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Impact of high- and low-flow nebulized saline on airway hydration and mucociliary transport

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Take-home Message (242/256 characters):

Nebulized isotonic saline, delivered with high-flow of humidified air in the perfused tracheal model, hydrates the airway surface and increases the mucus transport velocity without suppressing beating cilia as observed with hypertonic saline.

ABSTRACT

Nebulized drugs, including osmotic agents and saline, are increasingly used during non-invasive respiratory support including nasal high-flow therapy. The authors conducted an *in vitro* study to compare the hydration effect of nebulized isotonic 0.9% and hypertonic 7.0% saline on mucociliary transport.

In a perfused organ bath, 10 sheep tracheas were exposed to 7.5 mL nebulized 0.9% and 7.0% saline entrained into heated (38 °C) and humidified air delivered at high- and low-flow (20 and 7 L/min, respectively). Simultaneous measurements of the airway surface liquid height, mucus transport velocity, cilia beat frequency and surface temperature were made over time. The data was presented as mean±SD.

The airway surface liquid height increased significantly with both 0.9% and 7.0% saline: at low-flow by 37.2±10.0 µm and 152.7±10.9 µm, and at high-flow by 62.3±5.6 µm and 163.4±25.4 µm ($p<0.001$), respectively. Mucus velocity was increased by both 0.9% and 7.0% saline from a baseline of 8.2±0.8 mm/min to 8.8±0.7 mm/min and 17.1±0.5 mm/min with low-flow and at high-flow to 9.8±0.02 mm/min ($p=0.04$) and 16.9±0.5 mm/min ($p<0.05$), respectively. Ciliary beating did not change with 0.9% saline but declined from 13.1±0.6 Hz to 10.2±0.6 Hz and 11.1±0.6 Hz ($p<0.05$) with 7.0% saline at low- and high-flow, respectively.

The findings demonstrate that nebulized isotonic 0.9% saline like hypertonic 7.0% saline significantly stimulates basal mucociliary transport and the use of high-flow delivery had no significantly different hydration effects compared with low-flow. Hypertonic 7.0% saline suppressed ciliary beating indicating an increase in airway surface liquid osmolarity, which may have negative effects on the airway surface with frequent use.

INTRODUCTION

Large conducting airways are lined by a continuous ciliated epithelium with an overlying airway surface liquid (ASL) consisting of periciliary and mucus layers that protect the airway from desiccation and infection. The periciliary layer is composed of a less viscous fluid, which facilitates the beating of the motile cilia. Overlaying the periciliary layer is a mucous blanket that contains more viscous mucins secreted from specialized respiratory epithelial cells and glands. The mucus traps inhaled foreign particles and microorganisms which are moved in the mucus by the beating cilia to the larynx for swallowing. To prevent evaporation from the ASL and maintain a thermodynamic balance, inspired air is heated to body temperature and humidified to pressure saturation (BTPS) in the upper airway, which is known to be essential for mucociliary transport[1].

In muco-obstructive airway diseases, mucins are hyper-secreted into the ASL[2]. This results in an insufficiently hydrated ASL with compromised physiological viscoelastic properties which slow mucociliary transport and lead to mucostasis and cough[3]. Nebulized liquid formulations are increasingly being used in non-invasive respiratory support such as nasal high-flow therapy because of the simplicity in application and tolerance by patients related to humidification of the delivered gas[4]. However, aerosols entrained in a high-flow system of humidified air can be susceptible to condensation growth[5, 6], become diluted by high-flow and are thus less effective.

Various osmotic agents are used to decrease the viscosity of accumulated airway mucus[3]. Radioactive aerosols have been shown to be cleared more rapidly in cystic fibrosis patients following administration of nebulized hypertonic saline (HS) solutions in a concentration-dependent fashion[7]. However, increasing HS concentrations frequently leads to patients reporting pharyngeal irritation[7], likely to result in a lower tolerance of this therapy. Inhalation of nebulized isotonic saline (IS) improves clinical signs and lung function in children

with cystic fibrosis[8] and decreases breathlessness scores in chronic obstructive pulmonary disease patients[9].

Little is known about how nebulized IS and HS solutions are effective, the untested rationale being that the osmotic agents act through "moistening of the airway surface"[9], hydrating the ASL[10] and therefore resulting in improved mucociliary transport[7]. A recent multicentre trial in children with cystic fibrosis aged 3 to 6-years showed that the use of nebulized HS twice-daily for one-year had a positive effect on structural lung changes[11]. A double-blind, randomized controlled crossover trial in patients with primary ciliary dyskinesia found no significant improvement in quality of life after twice-daily inhalations of 7% HS or IS for 12-weeks[12]. However, adverse events were more frequent with HS. The authors believe the lack of improvement in quality of life could be due to the control treatment with IS also having therapeutic effects on cilia and ASL[13]. The authors also note that the oral administration of saline may not be the best method of delivery, as upper airways are also commonly affected in this heterogeneous disease. *In vitro* studies have shown that nebulized HS increases the ASL[10] and suggested that the deposition of HS causes osmotic changes that lead to an increase in transepithelial water flow through respiratory epithelial cells which act as osmometers for the regulation of ASL[14]. Administration of HS has been shown to result in the rapid (less than 5-minutes) reduction of the epithelial cell heights which persists for over 4-hours[10]. The cilia beat frequency (CBF) has been reported to decrease immediately after exposure to HS, not IS or hypotonic saline, and results in cilia stasis within 5-minutes[15]. This has been associated with histological changes in the epithelium, indicative of cell damage from fluid transport towards the surrounding medium. It is not known how those findings are applicable to nebulized saline delivered with high-flow.

The study was designed to test a hypothesis that both nebulized IS and HS solutions of sodium chloride (NaCl) hydrate the ASL and stimulate mucociliary transport when delivered with high-flows of humidified air.

METHODS

For full materials and methods, see the Supplemental Material: Part 1. Ten tracheas, harvested from healthy sheep immediately after slaughter, were placed in the experimental set-up (Figure 1) to measure changes on the airway surface in response to nebulized saline solutions, entrained into BTPS air at 7 L/min and 20 L/min, mimicking low- and high-flow delivery systems, respectively. Baseline measurements were made over the first 15-minutes with just BTPS air. As previously reported[1], mucociliary transport, in terms of mucus transport velocity (MTV) and CBF, on the airway surface was measured with infrared macro-imaging, which also allowed the authors to measure the airway surface temperature for monitoring thermodynamic changes. The ASL height was simultaneously and continuously monitored using a laser displacement sensor. The nebulized saline and the effect of relative humidity on the particles' size during high-flow delivery were characterized by measuring the mass median aerosol diameter (MMAD) with an optical particle sizer, when the nebulizer was connected to the humidification chamber outlet (Figure 1A). The MMAD is defined as the diameter at which 50% of the particles of an aerosol by mass are larger and 50% are smaller. Aerosol delivery with a flow of 7 L/min was not measured due to the technical limitation of the method given the high concentration of particles in the optical path and over-saturation of the signal.

Two tracheas were used for morphology to measure the potential effect of IS and HS on the tracheal mucosal height over time. The airway surface was directly exposed to IS and HS solutions, then a 4 µm section of each was placed in 10% formalin after 0, 5, 10 and 15-minutes, 24-hours prior to routine histological processing and preparation with haematoxylin and eosin stain for examination under a light microscope. The heights of three regions of intact mucosae (minimal artefact) on each tissue section were measured.

GraphPad Prism (V8.3.0) was used for statistical analysis of measurements. Significance testing was performed with a two-tailed paired t-test as appropriate, and a two-way ANOVA Tukey test where $p < 0.05$ was statistically significant. All data was tested for normality with D'Agostino & Pearson test and presented as mean ± SD.

RESULTS

Representative real-time changes in the airway surface parameters are presented in Figure 2. Mean changes of the airway surface parameters are presented in Figure 3.

Baseline. During baseline data collection, when the airway surface was exposed to BTPS air at high-flow (20 L/min), the average ASL height (3.3±3.3 µm), airway surface temperature (38.6±0.4 °C), MTV (8.5±0.9 mm/min) and CBF (13.2±0.5 Hz) were within normal ranges previously described[16] and were not significantly different from the baseline measures at low-flow (7 L/min) (Figure 3) (Supplemental Material: Part 2).

High flow with IS. When entraining IS into high-flow BTPS air, the average ASL height and MTV increased significantly, by 20-fold to 62.3±5.7 µm ($p < 0.001$) and 15% to 9.8±0.2 mm/min ($p < 0.05$), respectively. In contrast, the airway surface temperature declined significantly to 37.7±0.2 °C ($p < 0.05$) (Figure 3). CBF did not change significantly. Real-time data from a representative experiment (Figure 2A) showed that the ASL height increased rapidly in the first 5-minutes of IS nebulization before stabilizing.

High flow with HS. Exposing the airway surface to HS entrained into high-flow BTPS air resulted in a significant increase ($p < 0.001$) from baseline in the average ASL height (167.2±29.0 µm; a 55-fold increase) and MTV (16.9±0.5 mm/min; a two-fold increase) (Figure 3). There was a significant decline ($p < 0.05$) in the average airway surface temperature (to 36.4±0.4 °C) and CBF (to 11.1±0.6 Hz) compared to baseline values. The real-time data from

a representative experiment (Figure 2B) demonstrated that the ASL height increased continually over the recording period.

Low flow with IS. The introduction of nebulized IS into low-flow BTPS air resulted in a significant increase in the average ASL height (to 37.2±10.0 μm; $p < 0.001$) and average MTV (by 10% to 8.8±0.4 mm/min; $p < 0.05$). There were no significant changes to the average airway surface temperature or average CBF following IS administration (Figure 3).

Low flow with HS. Entraining HS into low-flow BTPS air resulted in similar findings to the high-flow results presented above. There was a significant increase ($p < 0.001$) in the average ASL height (by 40-fold to 124.7±16.6 μm) and average MTV (by 2-fold to 17.1±0.5 mm/min) with a significant decline in airway surface temperature (to 37.8±1.3 °C; $p < 0.05$) and CBF (to 10.2 ± 0.1 Hz; $p < 0.05$) (Figure 3).

Nebulized saline particle size. Mass median aerosol diameters (MMAD) of the nebulized IS and HS solutions are presented in Figure 4 and in Supplemental Material: Part 3. At high flow (20 L/min) the concentration of the nebulized saline had a significant effect on particle size with the MMAD of IS particles being smaller (1.1±0.3 μm) than those of HS particles (1.7±0.1 μm) when delivered with BTPS air. Increasing the flow rate from 20 to 40 L/min did not have a significant effect on the IS or the HS particle size. However, changing the carrier air temperature and humidity had a significant effect on particle sizes, except when nebulized IS was exposed to air with 80% and 60% relative humidity (Supplemental Material: Part 3).

Histology. There was a significant ($p < 0.05$) decrease in the mucosal height of the tracheas exposed to liquid HS from 64.4±4.5 μm to 35.2±3.7 μm after 15-minutes, a 45% decline (Figure 5), whereas there was no change in mucosal height for the tracheas immersed in IS after 15-minutes.

DISCUSSION

The results demonstrate that nebulized IS and HS significantly increased ASL height, stimulating mucociliary transport by increasing MTV, and the high-flow delivery of nebulized saline did not reduce this hydration effect. HS, relative to IS, increased ASL height three-fold (167.2±29.0 μm vs 62.3±5.7 μm when delivered at high-flow and to 124.7±16.6 μm vs 37.2±10.0 μm when delivered at low-flow) and doubled MTV but suppressed CBF. A summary of the key findings is schematically presented in Figure 6. The transitory ciliostatic effect from HS exposure could be due to ASL hyperosmolarity, removing water from the epithelial cells causing dysfunction. Contrary to HS, the nebulized IS can be considered optimal for hydration of the airway surface without suppressing ciliary function.

The basal mucociliary transport parameters, measured as MTV and CBF, were stable and comparable to previous reports[1, 16-18], indicates the thermodynamic balance during exposure to flowing air heated and humidified air to BTPS over the epithelium. There was a small but significant decline in airway surface temperature when nebulized saline was introduced in spite of the precise control of BTPS condition of air-flow that carried the aerosol. The temperature drop most likely occurred as a result of water condensation on the ASL surface according to Nusselt's film theory[19]. To determine the thermal effect, the heat transfer across the ASL was estimated (Supplemental Material: Part 4) and suggested that the underlying epithelium was insulated from the decreased surface temperatures by the large increases in the ASL height associated with the administration of the nebulized saline. The protecting effect of ASL on the tracheal epithelium is confirmed by a poor correlation between the airway surface temperature and the MTV ($R^2 = 0.15$) and CBF ($R^2 = 0.10$); this suggests there were no inhibitory effects on the basal mucociliary transport, which is known to be sensitive to changes in temperature[20].

Nebulized IS had an immediate effect on the ASL height, increasing it 20-fold, the finding consistent with a previous report that the ASL increased by 20 μm when human bronchial cells cultures were exposed to nebulized IS for 15-minutes[10]. The increases in ASL following administration of saline might be due to deposition of the NaCl particles and associated water, and/or as a result of osmotic activity drawing fluid from the underlying epithelial cells. An approximation of the ASL-height changes during nebulization of IS (Supplemental Material: Part 3) revealed that the measured ASL height could be achieved from just the nebulized IS particles depositing on the surface, with the ASL remaining iso-osmolar during the nebulization, and without triggering any large transepithelial water fluxes.

The changes in the ASL height were strongly correlated with MTV ($R^2 = 0.93$) that supports a role of hydration in mucociliary transport. Similar to a previous report[10], the administration of HS had an immediate effect on the ASL height; however, the increase was considerably greater than that seen with IS. Hyperosmolarity of the ASL has been shown to trigger the release of mediators and histamine[21], and mucin hypersecretion[22]. Mucin secretion and water absorption is a very rapid process; the individual mucin granules release their contents in only 0.1-second[23] and these absorb several 100-fold their mass in water, resulting in an increase in mucus volume within a second[24]. Although increases in the ASL have been associated with increased rates of mucociliary transport in the upper airways[25], such changes might be harmful in the smaller airways causing obstruction[24]. During exposure to HS, the ASL height did not stabilize in the measurement period (Figures 2D and 4B) and indicates that the ASL height may keep rising if nebulization continued, which could lead to excessive fluid in the airways, occlusion of small bronchi and cough. This would seem to be especially the case in patients with muco-obstructive disease[2] with mucin hypersecretion, which has been linked to increased ASL osmotic pressures and reduced mucociliary transport[26].

CBF did not change with IS administration, but it was decreased significantly with exposure of the epithelium to nebulized HS at both low- and high-flow rates. The decrease in the CBF with HS was associated with an increase in MTV. Although CBF has been reported to be directly related to MTV under physiological conditions[27], this relationship is not present in HS[1, 16] and during an exposure of the airway surface to unidirectional flow of cooler and drier air (Supplemental Material: Part 5). This disproportional relationship was explained by the shear thinning properties of mucus[1], where the sliding of the mucus layer during both effective and recovery strokes of the beating cilia allowed the MTV to far exceed the displacement cilia could impart[1, 16]. In the current study a substantial increase of ASL height by the nebulized HS was caused by an increased osmolarity, which is known to decrease CBF[28]. The hyperosmolarity could result in excessive fluid transport out of the cells, as reported previously[10, 29, 30] and demonstrated by the 43% decrease in mucosal height that was found following direct exposure of the tracheal epithelium to a HS solution (Figure 5). Damage to the epithelial cells[15], or dysfunction resulting from the cell shrinkage, might have contributed to the decreased CBF. Such changes to the epithelial cell layer might also explain the pharyngeal irritation side effect reported with HS nebulization[7].

Cilia movement is powered by dynein, a cytoskeletal motor protein that uses adenosine triphosphate (ATP) hydrolysis for energy. This generates force and movement on microtubules, leading to cilia bending [31]. The dynein ATPase activity of the axoneme regulates CBF and a reduction in CBF has been well established[32]. In a study on the effects of osmotic agents, Nadesalingam et al. (2015) [33] found that HS suppresses neutrophil extracellular trap formation and promotes apoptosis by reducing nicotinamide adenine dinucleotide phosphate oxidase activity. The authors suggest that neutrophil dehydration, caused by equimolar concentrations of choline chloride, a-mannitol and d-sorbitol, produced the same suppressive effect. However, a recent study by Mazzitelly et al. (2022) [34] found the opposite, concluding

that high salt concentration, rather than osmolarity, led to activation of the neutrophil response. Regardless of the mechanism, it can be speculated that ATP hydrolysis in ciliated epithelial cells may also be affected by HS and reduce CBF.

Smaller MMAD of IS relative to HS particles (Figure 4), which also did not change when flow was doubled from 20 to 40 L/min, may also indicate on advantage of delivery of the nebulized IS to distal airways during non-invasive respiratory support. Reduction of relative humidity of air in the breathing circuit may reduce MMAD of the nebulized IS (Supplemental Figure 1), which may further increase the efficiency of high-flow delivery of saline. Also, control of relative humidity in the high-flow system can potentially be used for targeted delivery as smaller particles tend to deposit more distally in the bronchial tree[35][31].

The study has several limitations. The excised trachea from healthy sheep may not truly represent the pathology in humans with mucus hypersecretion. However, the whole trachea maintained in the organ bath and ventilated with air conditioned to BTPS allowed the thermodynamic balance to be maintained on the mucosal surface, thereby excluding any effects of evaporation on the mucosal surface that may occur during inhalation of unconditioned air. While the results demonstrate that nebulized IS significantly increases the ASL and MTV in the basal mucociliary transport, the effects still need to be confirmed in studies on patients with a muco-obstructive airways disease. Nevertheless, it appears unlikely that the negative effect of HS on the ciliated cells could not be present in patients with muco-obstructive diseases based on the low tolerance reported in the literature[7]. The unidirectional flow did not truly represent the tidal breathing when heat and moisture are recovered during expiration, and kinetics on the nebulized particles would greatly depend on the breathing pattern. The use of unidirectional flow meant that relatively low-flow settings were used in the experimental protocol, which can be viewed as a major limitation. During normal tidal breathing of unconditioned room air via a mouth T-piece, tracheal or nasal cannula interface, flow is expected to exceed 20 L/min in

the trachea of an adult; however, no significant thermodynamic changes are observed on the ASL because of heat and moisture recovery following expiration[1]. To incorporate tidal breathing in this *in vitro* experimental set-up, it would require a complex lung simulator with heated and humidified air involving the complex control. The airway epithelium in the perfused organ bath is very sensitive to reduced temperature and humidity of air-flow, as previously demonstrated in a similar experimental model[14]. Flows of 7 L/min and 20 L/min allowed the authors to investigate the effect of a three-fold increased dilution of nebulized IS and HS delivered to the tracheal epithelium. Nebulization of saline at flows higher than 20 L/min and the effect of repeated nebulizations have not been tested in the model but these research questions could be addressed in future clinical studies. Deposition of the nebulized saline particles on a flat tracheal surface could be different from the deposition on the surface of the circular-shaped organ but the set-up allowed several key physiological parameters to be measured including real-time changes in ASL height. The histological examination was performed on tissue exposed to liquid forms of IS and HS to demonstrate the maximum effect of HS on the epithelial cells over a typical nebulization period, allowing for the maximum increase of the ASL to be estimated. According to the study protocol, the measurements were performed only during the nebulization period (15-minutes) and reabsorption of the increased ASL volume after nebulizing was not studied. There are reports of the ASL returning to initial levels within 60 minutes[10]. Finally, the effect of relative humidity of the carrier air transporting the nebulized saline on mucociliary transport and ASL was not studied but a reduction of humidity and the particle size changes through condensation growth[36, 37] was investigated. This may help to target deposition in the airway, particularly during delivery of the nebulized solutions with nasal high flow where larger particles are retained in the nasal passages[5](Supplemental Material: Part 3). The analysis showed that the carrier air condition had a significant effect on the particle size while the air-flow rate did not.

The study demonstrates that the high-flow vs low-flow delivery did not reduce the hydration effect of the nebulized saline and IS, like HS, significantly increases the ASL height and MTV. Although HS appeared to produce a greater increase of MTV and ASL height, these changes might have been associated with epithelial cell dysfunction and may inflict damage in the long term. The study may serve as a rationale for further investigations into the frequent use of nebulized IS or other physiological solutions that could be delivered with nasal high-flow or any alternative forms of non-invasive respiratory support in patients with muco-obstructive diseases and delayed mucus clearance; particularly when the upper airways are affected, which is common in cystic fibrosis and primary ciliary dyskinesia.

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DISCLOSURE

SK and ST are employees of Fisher & Paykel Healthcare Limited.

AUTHOR CONTRIBUTIONS

ST conceptualised the study. SK, MV, WHC and ST performed the experiments. SK analysed the data. SK, WHC, MV and ST interpreted the results of the experiment. SK prepared the figures. SK drafted the manuscript. SK and ST edited and revised the manuscript. SK, MV, WHC and ST approved the final version of the manuscript.

FIGURE LEGENDS

Figure 1. Experimental set-up to study the effects of nebulized isotonic (IS) (0.9% NaCl) and hypertonic (HS) (7.0% NaCl) saline delivered in air at body temperature and pressure saturated (BTPS) condition (38 °C, dew point 38 °C) at low- (7 L/min) and high-flow (20 L/min) rates over the airway surface. The sheep trachea was mounted flat in an organ bath with the epithelium facing upwards. The IS and HS solutions were nebulized with a vibrating mesh nebulizer (7.5 mL over a 15-minute period, in accordance with clinical practice) at (A) the humidification chamber outlet port with high flow to mimic delivery during non-invasive respiratory support and at (B) the organ bath with low flow. A high-speed infrared camera with macro lens was used to measure the airway surface temperature, mucus transport velocity (MTV) and cilia beat frequency (CBF) on the airway surface. A laser displacement sensor was used to measure changes in the airway surface liquid (ASL) height for the duration of the experiments.

Figure 2. Representative real-time changes in airway surface parameters with nebulized saline solutions delivered onto sheep tracheas at high flow (20 L/min) and low flow (7 L/min). Measurements of the airway surface liquid (ASL), mean airway surface temperature, cilia beat frequency (CBF) and mucus transport velocity (MTV) were made with a high-speed infrared macro-imaging camera and laser displacement sensor during a 15-minute period. Baseline data for each parameter was collected over the first 15-minute recording period when the mucosal surfaces of the tracheas were exposed to body temperature and pressure saturated (BTPS) air (38 °C, dew point 38 °C). Over the next 15-minute recording period, parameters were measured when the tracheas were exposed to (A) nebulized isotonic saline (IS) (0.9% NaCl) at high flow and (C) at low flow or (B) hypertonic saline (HS) (7.0% NaCl) nebulized at high flow or (D) at low flow.

Figure 3. Mean changes in airway surface parameters with nebulized saline solutions delivered onto sheep tracheas at different flow rates. Surface parameters measured were airway surface liquid (ASL) height, airway surface temperature, cilia beat frequency (CBF) and mucus transport velocity (MTV). Mean baseline values (white boxes) for each parameter were recorded with air at body temperature and pressure saturated (BTPS) air (38 °C, dew point 38 °C) at high- (20 L/min) and low-flow (7 L/min) particle delivery rates. Values with nebulized isotonic (0.9% NaCl) (light-grey boxes) and hypertonic (7.0% NaCl) (dark-grey boxes) saline solutions were also measured at low- and high-flow rates. * = significant difference.

Figure 4. Change in mass median aerosol diameter (MMAD) of nebulized isotonic (0.9% NaCl) and hypertonic (7.0% NaCl) solutions when delivered with body temperature and pressure saturated (BTPS) carrier air at high-flow rates (20 and 40 L/min) via a heated breathing circuit.

Figure 5. Representative histological images showing the mean (N=6) mucosal height (labelled length in images) of tracheal epithelial cells at baseline (t = 0) and after t = 5, 10 and 15 minutes of exposure to isotonic (0.9% NaCl) and hypertonic (7.0% NaCl) saline solutions. The bars on the images represent 50 µm. A figure demonstrates a reduction of mucosal height caused by the hypertonic saline solution.

Figure 6. A summary of the key findings. Nebulized isotonic 0.9% saline delivered with 20 L/min of humidified air increased the airway surface liquid (ASL) by ~500%, assuming the basal ASL thickness at baseline is 10 µm, and accelerated mucus transport velocity (MTV) by 5% without any effect on cilia beat frequency (CBF). Hypertonic 7.0% saline increased the

ASL by ~1600% and MTV by 80% but caused a reduction of CBF by 15%, which may indicate a ciliostatic effect.

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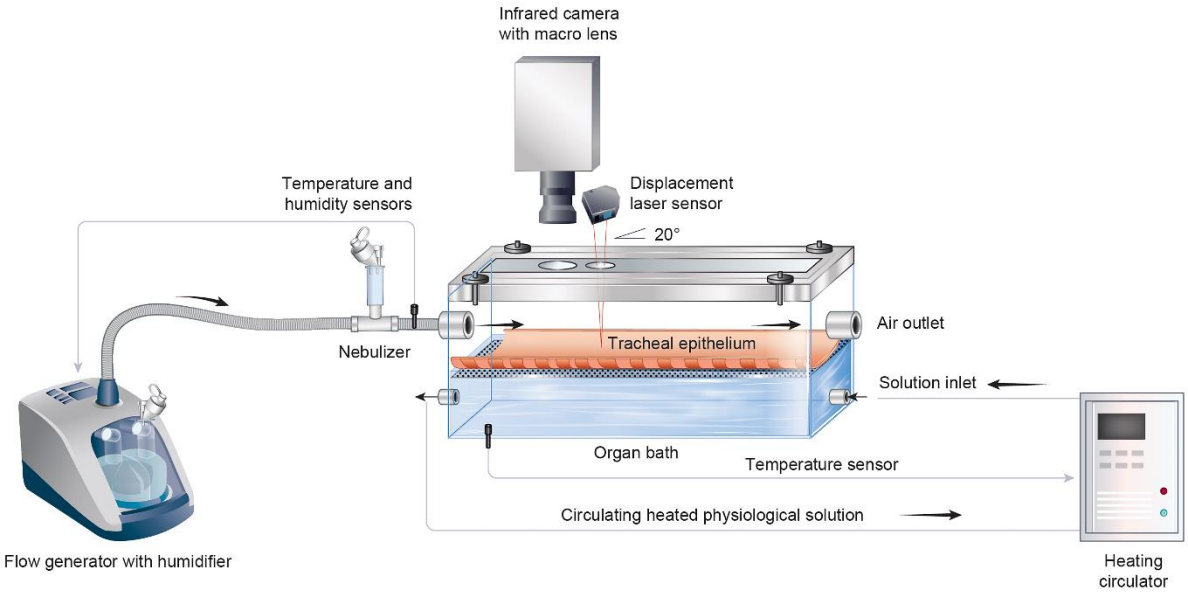
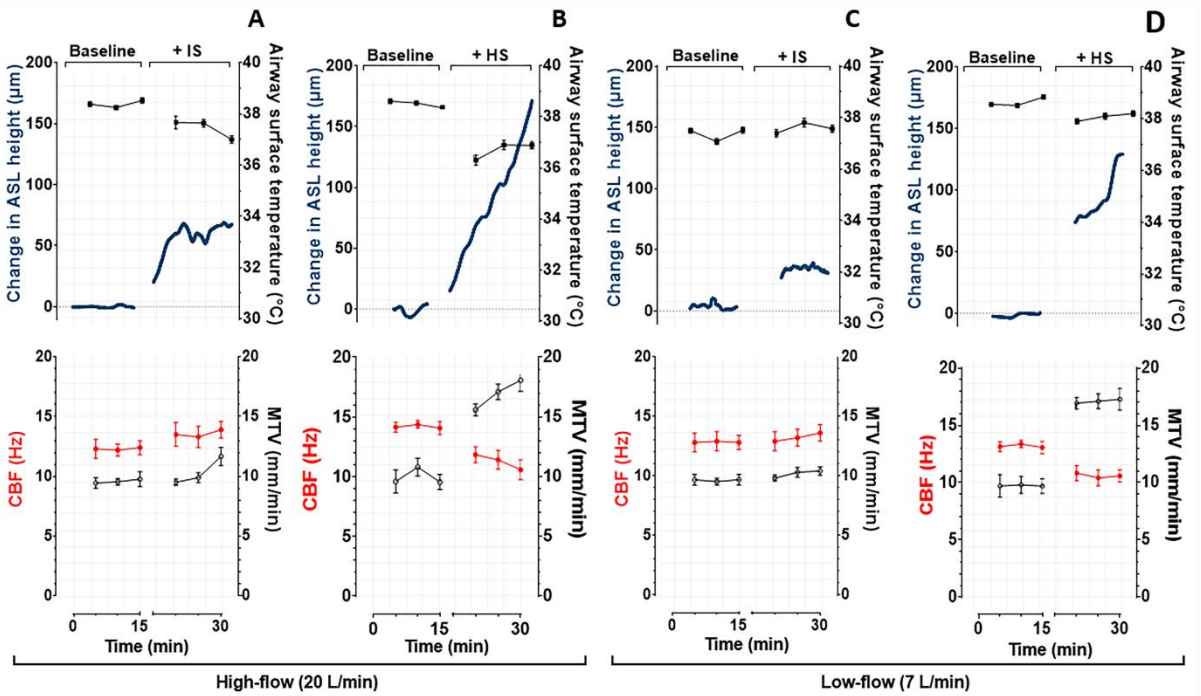
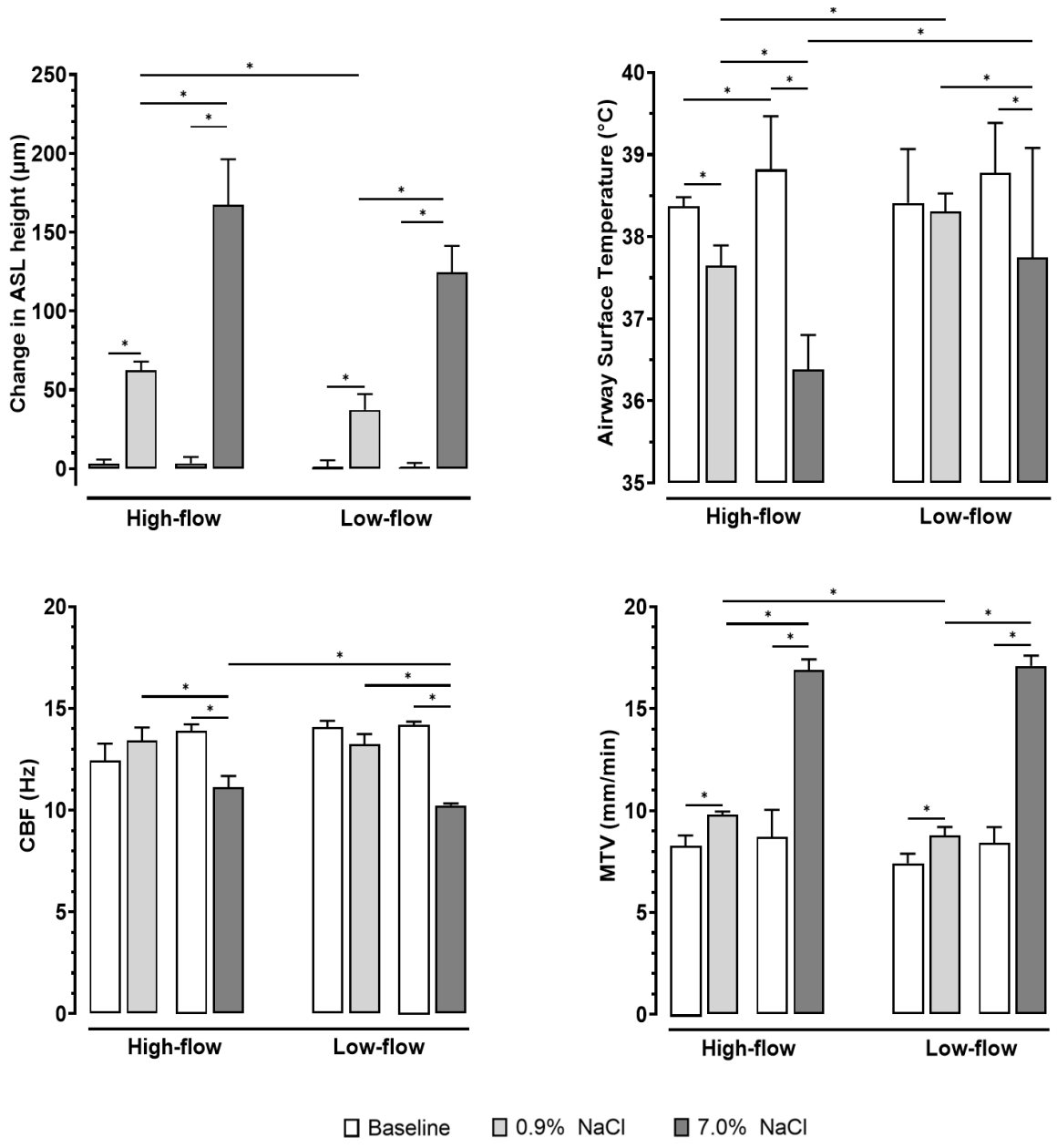
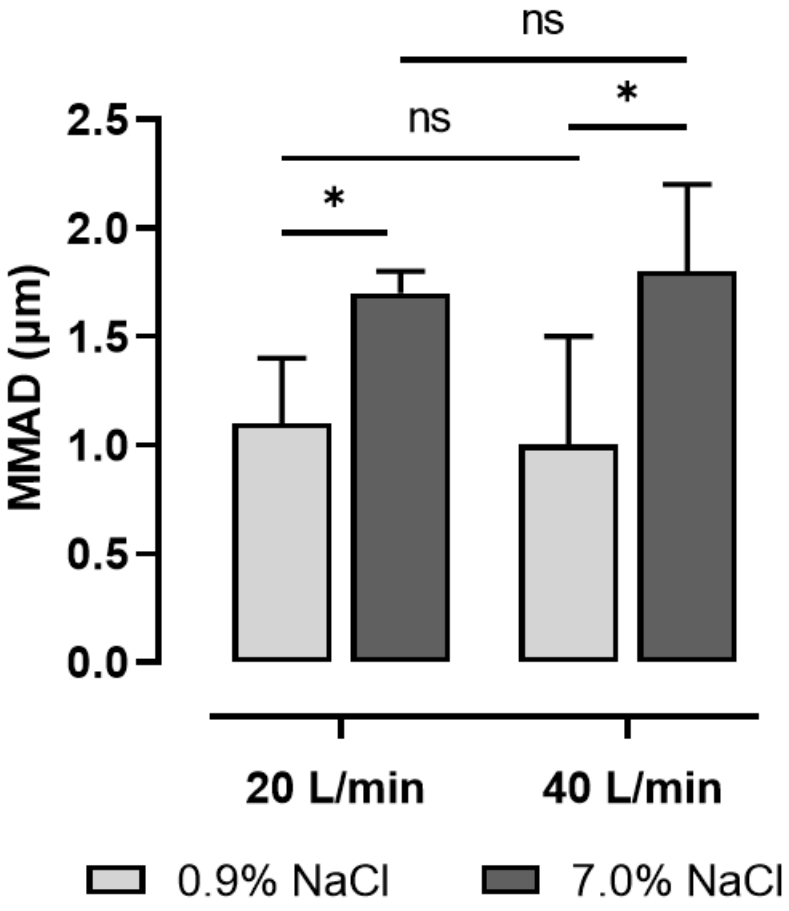
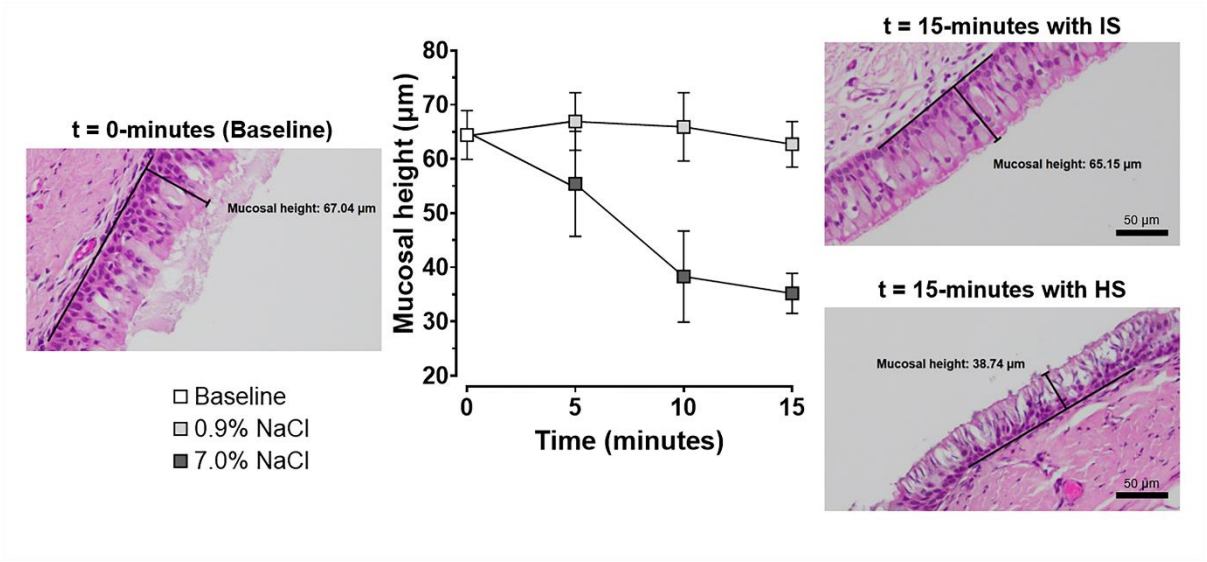
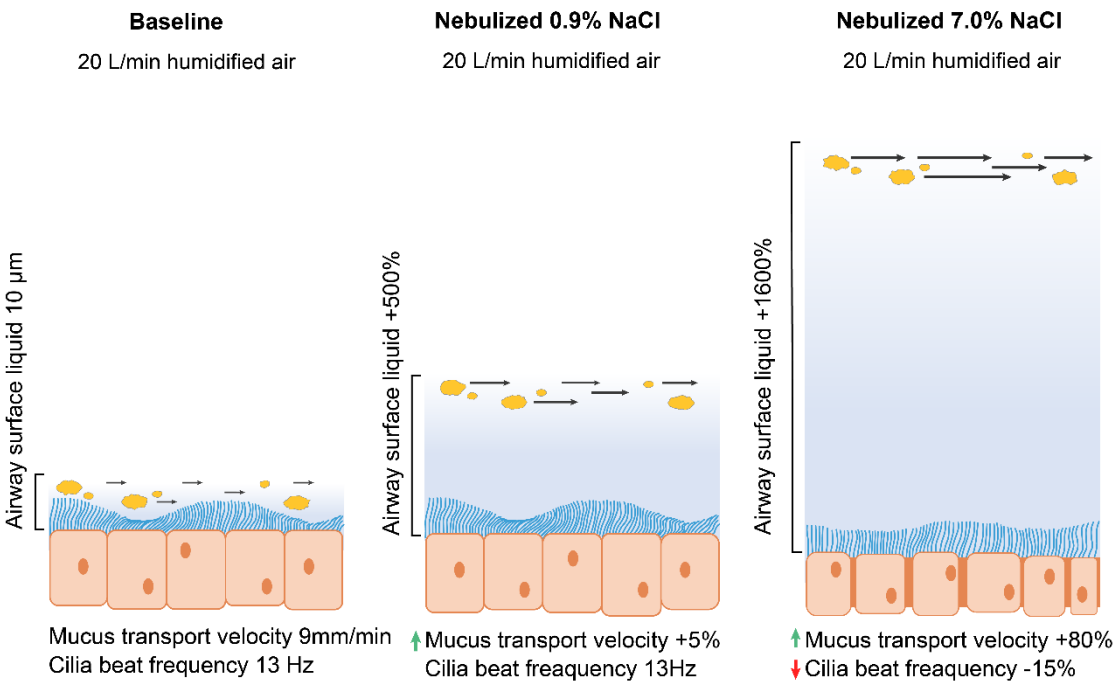

Infrared camera
with macro lens
Temperature and
humidity sensors
Displacement
laser sensor
20°
Air outlet
Tracheal epithelium
Nebulizer
Solution inlet
Organ bath
Temperature sensor
Circulating heated physiological solution
Flow generator with humidifier
Heating
circulator


A
B
C
D
Baseline
+ IS
+ HS
Change in ASL height (µm)
Airway surface temperature (°C)
CBF (Hz)
MTV (mm/min)
Time (min)
High-flow (20 L/min)
Low-flow (7 L/min)


Change in ASL height (µm)
250
200
150
100
50
0
High-flow
Low-flow
Airway Surface Temperature (°C)
40
39
38
37
36
35
High-flow
Low-flow
CBF (Hz)
20
15
10
5
0
High-flow
Low-flow
MTV (mm/min)
20
15
10
5
0
High-flow
Low-flow
Baseline
0.9% NaCl
7.0% NaCl


ns
ns
*
*
2.5
2.0
1.5
1.0
0.5
0.0
MMAD (µm)
20 L/min
40 L/min
0.9% NaCl
7.0% NaCl


t = 0-minutes (Baseline)
Mucosal height: 67.04 µm
Baseline
0.9% NaCl
7.0% NaCl
Mucosal height (µm)
80
70
60
50
40
30
20
0
5
10
15
Time (minutes)
t = 15-minutes with IS
Mucosal height: 65.15 µm
50 µm
t = 15-minutes with HS
Mucosal height: 38.74 µm
50 µm


Baseline
20 L/min humidified air
Nebulized 0.9% NaCl
20 L/min humidified air
Nebulized 7.0% NaCl
20 L/min humidified air
Airway surface liquid 10 µm
Airway surface liquid +500%
Airway surface liquid +1600%
Mucus transport velocity 9mm/min
Cilia beat frequency 13 Hz
Mucus transport velocity +5%
Cilia beat freaquency 13Hz
Mucus transport velocity +80%
Cilia beat freaquency -15%

Impact of high- and low-flow nebulized saline on airway hydration and mucociliary transport

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SUPPLEMENTAL MATERIAL

Part 1: Detailed materials and methods

Part 2: Summary of results

Part 3: Particle condensation growth measurements and mathematical modelling to determine volume changes from osmolarity and particle deposition on the tracheal surface with nebulized saline solutions

Part 4: Mathematical modelling to estimate the thermal effects from condensation on the surface and changes to the airway surface liquid (ASL) film height

Part 5: Effect of cooler and drier air on the ASL and mucociliary transport

Part 1: Detailed materials and methods

Samples

Ten tracheas, harvested from apparently healthy sheep immediately after slaughter, were collected from an abattoir and transported at room temperature to the laboratory. The tracheas were opened along the ventral mid-line and fixed flat with the epithelium positioned upward in a previously described[1] heated (38 °C) organ bath (Figure 1). Time from collection at the abattoir to the beginning of the laboratory experiments was around 30 minutes for each trachea.

Equipment

A high-flow generator with humidifier (Airvo^{TM} 2, Fisher & Paykel Healthcare, New Zealand) was used to create a unidirectional flow of heated and humidified air over the epithelial surface of the tracheas at 7 L/min (low-flow delivery) to mimic inhaler delivery or 20 L/min (high-flow delivery) to mimic nasal high-flow delivery. The temperature and dew point of the air were controlled using external sensors at the inlet of the organ bath (HMP110, Vaisala, Finland). To establish the effects of nebulized saline solutions, either IS (7.5 mL of 0.9% NaCl) or HS (7.5 mL of 7.0% NaCl) was nebulized into the air path using a vibrating mesh nebulizer (Solo, Aerogen, Ireland) over a 15-minute period, according to clinical practice. The nebulizer was connected to the humidification chamber (Figure 1A) throughout high-flow delivery to mimic nebulization during high-flow therapy via tracheostomy or nasal cannula. The nebulizer was connected directly to the organ bath (Figure 1B) to mimic low-flow delivery as would occur with a typical handheld nebulizer device.

The top cover of the organ baths contained heated calcium fluoride (CaF_2) optical windows, one of which was used to continuously measure the ASL with a displacement laser sensor (LK-G32 and LK-Navigator software, Keyence Corporation, Japan). This sensor is based on the light-scattering principle and has repeatability of up to 0.05 µm. Its analogue

signal was recorded using Keyence software (LK-Navigator, Japan). The other optical window was used for simultaneous infrared radiation (IR) high-speed macro-video imaging (SC7000 camera and L0905 lens, FLIR, US) to measure MTV, CBF and surface temperature, as reported in detail previously[2]. The IR macro-video imaging was carried out for 30 seconds, every 5 minutes, during the 30-minute experiment, at 100 frames/s with a field of view of 9.6 x 7.7 mm.

Baseline measurements were made over the first 15 minutes with air heated to 38 °C and fully saturated with water, so-called "body temperature pressure saturated" (BTPS), on all tracheas (N = 10). Thereafter measurements were made at 15-minute intervals with exposure to IS (N = 5) and HS (N = 5).

GraphPad Prism (V8.3.0, US) was used for statistical analysis of measurements. Significance testing was performed using a two-tailed paired t-test, and two-way ANOVA Tukey test where $p < 0.05$ was statistically significant. All data was tested for normality with the D'Agostino & Pearson test.

The mass median aerosol diameter (MMAD) was measured with an optical particle sizer (Model 3330, TSI, Germany) at flows of 20 and 40 L/min with the nebulizer connected to the humidification chamber outlet (Figure 1A), using a restrictor to prevent the optical receiver from becoming over-saturated. This enabled investigation into the effect of relative humidity on the size of particles during high-flow treatment. Nebulized saline was entrained into the following carrier air conditions to see the effect of air conditions on MMAD: temperatures (37 °C, 41 °C and 47 °C) and dew points (28 °C, 33 °C and 37 °C) corresponding to relative humidity (60%, 80% and 100%). Low-flow particle delivery (7 L/min) generated particles in too great a concentration for the optical receiver in the particle sizer to provide meaningful measurements and therefore was not tested. The particle sizer sampled gas at the end of the standard nasal cannula interface (OPT944, Fisher & Paykel Healthcare, New Zealand) used in clinical practice during nasal high-flow (NHF) therapy (Airvo 2, Fisher &

Paykel Healthcare, New Zealand). The particle count was based on particle sizes of between 0.3 and 10 µm, where the MMAD is the spherical aerodynamic equivalent diameter with the same physical properties at which half the mass lies below the stated diameter.

For histology, tracheas were collected within 20 minutes of killing at the local abattoir, the "plucks" from four freshly-killed adult Barbados black-belly sheep were removed and N = 2 tracheas were submerged in 1 L of IS (0.9% NaCl) solution and N = 2 in 1 L of HS (7.0% NaCl) solution. The tracheal segments were sharply excised into 4 µm sections and placed in 10% formalin after 0, 5, 10 and 15 minutes for fixation for a minimum of 24 hours prior to routine histological processing and preparation with haematoxylin and eosin stain. Sections were examined using a light microscope (Olympus BX51, Japan) at 200x and the heights of three regions of intact mucosae (minimal artefact) of each tissue section were measured using an Olympus DP27 camera and cellSens imaging software to determine a mean height.

Part 2: Summary of results

The table below summarizes all the experimental air conditions and nebulized saline delivery and the resulting mucociliary transport, surface temperatures and airway surface liquid levels.

Supplemental Table 1: Mean airway surface measurements and particle size distributions measured over a 15-minute period while the sheep tracheas were exposed to different nebulized saline solutions: airway surface liquid (ASL), mucus transport velocity (MTV), cilia beat frequency (CBF) and mass median aerosol diameter (MMAD). ^ dew point temperature measurements could not be made due to the proximity of the nebulizer to the dew point temperature sensor. ~ low-flow nebulization generated particles in too great a concentration for the optical receiver in the particle sizer to provide meaningful measurements and therefore was not tested. * values significantly different to measurements made under body temperature and pressure saturated (BTPS) conditions ($p < 0.001$).

Parameters	**High-flow Nebulizing (20 L/min)**			**Low-flow Nebulizing (7 L/min)**		
	BTPS	**Isotonic**	**Hypertonic**	***BTPS***	**Isotonic**	**Hypertonic**
Air temperature (°C)	*38.1 (0.6)*	38.2 (0.1)	*38.1 (0.6)*	*38.1 (0.6)*	38.0 (1.9)	38.1 (0.2)
Dew point (°C)	*37.3 (0.5)*	37.2 (0.5)	*37.3 (0.5)*	*37.3 (0.5)*	^	^
Airway surface temperature (°C)	*38.6 (0.4)*	37.7(0.2)	*38.6 (0.5)*	38.6 (0.2)	38.3(0.2)	37.8 (1.3)
ASL height (µm)	*3.3 (3.3)*	62.3 (5.6)*	*167.2 (29.0)*	0.8 (3.7)	37.2 (10.0)*	124.7 (16.6)
MTV (mm/min)	*8.5 (0.9)*	9.8 (0.2)*	*9.4 (2.0)*	7.9 (0.6)	9.8 (0.7)*	17.1 (0.5)*
CBF (Hz)	*13.2 (0.6)*	13.4 (0.6)	*13.1 (0.6)*	14.2 (0.2)	13.3 (0.5)	10.2 (0.1)*
MMAD (37 °C, dew point 28 °C)	-	0.4 (0.1)	-	-	-	-
MMAD	-	0.5 (0.1)	-	-	-	-

(37 °C, dew point 33 °C)						
MMAD (37 °C, dew point 37 °C)	-	1.1 (0.3)	-	-	-	-
MMAD (41 °C, dew point 37 °C)	-	1.0 (0.4)	-	-	-	-
MMAD (47 °C, dew point 37 °C)	-	0.8 (0.4)	-	-	-	-

Part 3: Particle condensation growth measurements and mathematical modelling to determine osmolarity changes on the tracheal surface with nebulized saline solutions

Particle condensation growth[3] appears to be an important factor in determining the deposition rates of nebulized saline solutions onto different parts of the airways. Particles larger than 5 μm in aerodynamic diameter tend to deposit in the upper airway while smaller particles (< 2 μm) are more likely to deposit in the lower airway[4]. Nebulized NaCl reduces the water vapour pressure on the particle surface, resulting in condensation growth at relative humidities at or below saturation conditions[5]. Condensation growth offers an opportunity for adjusting particle concentration and sizes, allowing for target deposition in the airways[6]. In this study particle sizes were measured from nebulized isotonic and hypertonic saline solutions with different carrier air conditions and flow rates to determine what effect the carrier air conditions have on particle size and condensation growth.

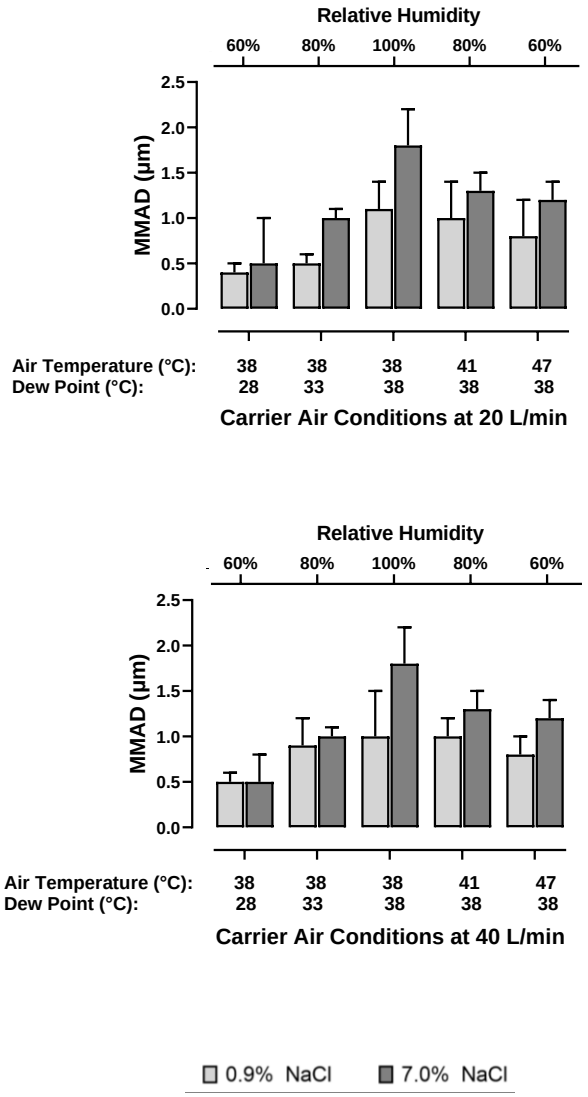
Method

Measurement of particle sizes at temperatures (37 °C, 41 °C and 47 °C) and dew points (28 °C, 33 °C and 37 °C) corresponding to relative humidity (60%, 80% and 100%) was performed with an optical particle sizer (Model 3330, TSI, Germany) at flow rates of 20 and 40 L/min with the nebulizer connected to the humidification chamber outlet (Figure 1A), using a restrictor to prevent the optical receiver from becoming over-saturated; this enabled

investigation into the effect of relative humidity on the size of particles during high-flow treatment. Low-flow particle delivery (7 L/min) generated particles in too great a concentration for the optical receiver in the particle sizer to provide meaningful measurements and therefore was not tested. The particle sizer sampled gas at the end of the standard nasal cannula interface (OPT944, Fisher & Paykel Healthcare, New Zealand) used in clinical practice during nasal high-flow (NHF) therapy (Airvo 2, Fisher & Paykel Healthcare, New Zealand). The particle count was based on particle sizes of between 0.3 and 10 μm, where the mass median aerodynamic diameter (MMAD) is the spherical aerodynamic equivalent diameter with the same physical properties at which half the mass lies below the stated diameter.

Results

Particle sizes were determined for the nebulized isotonic and hypertonic saline solutions carried by different air conditions and flow rates and are presented in Supplemental Figure 1. The effect of condensation growth is apparent in the larger particle size created when the carrier air was 38 °C with dew point 38 °C (100% relative humidity), also called “body temperature pressure saturated” (BTPS) air.



Supplemental Figure 1: Effect of nebulized 0.9% NaCl (light grey) and 7.0% NaCl (dark grey) on the mass median particle diameter (MADD) under different carrier air conditions at 20 L/min (top) and 40 L/min (bottom) delivered by a nasal high-flow system. MMADs from NaCl concentrations were significantly different across the carrier air conditions and flow rates.

A two-way ANOVA Tukey test was performed to analyse the effect of flow rate and carrier air conditions on the MMAD with nebulized 0.9% NaCl and 7.0% NaCl. This revealed that there was not a statistically significant interaction between the effects of the flow rate and carrier air condition ($F(4, 20) = 0.65$, $p = 0.64$ for 0.9% NaCl and $F(4, 20) = 1.091$, $p = 0.39$ for 7.0% NaCl).

Simple main effects analysis showed that the flow rate did not have a statistically significant effect on the MMAD for either 0.9% NaCl or 7.0% NaCl ($p = 0.46$, and $p = 0.67$, respectively). However, such analysis revealed that the carrier air conditions did have a statistically significant effect on the MMAD with 0.9% NaCl and 7.0% NaCl ($p = 0.01$, and $p < 0.0001$, respectively).

Supplemental Table 2: Two-way ANOVA analysis of mass median particle diameter (MMAD) changes with NaCl concentration, flow rate and carrier air conditions.

[NaCl]	ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
0.9%	Interaction	0.2220	4	0.05550	F (4, 20) = 0.6453	0.6366
	Air conditions	1.410	4	0.3525	F (4, 20) = 4.099	0.0138
	Flow rate	0.04800	1	0.04800	F (1, 20) = 0.5581	0.4637
	Residual	1.720	20	0.08600	-	-
7.0%	Interaction	0.2880	4	0.07200	F (4, 20) = 1.091	0.3878
	Air conditions	3.468	4	0.8670	F (4, 20) = 13.14	< 0.0001
	Flow rate	0.01200	1	0.01200	F (1, 20) = 0.1818	0.6744
	Residual	1.320	20	0.06600	-	-

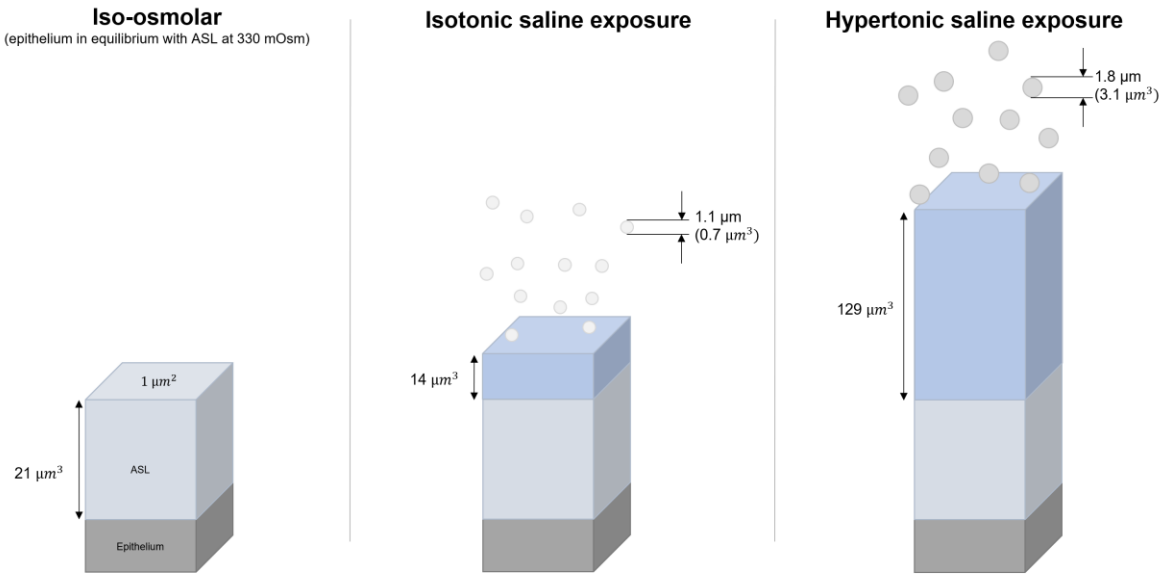
Volume change from particle deposition or osmolarity calculations

Using the particle size generated by each NaCl solution under BTPS conditions, the volume change on the ASL, determined from the ASL level measured by the displacement sensor, can be approximated as either being solely from the particle deposition or a combination of deposition and osmotic action.

Iso-osmolarity has been established as 330 mOsm[7] with a mean ASL level of 21 µm, made up of the periciliary layer (approximately 7 µm) and mucus layer[8].

Exposure to isotonic and hypertonic saline resulted in an ASL-height change. As shown below, the authors determined how much particle deposition is required under each condition to result in the measure change to the ASL height. Using the particle size generated at 20 L/min

with carrier air at BTPS conditions, the following illustrations show the change in volume (blue) over a 1 μm^2 surface area.



To determine the number of particles generated during nebulization, the authors assumed no losses, where the volume of NaCl solution placed in the Aerogen nebulizer (7.5 mL) over a 15-minute period was the volume nebulized into the particle size measured above (Supplemental Figure 1) for each NaCl concentration (0.9 and 7.0%) at 20 L/min with BTPS carrier air.

$$\frac{7.5\ mL}{15\ min} = 0.5 mL/min = 5x10^{11}\ \mu m^3/min$$

Accordingly, the particles generated from the isotonic (0.9% NaCl) and hypertonic (7.0% NaCl) solutions over an assumed tracheal surface area of 30 cm long and 10 cm wide for 15 minutes are calculated below, respectively:

$$\frac{5 \times 10^{11}\ \mu m^3}{min}\frac{particle}{0.7\ \mu m^3}\frac{1}{3\ \times 10^{10}\ \mu m^2}\frac{15\ min}{1} = 356\ particles/\mu m^2$$

$$\frac{5 \times 10^{11}\ \mu m^3}{min}\frac{particle}{3.1\ \mu m^3}\frac{1}{3\ \times 10^{10}\ \mu m^2}\frac{15\ min}{1} = 81\ particles/\mu m^2$$

The number of particles that would need to be deposited on the 1 μm^2 surface area to achieve the measured change in ASL level when the epithelium was exposed to isotonic (0.9% NaCl) nebulized saline is calculated as:

$$\frac{particle}{0.7\ \mu m^3}\frac{14\ \mu m^3}{\Delta\ ASL\ level} = 20\ particles\ deposited\ to\ change\ the\ ASL\ level$$

When the epithelium was exposed to isotonic saline, 6% (20/356) of the particles generated would need to be deposited over a 1 μm^2 surface area to achieve the change in ASL height measured. This value is approximately in line with values reported in Ari et al.[9], where the amount of particles emitted by the Aerogen nebulizer when placed before the humidifier in a similar experimental set-up was 7%.

With hypertonic saline (7.0% NaCl), the number of particles that would need to be deposited over a 1 μm^2 surface area to achieve the measured change in ASL level is calculated as:

$$\frac{particle}{3.1\ \mu m^3}\frac{129\ \mu m^3}{\Delta\ ASL - thickness} = 42\ particles\ deposited\ to\ change\ the\ ASL\ level$$

As a portion of the total particles generated, 52% (41/82) of particles would need to be deposited to achieve the change in ASL height measured during exposure to hypertonic (7.0%) nebulized saline. This number is much greater than those seen in the literature, and, therefore, it appears that the large change in ASL level is a combination of a volume change caused by particle deposition and osmotic action, drawing water into the ASL from the underlying epithelium.

Further calculations were performed to determine the change in ASL level attributed to particle deposition, assuming 7% of particles can be deposited onto the ASL surface to cause a change in the ASL height, and the ASL-level change resulting from osmotic action.

Assuming a 7% particle deposition, the change in ASL level would be:

$$\frac{7}{100}\,\frac{81\ particle}{\mu m^2}\,\frac{3.1\ \mu m^3}{particle} = 18\ \mu m$$

If 18 μm of the ASL-height change is attributed to particle deposition, 111 μm of the ASL-level change is from osmotic action.

Assuming the particles generated by the hypertonic (7.0% NaCl) solution have the same concentration, and the ASL remains iso-osmolar, the number of particles deposited into the 111 μm ASL-height change is:

$$\frac{70\ mg\ NaCl}{1000\ mL}\,\frac{1\ mL}{1\ \times 10^{12}\ \mu m^3}\,\frac{3.1\ \mu m^3}{particle} = 2.2\ \times\ 10^{13}\ mg\ NaCl/particle$$

$$\frac{70\ mg\ NaCl}{1000\ mL}\,\frac{1\ mL}{1\ \times 10^{12}\ \mu m^3}\,\frac{111\ \mu m^3}{ASL\ level} = 7.8\ \times\ 10^{12}\ mg\,\frac{NaCl}{ASL} - thickness$$

$$\frac{7.8\ \times\ 10^{12}\ mg\ NaCl/ASL\ level}{2.2\ \times\ 10^{13}\ mg\ NaCl/particle} = 35\ particle$$

Therefore, 35 particles of 7.0% NaCl concentration need to be deposited onto the ASL surface to cause an osmotic change on the ASL height, increasing it by 111 μm. This is still 43% of the total number of particles emitted, much greater than the amount of 7% shown in past literature. As such, osmotic action appears to play a part in drawing water into the ASL to achieve the change in ASL height recorded during the study.

Conclusions

The carrier air condition had an effect on the particle size while the air-flow rate did not have a significant effect on the particle size. The saline concentration and relative humidity had a significant effect on particle sizes in all cases, except when nebulized isotonic saline was exposed to warmer air (heated above 37 °C) with 80% and 60% relative humidity (dew point 37 °C). Condensation growth of the particles, when carried by BTPS air, significantly reduced their salinity, resulting in very minor increases to the osmolarity of the ASL.

Part 4: Mathematical modelling to estimate the thermal effects from condensation on the surface and changes to the airway surface liquid (ASL) film height

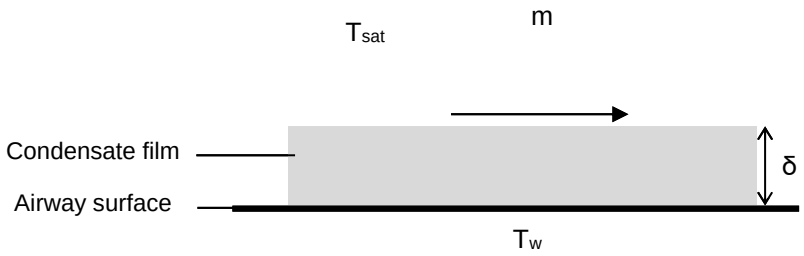
The airway surface is known to be sensitive to changes in temperature. By measuring the surface temperature using infrared-imaging, the authors saw a drop in surface temperature when the airway surface was exposed to the nebulized saline solutions. A larger decrease in temperature was seen when nebulized saline was entrained into body temperature and pressure saturated (BTPS) air at high flow (20 L/min).

Presented below is a mathematical model to enable a better understanding of the thermal effects that result from particles condensing onto the airway surface, and how the changes in the airway surface film height play a part in the heat transfer.

When heated and humidified air comes into contact with a surface that has a lower temperature than the dew point temperature of the air, it condenses into a liquid releasing latent heat. The heat transfer accompanied by condensation is termed “condensation heat-transfer”. When considering what happens to the water vapour in the air coming into contact with the airway surface, the term “film-wise condensation” describes the heat transfer taking place. In film-wise condensation, a saturated single-component (water) vapour condenses and forms a continuous film on the airway surface where the heat transfer is controlled by conduction in the liquid film.

Heat-transfer estimate equations

When condensation occurs on the airway surface, the film height on the surface is thin and so the curvature of the cylindrical airway in the trachea can be disregarded. A schematic diagram of the film-wise condensation on the airway surface is shown below (Figure 1) with the parameters in the following calculations used to estimate the heat transfer at the gas-liquid interface when air of different conditions comes into contact with the airway surface.



where T_w is the wall temperature (°C), T_{sat} is the saturation temperature of the air (°C), δ is the film height (mm) and m is the air-flow rate (L/min).

Assuming friction forces are negligible on the surface and the movement along the airway surface by mucus transport velocity (MTV) is negligible compared to the air-flow rate (m), the boundary-layer conditions can be approximated as:

Continuity:

$$\frac{\partial u}{\partial x} + \frac{\partial v}{\partial y} = 0 \qquad \text{(Equation 1)}$$

Conservation of momentum:

$$\rho_l u \frac{\partial u}{\partial x} + \rho_l v \frac{\partial u}{\partial y} = \mu_l \frac{\partial^2 u}{\partial y^2} + (\rho_l - \rho_g) m \qquad \text{(Equation 2)}$$

Conservation of energy:

$$c_{pl}\rho_l u \frac{\partial T}{\partial x} + c_{pl}\rho_l v \frac{\partial T}{\partial y} = k_l \frac{\partial^2 T}{\partial y^2} \qquad \text{(Equation 3)}$$

where ρ_l is the density of water, ρ_g is the density of vapour, μ_l is the viscosity coefficient at of water, c_{pl} is the specific heat at constant pressure of the water, k_l is the thermal conductivity of water and m represents the mass flow rate of the air.

Using the schematic diagram shown above, the boundary conditions are as follows:

$$u = v = 0,\ T = T_w \text{ at } y = 0 \text{ (the airway surface)}$$

$$\mu_l \frac{\partial u}{\partial y} = 0, T = T_{sat} \text{ at } y = \delta \text{ (the condensate film surface)}$$

As the film is considerably thin and the velocity of the film (MTV) is small, the transport momentum due to convection can be disregarded. Therefore, the left side of the conservation of momentum equation (Equation 2) can be approximated to zero, resulting in:

$$\mu_l \frac{\partial^2 u}{\partial y^2} = -(\rho_l - \rho_g)m$$

By integrating the above equation and determining the integration constants from the boundary conditions, the velocity distribution in the liquid film can be described as:

$$u = \frac{(\rho_l - \rho_g)m}{2\mu_l}(2\delta - y^2)$$

Because the transport momentum can be approximated as zero, similarly the energy transport due to convection can be estimated as being zero, which results in the conservation of energy equation (Equation 3) becoming:

$$\frac{\partial^2 T}{\partial y^2} = 0$$

By integrating the above equation, the temperature distribution in the film can be defined as:

$$T = T_w - (T_w - T_{sat})\frac{y}{\delta}$$

When considering the energy balance of the condensing vapour over the liquid film flowing from position $x = 0$ to $x = x$, using the latent heat of vapourization h_{fg}, the total amount of

latent heat released during condensation relative to the total heat transferred through the airway surface can be determined as:

$$h_{fg}\rho_l \int_0^{\delta} u dy = \int_0^{x} k_l \left(\frac{\partial T}{\partial y}\right)_{y=0} dx$$

The local heat-transfer coefficient at position x (h_x) can be determined using the equation that defines the temperature distribution in the film ($T = T_w - (T_w - T_{sat})\frac{y}{\delta}$) and the energy balance of the condensing vapour over the liquid film ($h_{fg}\rho_l \int_0^{\delta} u dy = \int_0^{x} k_l \left(\frac{\partial T}{\partial y}\right)_{y=0} dx$) to give:

$$h_x = \frac{-k_l \left(\frac{\partial T}{\partial y}\right)_{y=0}}{(T_w - T_{sat})} = \frac{k_l}{\delta}$$

From the above equation, it is evident that the heat transfer is controlled by thermal conductivity (k_l) (heat conduction) and height of the film (δ).

Calculations

The following table shows the heat-transfer (h_x) estimates determined from the measurements used in the study, where the surface temperature of the film (T_{sat}) was used to adjust the thermal conductivity of the liquid film (k_l) under each condition and the measured relative change in film-height level was used for the film-height measurement (δ).

Air condition	**T_{sat} (°C)**	**k_l (W/mK)**	**δ (mm)**	**h_x (W/mm)**
High-flow nebulized isotonic saline	37.7	0.6253	0.0368	17.0
High-flow nebulized hypertonic saline	36.4	0.6235	0.1672	3.7
Low-flow nebulized isotonic saline	37.8	0.6255	0.0578	10.8
Low-flow hypertonic saline	36.4	0.6235	0.1592	3.9

Conclusions

In general, the study showed that thinner airway surface films had larger heat transfers than thicker airway surface films. The airway surface film height was found to be more dependent on the NaCl concentration than flow rate where isotonic saline resulted in thinner airway surface films compared with exposure to hypertonic saline.

The change in surface temperature had little effect on the calculated heat transfer where the largest heat transfer reported (17 W/mm) resulted from a 0.9 °C surface temperature decrease when exposed to nebulized isotonic saline with high-flow carrier air. However, the largest surface temperature drop (2.2 °C) was recorded when nebulized hypertonic saline was used to deliver with high-flow air, resulting in just 3.7 W/mm heat transfer. The larger changes in surface temperature appeared to be more dependent on the flow rate where high flow caused more evaporative cooling than those recorded with low flow.

Flow rate and the height of the airway surface film appeared to have the largest effect on the calculated heat transfer where the airway surface film height was most affected by the osmotic gradient, which was larger when nebulized hypertonic saline was introduced. While the heat-transfer effects seemed to be fewer than when hypertonic saline was used, these thermal effects may have been overshadowed by the larger osmotic action seen by the change in ASL level in Figure 3 of the main text section.

Part 5: Effect of cooler and drier air on the ASL and mucociliary transport

To determine the effects of air colder and drier than body temperature pressure saturated (BTPS) air on the ASL height and mucociliary transport, the authors used infrared video microscopy and a displacement sensor to study sheep tracheas exposed to air at BTPS followed by air that was colder and drier (31 °C) with 60% relative humidity for 15 minutes. An understanding of the effects of temperature and humidity on the ASL and mucociliary transport is essential to describe the functioning of the tracheal mucosa in intubated patients.

Methods

Five tracheas, harvested from apparently healthy sheep immediately after slaughter, were collected from an abattoir and transported at room temperature to the laboratory. The tracheas were opened along the ventral mid-line and fixed flat with the epithelium positioned upward in a previously described[1] heated (38 °C) organ bath (Figure 1). Time from collection at the abattoir to the beginning of the laboratory experiments was around 30 minutes for each trachea.

An air-flow generator with humidifier (Airvo 2, Fisher & Paykel Healthcare, New Zealand) was used to create a unidirectional flow of heated and humidified air over the epithelial surface of the tracheas at 20 L/min (high-flow delivery) to mimic nasal high-flow delivery. The temperature and dew point of the air were controlled using external sensors at the inlet of the organ bath (HMP110, Vaisala, Finland).

The top cover of the organ baths contained heated CaF_2 optical windows, one of which was used to continuously measure the ASL with a displacement laser sensor (LK-G32 and LK-Navigator software, Keyence Corporation, Japan). This sensor is based on the light-scattering principle and has repeatability of up to 0.05 µm. Its analogue signal was recorded using Keyence software (LK-Navigator, Japan). The other optical window was used for simultaneous IR high-speed macro-video imaging (SC7000 camera and L0905 lens, FLIR, US) to measure

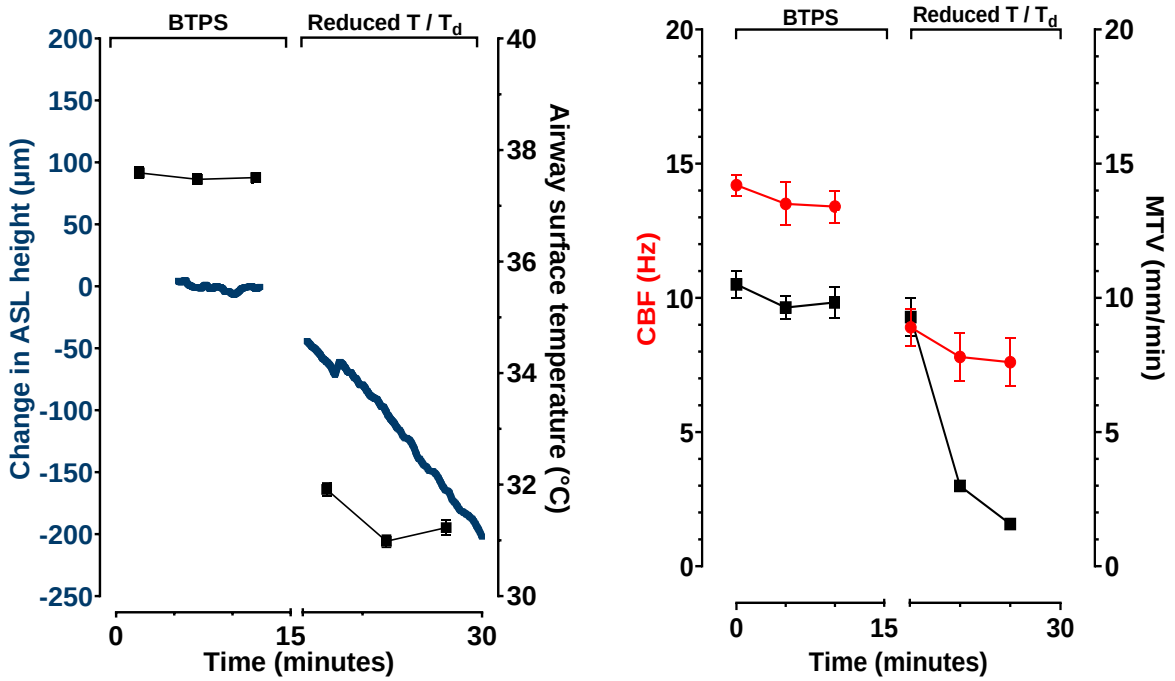
MTV, CBF and surface temperature, as reported in detail previously[2]. The IR macro-video imaging was carried out for 30 seconds, every 5 minutes, during the 30-minute experiment, at 100 frames/s with a field of view of 9.6 x 7.7 mm.

Baseline measurements were made during the first 15 minutes with air heated to 38 °C and fully saturated with water (BTPS) on all tracheas (N = 10). Thereafter measurements were made at 15-minute intervals with exposure to reduced temperature and dew-point air conditioned to 31 °C and 60% relative humidity (23 °C dew point).

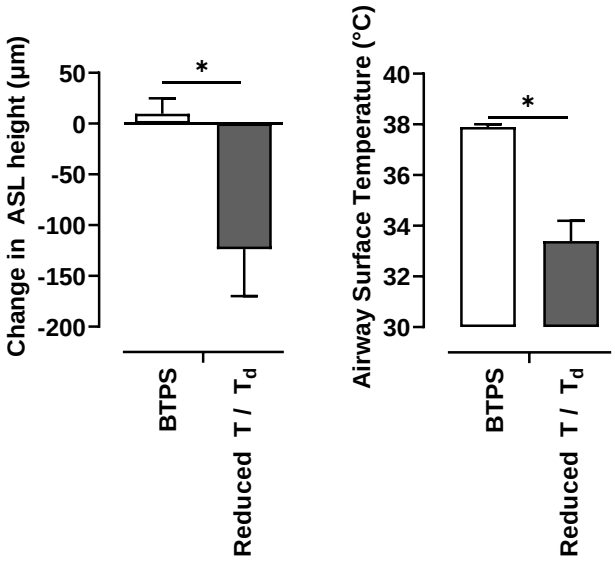
GraphPad Prism (V8.3.0, US) was used for statistical analysis of measurements. Significance testing was performed using a two-tailed paired t-test, and two-way ANOVA Tukey test where $p < 0.05$ was statistically significant. All data was tested for normality with the D'Agostino & Pearson test.

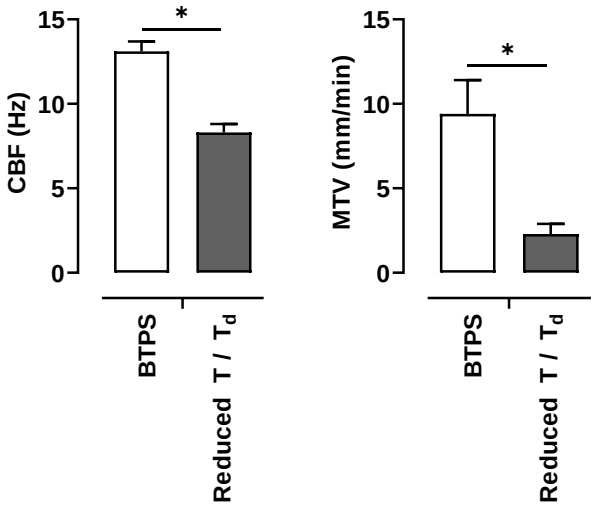
Results

During the baseline period (t = 0 to 15 minutes), the mean surface temperature (37.9 ± 0.1 °C (Figure 2), MTV (8.2 + 0.8 mm/min) and CBF (13.1 + 0.6 Hz) (Figures 1 and 2) were within normal ranges previously described[1]. Air with reduced temperature (31 °C) and humidity (dew point 23 °C) at the high-flow rate (20 L/min) significantly ($p < 0.05$) lowered the airway surface temperature (31.4 + 0.5 °C), MTV (4.6 + 4.1 mm/min) and CBF (8.1 + 0.7 Hz) after 15 minutes. The drop in surface temperature and mucociliary transport was accompanied by a substantial reduction in the change in ASL thickness (-123.6 ± 46.3 µm).



Supplemental Figure 2. Representative real-time airway surface liquid (ASL) heights (blue) and 5-minute mean airway surface temperatures (black), cilia beat frequency (CBF) (red) and mucus transport velocity (MTV) (black) measured over a 30-minute period on the airway surface exposed to body temperature and pressure saturated (BTPS) air (38 °C, dew point 38 °C) for the first 15 minutes, followed by 15 minutes of exposure to reduced temperature (T) and dew point (T_d) air conditioned to 31 °C and 60% relative humidity (23 °C dew point).





Supplemental Figure 3. Mean effect of reduced temperature (T) and dew point (T_d) air conditioned to 31 °C and 60% relative humidity (23 °C dew point) compared to body temperature and pressure saturated (BTPS) air (38 °C, dew point 38 °C) baseline conditions (white) on the change in the airway surface liquid (ASL) height, airway surface temperature, cilia beat frequency (CBF) and mucus transport velocity (MTV) on the airway surface (5 tracheas). Airway surface temperatures, CBF and MTV were measured with infrared video-imaging while simultaneous measurements of the ASL were made with a displacement laser sensor. Mean ASL heights presented above are from the last 5 minutes of the experiment. * = significant difference.

Discussion

The response to low temperature and humidity air was consistent with previous reports showing impaired mucociliary transport[1] and a desiccated ASL[10]. Of note is the magnitude of the change in the ASL thickness, which decreased by approximately 100 μm, suggesting the change in the ASL thickness in response to cooler and drier air causes not only the ASL to be dehydrated but also the epithelial cells below to become desiccated.

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