementary Data) than at a particular condition, while mean PTP data in response to a hydration stimulus tended to be most reproducible, as indicated by the PTP intrasubject data shown in the Supplementary Data.

Statistics

All error bars represent 95% confidence intervals based on standard deviation values. Significance of differences in individual and collective aerosol numbers was determined by twin-tailed t test. We calculated statistical significance of differences using a multiway analysis of variance (ANOVA) test for each set of variables. This allowed for evaluating the influence of multiple factors on the mean within a 95% confidence interval. p-Values were calculated for each unique set of variables compared to baseline values. Each p-value below 0.05 was considered to be statistically different.

Theoretical analysis

The computational model used in this study was adapted from the model developed by Sandefur et al.,[40] referred to as the Sandefur, Boucher and Elston (SBE) model. It

Table 1. Inclusion and Exclusion Criteria for the HS Phonation Threshold Pressure Study at New York University

Inclusionary (must meet all to participate)	*Exclusionary (not qualified if any are met)*
Aged 18–35 years old	Younger than 18 or older than 35 years of age
Able to provide informed consent and comply with study procedures	Currently have any of the following: Asthma Acid reflux Severe seasonal allergies or hay fever Chronic congestion Chronic cough Deviated septum Cancer Autoimmune disease(s) Diabetes Cardiovascular disease Psychological/Psychiatric condition
Willing to maintain stable body weight and follow his/her habitual diet and physical activity patterns throughout the trial	Currently on any of the following types of medication: Mucus thinners Bronchodilators Lipid-lowering medications Antihistamines Prolonged use of anti-inflammatories
Judged by the investigator to be in general good health on the basis of disclosure by the participant through the qualtrics survey responses	Currently smoking e-cigarettes/vaping, cigarettes and tobacco, etc., or former smoker having quit less than 2 years ago
	Smoking marijuana biweekly or daily
	Consuming more than three alcoholic drinks in one sitting biweekly or daily
	Consuming more than four cups of caffeine daily
	Have a history of substance abuse within the last 2 years
	Are pregnant, planning to become pregnant, or breastfeeding
	Have a fever or persistent cough or lung infection within the last 2 weeks
	Have experienced severe COVID symptoms that affected your breathing for a prolonged period of time
	Have any conditions that may affect your breathing

represents water and solute exchanges between the cell cytosol, the ASL, and the basolateral compartment. Volume (or height) and concentration profiles are obtained by solving time-dependent ordinary differential equations expressing conservation of mass and electric charge. The solutes included in the model are Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}, and mannitol.

The last three solutes, which were not represented in the SBE model, are taken to be transported exclusively across paracellular pathways, that is, between the basolateral compartment and the ASL fluid.[40] Paracellular fluxes are calculated using the Goldman-Hodgkin-Katz equation, which does not include effects of solute drag, that is potential convective motion. We assume that the paracellular permeability of solute S (S = K^+, Ca^{2+}, Mg^{2+}, mannitol) scales with that of Na^+ according to the S-to-Na^+ diffusivity ratio. The SBE model does not include any effect of water or solute movement perpendicular to evaporative water flow, as may occur through mucociliary clearance. That may be of particular significance in the larynx beyond standard mucociliary clearance times, $\sim$20 minutes of a well-hydrated human trachea.

As in the SBE model, the ion transporters included in this study are CFTR, Ca^{2+}-activated Cl^- channels (CaCC), epithelial Na^+ channels (ENaC), and voltage-gated K^+ channels (BK) at the apical membrane, and basolateral Cl^- channels (Clb), Ca^{2+}-activated K^+ channels (CaCK), Na^+-K^+-$2Cl^-$ cotransporters (NKCC), and Na^+-K^+-ATPase pumps (NKA) at the basolateral membrane. Concentrations in the basolateral compartment are fixed; values are given in Figure 1.

Transcellular fluxes are calculated using the SBE model equations and parameter values, with the exception of the cotransporter NKCC, which is represented using the formulation of Garcia et al.[39] In the simulations with salt addition, we assume an instantaneous 1 μm increase in ASL height, as well as instantaneous dilution or concentration of solutes in the ASL fluid (using mixing rules). We ignore water evaporation from the free surface of the ASL, and assume water volume within the mucus layer unchanged, such that mucosal thickness is not included in our simulations of ASL height.

The model was solved using MATLAB (R2022a; The MathWorks, Inc., Natick, MA) and the computer code is available upon request.

Results

Clinical study of laryngeal hydration by hypertonic endogenous airway salts

We conducted the human clinical study as described in the Methods section and Supplementary Data. As an

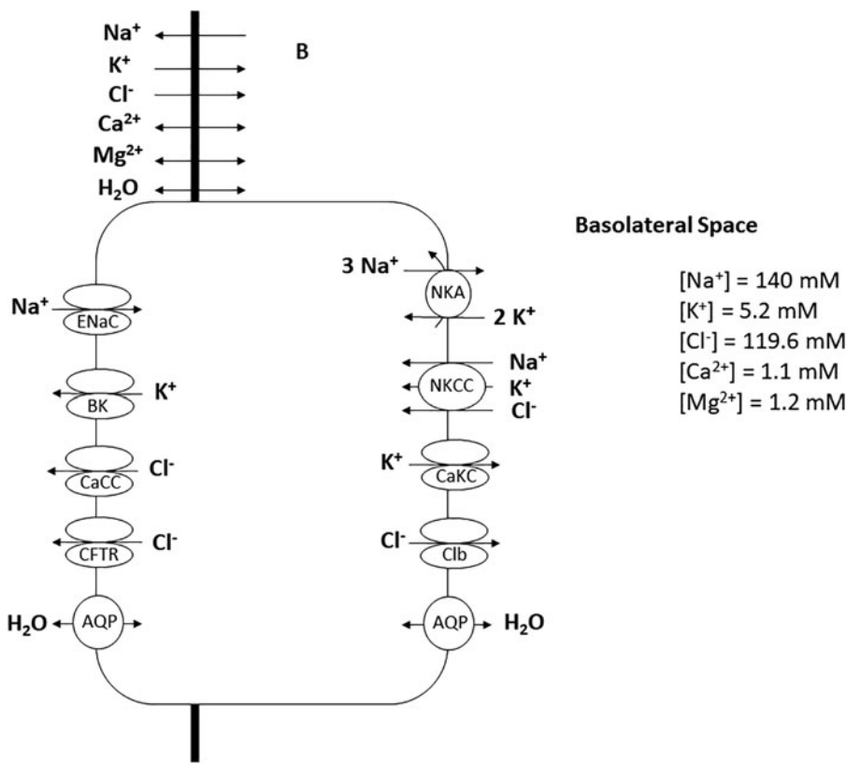

FIG. 1. Schematic diagram of transport pathways included in the model. The apical membrane expresses aquaporin water channels (AQP), CFTR, CaCC, BK, and ENaC. The basolateral membrane expresses AQP, Cl^- channels (Clb), CaCK, NKCC, and NKA. The paracellular pathway is permeable to water and ions. Concentrations in the basolateral compartment are specified as shown. BK, voltage-gated K^+ channels; CaCC, Ca^{2+}-activated Cl^- channels; CaCK, Ca^{2+}-activated K^+ channels; Clb, basolateral Cl^- channels; ENaC, epithelial Na^+ channels; NKA, Na^+-K^+-ATPase pumps; NKCC, Na^+-K^+-$2Cl^-$ cotransporters.

indicator of laryngeal hydration, we measured PTP values preadministration and postadministration of the four hypertonic chloride salt aerosols relative to placebo control. The results at low frequency are shown in Figure 2.

Normalized PTP diminishes ($p < 0.05$) relative to baseline by ~10%–20% for each of the hypertonic salt (HS) aerosols at peak decline (Fig. 2A–D). For the permeating cation salts (Fig. 2A, B), peak PTP decline occurs between 30 and 45 minutes, after which PTP begins a gradual increase toward baseline. For the nonpermeating cation salts (Fig. 2C, D), PTP tends to decline over the course of 2 hours of measurement. Relative to the placebo control, only NaCl (Fig. 2A) and $MgCl_2$ (Fig. 2D) show significant PTP suppression beyond the first 15 minutes ($p < 0.05$), with $MgCl_2$ showing longest and most significant decline (21% relative to baseline at 2 hours postinhalation), and with significantly lower PTP relative to the placebo ($p = 0.01$).

Figure 3 presents normalized PTP values measured at low, medium, and high frequency preadministration and postadministration of the four hypertonic salt aerosols relative to the placebo control. At both low and high frequencies (Fig. 3A, B), the placebo control PTP remains unity ($p < 0.05$) over the 2 hours of the study, while at the intermediate frequency (Fig. 3C), the placebo control PTP declines significantly below unity ($p < 0.05$), suggesting acoustical instability at the intermediate frequency, as has been previously reported.[42] At low frequency, the order of decline of PTP within the first 30 minutes postdosing follows the order of magnitude of the osmolarity of salt at 5% weight fraction, notably NaCl, $MgCl_2$, $CaCl_2$, and KCl (1710, 1575, 1350, and 1340 mEq/L).

Beyond 60 minutes, PTP is statistically indistinguishable from placebo ($p > 0.05$) for all salts at high frequency (Fig. 3B), unlike the case of low frequency (Fig. 3A), where $MgCl_2$ shows a long duration of PTP suppression, suggesting the possibility that the PTP suppression after 60 minutes arises from submucosal hydration to which the shorter wavelength phonation disturbance at the higher frequency is insensitive.

To interpret our results, we computed (Fig. 1) the time evolution of ASL height, and concentrations of principal salt

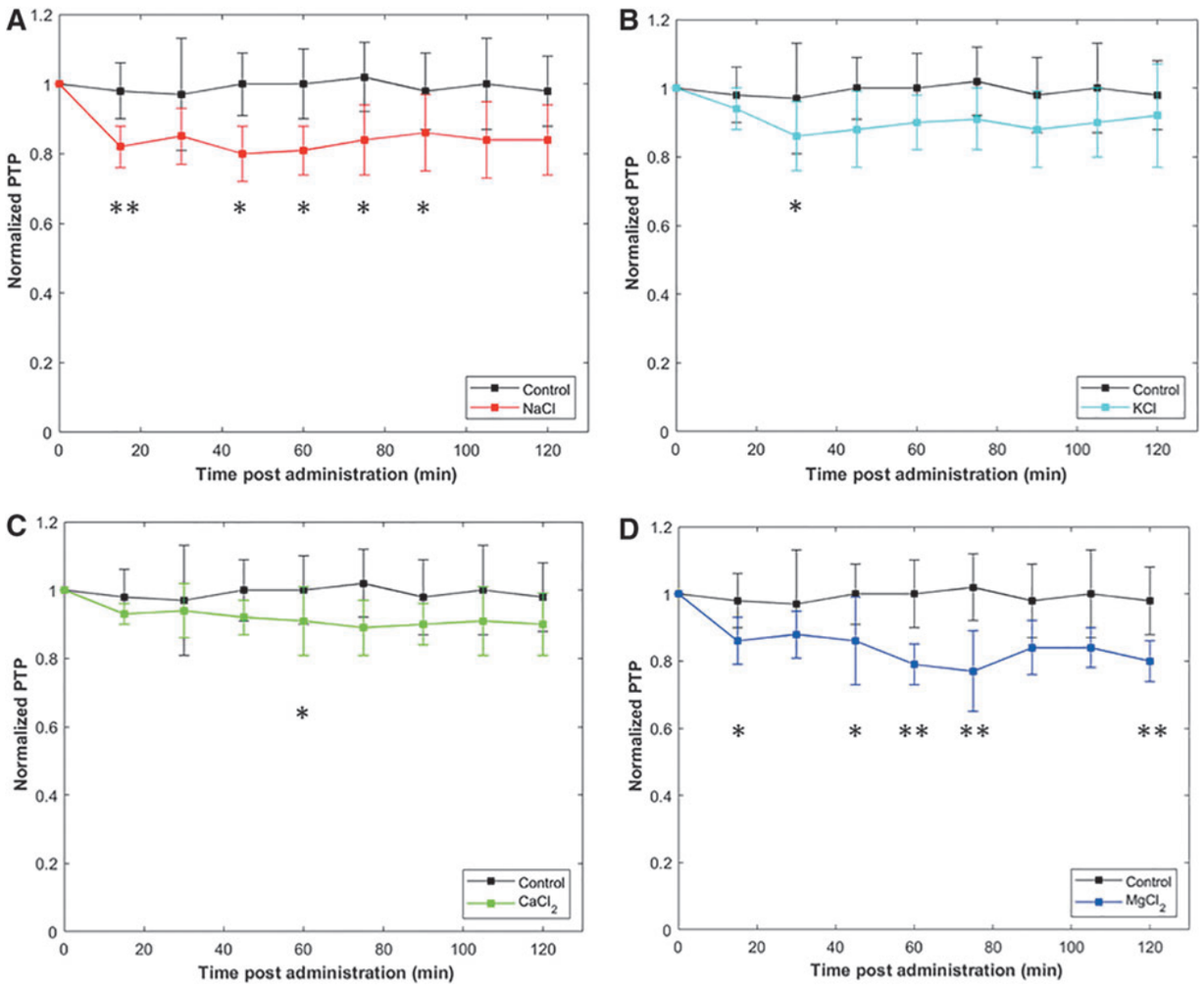

FIG. 2. Normalized PTP values versus time postadministration of the four hypertonic salt aerosols relative to the placebo control. Actual PTP after vocal loading exercise ranged per subject from 3.5 to 5.3 cm H_2O. Normalized values are reported relative to each subject's postvocal loading exercise PTP (before nasal salt administration). **(A)** NaCl. **(B)** KCl. **(C)** $CaCl_2$. **(D)** $MgCl_2$. PTP, phonation threshold pressure.

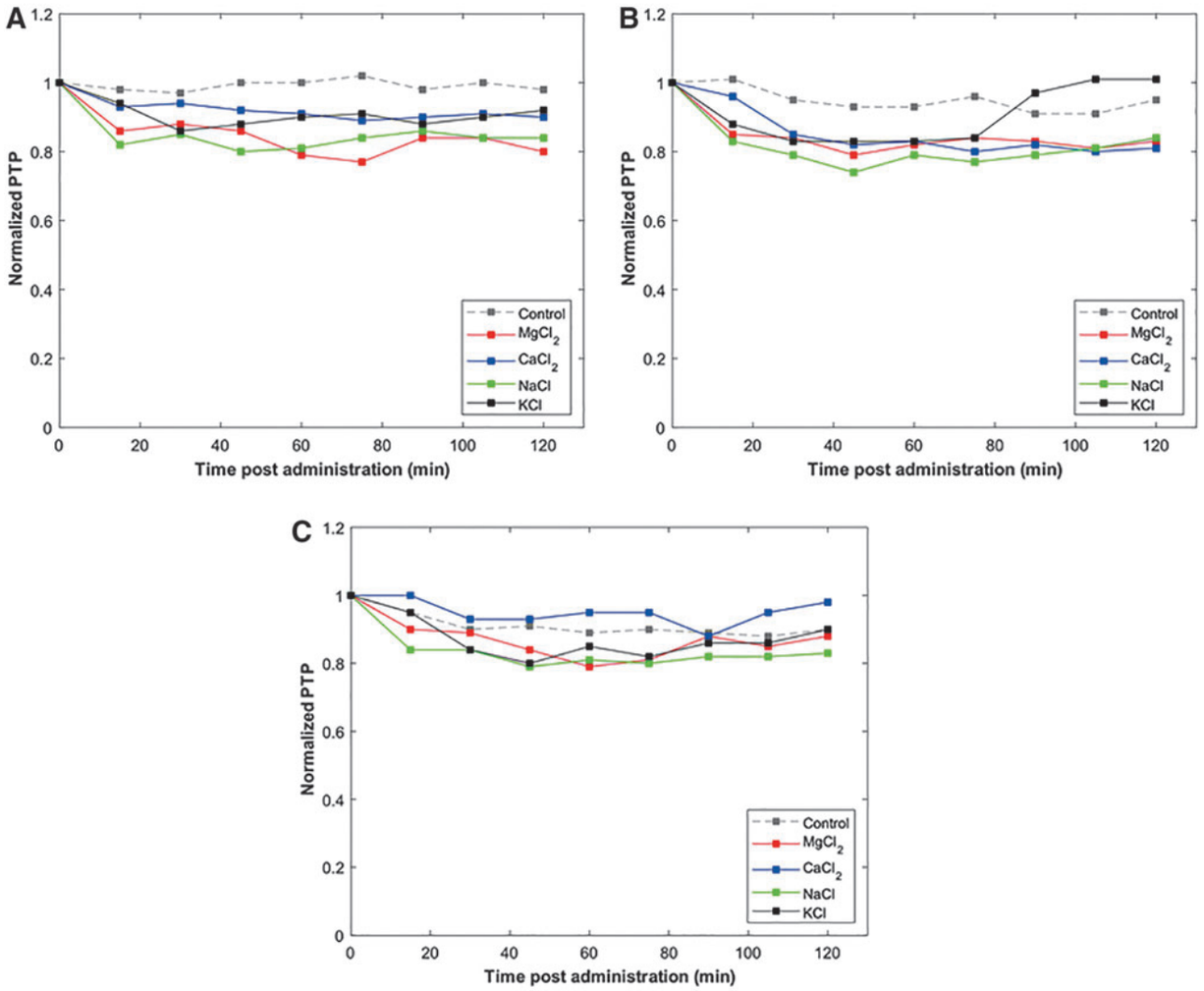

FIG. 3. Normalized PTP values versus time postadministration of the four hypertonic salt aerosols relative to the placebo control at **(A)** low, **(B)** high, and **(C)** medium frequencies.

ions within ASL and adjacent epithelial cells, assuming laryngeal/tracheal deposition of 6 mg each of salts used in the clinical trial. We specifically determined the time evolution of ASL height (Fig. 4A) and intracellular chloride (Fig. 4B), sodium (Fig. 4C), and potassium (Fig. 4D) concentrations postdeposition of each of the four hypertonic salts on a human larynx and trachea, in relation to the relatively fast (<5 minutes) regulation of ASL hyperosmolarity (Fig. 4E). Recognizing the importance of CFTR flux to ASL acidification/alkalinization,[43] and the role of TRPV receptor activation in the provocation of cough and bronchoconstriction,[44] we also determined ASL chloride concentration for the four airway salts (Fig. 4F), comparing these results with the case of a nonanion-permeating osmolyte (mannitol) commonly used to provoke cough and bronchoconstriction in hypersensitive airways.

Figure 4A reveals that aerosol deposition promotes a rapid increase in ASL height by osmotic water movement from tracheal and laryngeal epithelial cells into the PCL in response to ASL hypertonicity. The initial degree of ASL hydration is entirely determined by the degree of osmolarity. That is, at 5% weight fraction, the decreasing order of salt osmolarity is NaCl, $MgCl_2$, $CaCl_2$, and KCl. ASL height returns to its value immediate postdeposition, $\sim$50 minutes after NaCl delivery. This same ASL height is not reached until $\sim$110 minutes for $MgCl_2$ delivery.

The reason for this longer hydration can be surmised from the numerically generated flux values shown in Table 2. Sodium, potassium, and chloride exit the ASL by transport across both paracellular pathways and epithelial cells through ion-specific channels, co-transporters, and pumps. Calcium and magnesium exit the ASL only by the paracellular route, at a much slower rate (see yellow cells of Table 2). The rates at which ASL sodium, potassium, and chloride concentrations are restored post-topical delivery of the HS aerosol are all influenced by transcellular exchange, which does not occur for the divalent cations.

Figure 4B–D present the simulated cytosolic ion kinetics post-HS delivery. As with ASL height elevation, the immediate spike in intracellular ion concentration reflects that,

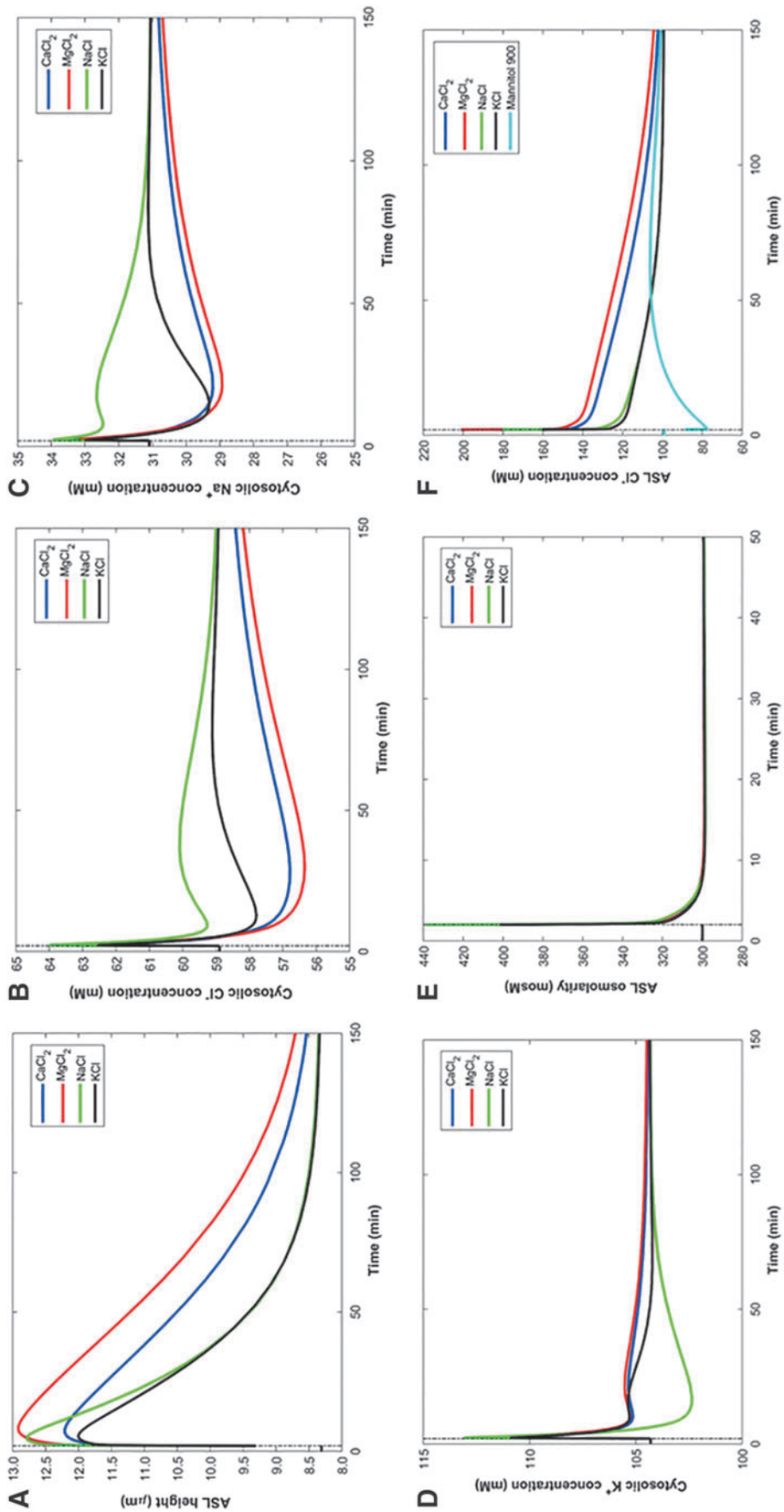

FIG. 4. Numerical simulations of ASL water and ion transport after the deposition of 6 mg of the four 5% (by weight) hypertonic salt solutions. In our simulations, deposition occurs instantaneously at 2 minutes. (**A**) ASL height versus time. (**B**) Cytosolic chloride concentration versus time. (**C**) Cytosolic sodium concentration versus time. (**D**) Cytosolic potassium concentration versus time. (**E**) ASL osmolarity versus time. (**F**) ASL chloride concentration versus time and comparison with topical deposition of 900 mOsm mannitol. ASL, airway surface liquid.

Table 2. Numerically Simulated Ionic Fluxes

	At rest	*$CaCl_2$*	*$MgCl_2$*	*NaCl*	*KCl*
Fluxes, μmol/(s·m^2)					
Na^+ apical	**–0.681**	–0.552	–0 .534	–0.821	–0.560
Na^+ basolateral	**+0.681**	+0.629	+0.621	+0.752	+0.623
Na^+ paracellular	**+0.681**	+0.619	+0.616	+0.437	+0.720
K^+ apical	**+0.015**	+0.013	+0.013	+0.015	–0.007
K^+ basolateral	**–0.015**	+0.005	+0.011	0.000	–0.013
K^+ paracellular	**–0.015**	–0.009	–0.008	–0.002	–0.371
Cl^- apical	**+1.071**	+1.179	+1.200	+0.993	+1.123
Cl^- basolateral	**–1.071**	–1.086	–1.088	–1.046	–1.080
Cl^- paracellular	**–1.071**	–1.347	–1.361	–1.361	–1.340
Ca^{2+} paracellular	**0.000**	–0.118	–0.001	0.000	+0.001
Mg^{2+} paracellular	**0.000**	–0.001	–0.123	0.000	+0.001
Apical membrane potential (mV)	**–24.7**	–33.4	–34.4	–28.3	–29.8

Transcellular fluxes are taken to be positive when solutes are carried out of the cell. Paracellular fluxes are taken to be positive if directed from the basolateral compartment to the ASL. All fluxes are averages integrated over the 15 minutes postdeposition of the hypertonic salts. The apical membrane potential is the value at peak ASL height. The *yellow* cells represent primary deposited cation efflux rates from ASL, while the *green* cells highlight the hyperpolarization of the apical membrane consequent to the (nonpermeating) divalent cation deposition.
ASL, airway surface liquid.

as water is drawn by osmosis out of the cell, intracellular Cl^-, Na^+, and K^+ increase. This enhances ion efflux (or reduces ion influx), which quickly reduces intracellular ion concentrations.

The rate of ion transport is also sensitive to changes in the membrane potential, as further discussed below. Cytosolic sodium concentration remains elevated (Fig. 4C) in the case of NaCl delivery, given the high extracellular concentration of sodium (similar elevations of intracellular sodium occur in epithelial cells exposed to a high sodium diet[45]). Cytosolic chloride concentration remains somewhat elevated in the NaCl case owing to smaller apical membrane polarization associated with the high sodium flux into the cell. Cytosolic potassium concentration (Fig. 4D) actually diminishes, reflecting stimulation of K^+ efflux across basolateral channels due to the relatively small membrane polarization.

For each of the other salt aerosols, the absence of sodium in the deposited solution leads to diminution of sodium within the cell (Fig. 4C, see also Table 2 for a summary of the time-averaged ion fluxes). Reduced apical entry of Na^+ (relative to the NaCl case) further polarizes the apical membrane (note particularly the green cells of Table 2), which favors higher Cl^- efflux through CFTR channels. Conversely, hyperpolarization lowers apical K^+ efflux, reducing the fall in cytosolic potassium (Fig. 4D). In the case of the nonpermeating cation aerosols $CaCl_2$ and $MgCl_2$, intracellular chloride levels remain suppressed through 2.5 hours, reflecting enhanced chloride transport out of the cell (Table 2) owing to hyperpolarization. In the KCl case, intracellular chloride concentration returns to baseline within 45 minutes, reflecting shorter ASL hydration time (Fig. 4A). Figure 4F presents ASL chloride concentrations post-topical delivery, including comparison with mannitol.

Among the four salts, ASL chloride concentrations are most elevated for the divalent salts, in part, as a consequence of the relatively higher flux of chloride through the CFTR channels in $CaCl_2$ and $MgCl_2$ cases (Table 2). Also contributing are chloride concentration differences in the added salt ($[Cl^-]$ 1050 mM for $MgCl_2$ and 900 mM for $CaCl_2$ vs. 855 mM for NaCl and 670 mM for KCl). We chose to simulate mannitol topical delivery at 900 mOsm/L, a hypertonic concentration commonly used in cough provocation testing (representing 3×isotonic, whereas the HS aerosols are ∼5×isotonic). ASL chloride concentration abruptly falls on topical mannitol delivery, reflecting the immediate dilution of chloride in the ASL that occurs postdeposition of the aerosol, a phenomenon that will occur with any nonanion permeating osmolyte.

Hypertonic aerosol hydration and coordination of CFTR flux and ASL height

We simulated numerically the airway hydration and ion kinetics for three hypertonic aerosols with and without the permeating ions—NaCl, $MgCl_2$, and mannitol at 900 mOsm/L each. We sought to determine the propensity in each case for CFTR chloride flux to rise or fall with ASL height, recognizing that coordination tends to alkalinize ASL, while lack of coordination tends to acidify, owing to bicarbonate fluxes through the CFTR channel (43). Our results are presented in Figure 5.

Mannitol deposition leads to relatively high ASL height with relatively prolonged time to peak (Fig. 5A). Lacking the permeating anion, mannitol deposition engenders a high chloride efflux (Fig. 5B) from epithelial cells in the first minutes postdeposition, as has been observed *in vitro* with chloride-free extracellular media.[43] This chloride flux slows down the decrease in ASL osmolarity (which otherwise falls rapidly in the first few minutes) (Fig. 5C), which is predicted to return to 300 mOsm 23 minutes postdeposition, versus 8 minutes for the other two salts (Fig. 5C).

In response, water moves by osmosis out of epithelial cells and into the ASL, leading to peak ASL height at around 25 minutes (Fig. 5A). With the hydration of the ASL increasing significantly over the period of 10–25 minutes postdeposition, CFTR chloride flux falls (Fig. 5B), a combination that has been shown *in vitro* to promote acidification of the ASL, given the role of the CFTR channel in bicarbonate efflux.[43] Hypertonic NaCl delivery leads to an immediate fall in CFTR chloride flux into the ASL on

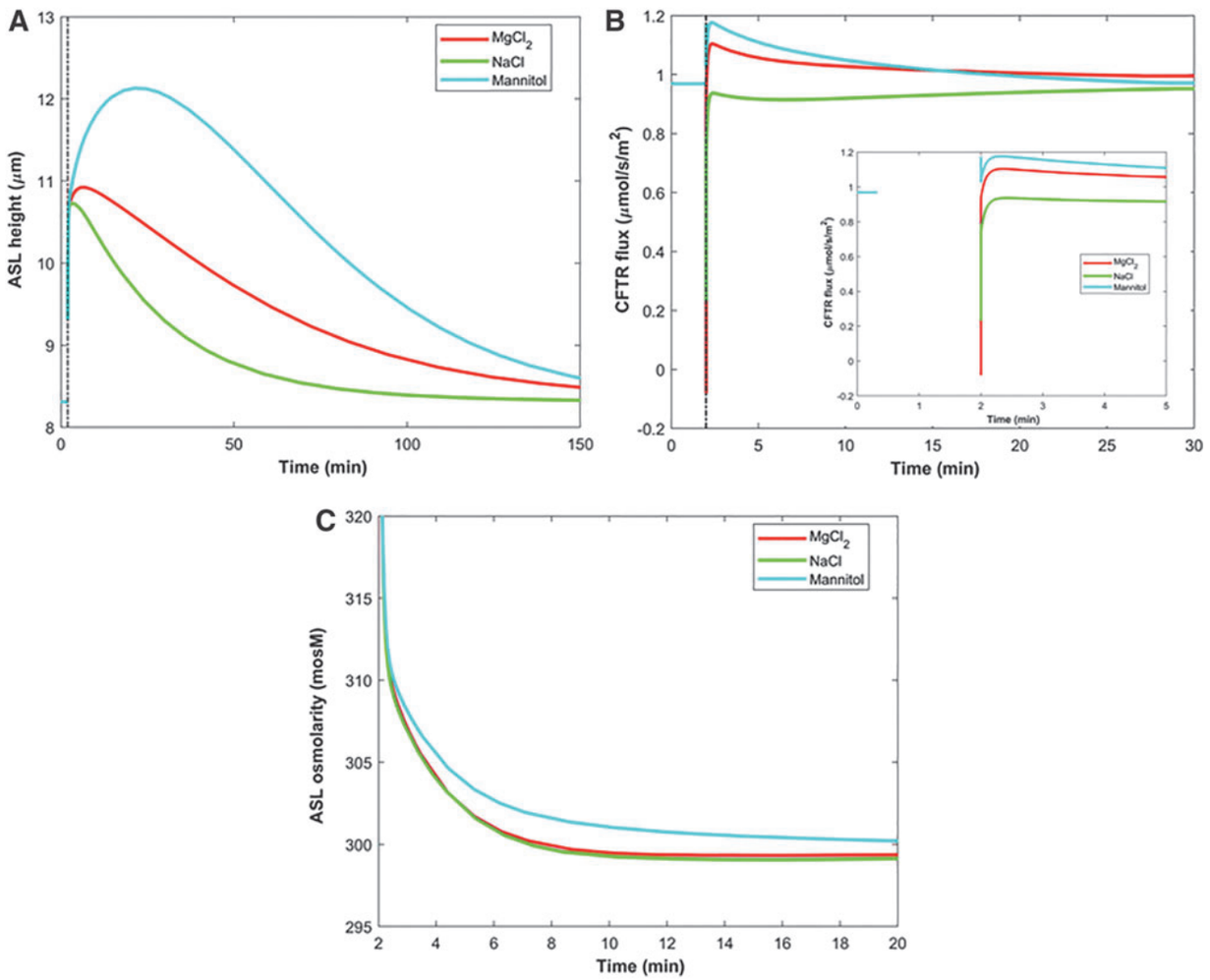

FIG. 5. Numerical simulations of ASL water and ion transport after the deposition of 6 mg of 900 mOsm NaCl, $MgCl_2$, or mannitol. **(A)** ASL height versus time. **(B)** CFTR chloride flux versus time. **(C)** ASL osmolarity versus time.

deposition, owing to the increase in ASL chloride concentration. Lowered polarization of the membrane relative to baseline (predeposition) reduces chloride flux as ASL height rapidly climbs, a combination of phenomena that again appear to increase acidification of the ASL.[43]

Hypertonic $MgCl_2$ leads to a higher CFTR flux starting after $\sim$1 second postdeposition relative to baseline and NaCl (Fig. 5B), owing to hyperpolarization of the apical membrane. It also prolongs hydration of the ASL (Fig. 5A), as does mannitol. However, peak hydration (Fig. 5A) occurs ($\sim$6 minutes) near peak ($\sim$2.5 minutes) CFTR chloride flux (Fig. 5B), and diminishing ASL height (Fig. 5A) is coordinated with diminishing CFTR chloride flux (Fig. 5B), circumstances that alkalinize rather than acidify.[43]

We simulated ASL hydration for other hypotonic (NaCl) and isotonic (mannitol and mixtures of mannitol and NaCl) aerosols known to provoke cough,[46] as shown in Figure 6A. Isotonic mannitol still promotes the lack of coordination between CFTR flux and ASL height rise and thus presents the same inherent features of the hypertonic mannitol studied in Figure 5A–C. Hypotonic NaCl promotes immediate swelling of epithelial cells and loss of ASL volume, leading to an augmentation of the overall pressure on epithelial cells, and, as demonstrated elsewhere,[6] secretion of ATP, which provokes cough by P2X3 receptor activation.[6]

This increase in ATP leads to further acidification risk. Others have shown that prolonged elevation of extracellular ATP results in elevated cytosolic calcium[47] and reduction of CFTR channel expression[48] by $\sim$87% following 12 hours of elevated intracellular calcium.[47] Figure 6B therefore presents the simulated ASL Cl^- concentration following an 87% reduction in CFTR permeability. ASL chloride concentration falls by 10%–15%, a degree of diminution of Cl^- concentration that has been observed experimentally in the exhaled breath condensate of chronic cough[49] and cystic fibrosis patients[50] alike, while cytosolic chloride concentration increases by $\sim$30%, reflecting decreased chloride and bicarbonate secretion into the ASL following CFTR downregulation.

Discussion

Voice is a sensitive diagnostic for laryngeal hydration, and has been studied for many years.[51] While the experience

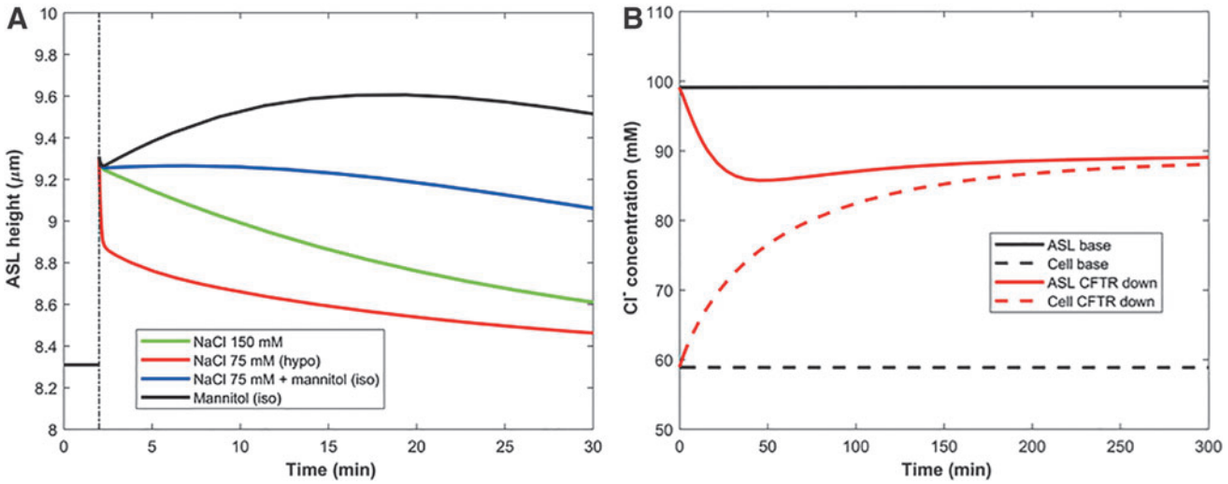

FIG. 6. **(A)** Numerical simulations of ASL height after the deposition of 6 mg of isotonic NaCl, hypotonic NaCl, isotonic 50:50 NaCl: mannitol, and isotonic (300 mM) mannitol. **(B)** Numerical simulations of ASL and cytosolic chloride concentration after 87% reduction in CFTR chloride permeability.

of voice dependence on hydration is as common as singing, a clear understanding of how voice production varies with airway hydration remains elusive.[23] A direct correlation between airway hydration and viscosity of vocal fold tissue has been hypothesized as a possible origin of hydration dependence of the pressure required to make sound, that is PTP,[52] theorizing that as hydration lowers viscous resistance to vocal fold wave propagation, the stress of vocal production diminishes.[24,25]

Our human clinical trial results confirm that PTP falls immediately postinhalation for each of the hypertonic salts (Fig. 2) and in direct proportion to ASL volume and/or specific salt osmolarity (Fig. 4A). At least in the first 15 minutes postinhalation, PTP reduction therefore appears less a reflection of hydration impact on vocal fold viscosity than on the viscous resistance in the superficial mucosal layer of the ASL. PTP suppression following inhalation of the hypertonic salts was observed beyond 30 minutes only at the low frequency of voice (Fig. 3), not at the high frequency, and only for the salts (NaCl and $MgCl_2$) with the highest osmolarity (Fig. 2A, D).

Highest frequency vocal fold waves have shortest wave length, therefore weakest tissue penetration, the insensitivity of the high-frequency PTP values on topical hydration indicates that after 30 minutes of topical delivery, tissue hydration may be most responsible for PTP suppression (in the low frequency voice range). The longest hydrating salt with the highest osmolarity ($MgCl_2$) yields the longest PTP suppression (beyond 2 hours) (Fig. 2D). Assuming this longer term suppression of PTP reflects a diminution of vocal fold tissue viscosity, and given that vocal fold viscosity dependence on hydration has been reported to range between 0.1 and 1.0 Poise,[53] our findings (Fig. 2D) suggest that Mg^{2+} topical hydration reduces vocal fold viscosity, ~20% of the maximal reported hydration range.

It has been observed that the impact of airway hydration on cough, otherwise provoked by the breathing of dry air,[54] associates with its impact on phonation, and indeed strategies to reduce dysphonia have proven helpful in the reduction of cough.[55] Mechanistically, it has recently been proposed that water evaporates from upper airway mucus by transpiration,[6] a process that delivers stresses on underlying epithelia as a leaf produces pressures capable of lifting water from distant roots.[56] These stresses can promote inflammation and ATP secretion on the mouth breathing of dry air, provoking cough.[6]

Prolonged elevated ATP further has a consequence of reducing CFTR activity, a hallmark of airway dehydration, as observed in reduced chloride ion concentration in exhaled breath condensate of cystic fibrosis patients,[49] and of sufferers of chronic cough.[48] In the numerical analysis of this study, we have shown that such reduction of CFTR activity produces conditions that acidify the airways (Fig. 6B) similar to the inhalation of hypertonic mannitol or NaCl. Recognizing that acidity activates TRPV channels to promote cough and bronchoconstriction,[44] it may be that this second pathway to the provocation of cough by the breathing of dry air, through the acidification of the upper airways, links provocation of cough by hyperpnoea[54] with cough provocation by hypertonic NaCl and mannitol.[57]

Our numerical simulations predict that hypertonic aerosols of NaCl and mannitol acidify the airways either by the presence of the predominant permeating cation (Na^+) or by the absence of the permeating anion (Cl^-). Lacking the permeating anion, hypertonic aerosols drive an increase in ASL volume, with a decrease in CFTR flux (Fig. 5A, B). Possessing the permeating cation, hypertonic aerosols drive an immediate fall in CFTR flux followed by a rapid rise in ASL volume (Fig. 5A, B). Both behaviors provoke acidification.[43]

These behaviors do not occur with compositions that include the permeating anion, while avoiding the permeating cation ($MgCl_2$ and $CaCl_2$) (Fig. 5A, B). We further predict that the divalent cations (Ca^{2+} and Mg^{2+}) clear more slowly from the ASL postdelivery than the monovalent cations (Na^+, K^+) (Table 2), leading to longer acting airway hydration for these nonpermeating cation salts (Fig. 4). This phenomenon (and the relatively high osmolarity of 5%

$MgCl_2$ vs. 5% $CaCl_2$) appears to explain the relatively long reduction (beyond 2 hours) of PTP postdelivery of $MgCl_2$ (Fig. 2A) in our clinical study.

The principal results of our clinical study (Figs. 2 and 3) indicate that hypertonic aerosol solutions can be delivered rapidly and simply to the upper airways by generating large (8–15 μm) rather than small (3–6 μm) droplet sizes. Aerosols of isotonic saline, and other isotonic and hypotonic solutions, have frequently been delivered to the airways in attempts to hydrate the larynx for various diagnostic, therapeutic, and vocal relaxation purposes.[21,23,59]

Such aerosols have generally been designed[58] with MMAD in the range of 3–6 μm, leading to penetration of aerosols into the central and lower airways, and typically requiring for effectiveness 15 minutes or longer of delivery through a nebulizer.[21] This study design of the hydrating aerosol with MMAD 13 μm targets deposition of the aerosol to the upper airways,[34,35,60] while delivering greater mass per unit of time, given that mass per droplet grows with the cube of the diameter of the droplet. This allows delivery of an effective dose rapidly from a simple hand-held spray pump device (in this study, $\sim$20 seconds relative to a 15-minute nebulizer time).

The results of our study indicate that four nasal inhalations effectively hydrate the larynx, with a laryngeal/tracheal dose of 5% salt $\sim$300 μg (5% of 6 mg deposited dose) following four nasal inhalations.[34] Magnesium has been delivered as an isotonic (sulfate) salt with inhaled doses of 2.5–10 mg (10–100 times greater doses than in this study), in over 25 published human clinical trials,[61,62] as a potential bronchodilator, with modest or insignificant benefit lacking the hydration benefit inherent in the hypertonic aerosol.

Magnesium is stored in the body largely in bone, while also in other body tissues and naturally processed by the body at doses far above those reached by inhalation of HS aerosols in this study, for example, magnesium is taken in pill form as an oral supplement at daily levels of 65–300 mg/day, while injected magnesium is commonly administered at doses of 2 g.[63] Calcium chloride and potassium chloride are commonly delivered to the upper airways in salt solutions such as Ringer's solution currently on the U.S. market today as Flo Mist.

Further research is needed to clarify the practicality of a ''PTP diagnostic'' for assessing laryngeal dehydration, as would be valuable in the determination of potential dehydration origins of upper airway inflammation and dysfunction. In our clinical study, PTP values fell following an initial vocal load task, as has been reported elsewhere.[42] At the high and low frequencies of voice (Fig. 3A, B), PTP remained steady for the placebo control over the 2 hours of phonation testing, suggesting that, at least when data collection is performed in the morning hours, shortly after awakening and without significant pretesting phonation, a period of 45 minutes of vocal exercise is sufficient to reach a steady PTP value with normal phonation (what we have characterized as ''at rest''). PTP values did fall over time for the placebo in the case of the intermediate pitch (Fig. 3C).

Singers are familiar with the concept of a voice break when dealing with the zona di passaggio, the zone of acoustical instability in the voice, which results in a moment of irregular vibration of the vocal folds. This level of instability in the vibration of folds happens as a voice user ascends in pitch throughout the range, and could be a determining factor in the unreliability of the intermediate pitch, as has been observed elsewhere.[42]

With elevated phonation and other high minute-volume conditions such as strenuous exercise, the parameters used in this study to assess laryngeal hydration by PTP measures will inevitably differ, and should be determined if PTP is to serve as a practical laryngeal hydration diagnostic. Further research is also needed to measure ASL acidity postexposure to dry air and delivery of hypertonic aerosols, and to clarify the role of acidity in the provocation of cough and bronchoconstriction by hyperosmolar aerosols such as mannitol and NaCl.

Many contemporary factors contribute to dehydration of the human larynx and trachea, and appear to contribute to the worsening of human respiratory health over recent decades. Hypertonic aerosols targeted to the upper airways may be useful for the diagnosis, prophylaxis, and treatment of such dehydrated human airways. Recognition of the composition specificity of hypertonic aerosol impact on upper airway function and dysfunction may eventually lead to a broader set of options for the management of cough, asthma, and other respiratory illnesses worsened by today's environmental and human behavior trends.

Authors' Contributions

A.F.Z. led the design and execution of the clinical trial research, interpreted results of experiments, participated in the drafting of the article, and co-approved the final version of article. A.E. designed the theoretical research, performed all numerical simulations, interpreted results, participated in the drafting of the article, and co-approved the final version of article. D.B. participated in the interpretation of results of experiments and in the drafting of the article. D.A. participated in the conception of the research, and in the interpretation of results of the experiments. D.A.E. led the conception of the research, participated in the design of the research, led interpretation of results and drafting of the article, and co-approved the final version of article.

Author Disclosure Statement

D.B., D.A., and D.A.E. are, respectively, the employee, board member, and founder of the company that produces the inhaler used in the clinical trial. All other authors have no conflicts of interest.

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Supplementary Material

Supplementary Data

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