Variability in tracheal mucociliary transport is not controlled by beating cilia in lambs in vivo during ventilation with humidified and nonhumidified air

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Abstract

Mucociliary transport in the respiratory epithelium depends on beating of cilia to move a mucus layer containing trapped inhaled particles toward the mouth. Little is known about the relationship between cilia beat frequency (CBF) and mucus transport velocity (MTV) in vivo under normal physiological conditions and when inspired air is dry or not fully humidified. This study was designed to use video-microscopy to simultaneously measure CBF and MTV in the tracheal epithelium through an implanted optical window in mechanically ventilated lambs. The inspired air in 6 animals was heated to body temperature and fully saturated with water for 4 hours as a baseline. In another series of experiments, 5 lambs were ventilated with air at different temperatures and humidities and the mucosal surface temperature was monitored with infrared macro-imaging. In the baseline experiments, during ventilation with fully humidified air at body temperature, CBF remained constant, mean 13.9 ± 1.6 Hz but MTV varied considerably between 0.1 and 26.1 mm/min with mean 11.0 ± 3.9 mm/min, resulting in a maximum mucus displacement of 34.2 μm/cilia beat. Fully humidified air at body temperature prevented fluctuations in the surface temperature during breathing indicating a thermodynamic balance in the airways. When lambs were ventilated with dryer air, the mucosal surface temperature and MTV dropped without a significant change in CBF. When inspired air was dry, mainly latent heat (92%) was transferred to air in the trachea, reducing the surface temperature by 5 °C. Reduced humidity of the inspired air lowered the surface temperature and reduced MTV in the epithelium during ventilation.

cilia; humidity; mucociliary transport; mucus; video-microscopy

INTRODUCTION

Mucociliary transport in conducting airways is a continuous process in which beating cilia propel mucus containing trapped inhaled particles along the respiratory epithelium (1) until they reach the mouth and are swallowed. During breathing, inspired air is heated and humidified to body temperature and fully saturated with water through heat and water exchange with the respiratory mucosal surface (2). During expiration, partial recovery of heat and water occurs when the warm humid air passes over the relatively cooler airway surface liquid, such that water vapor condenses onto the epithelium. Blood micro-circulation helps to maintain the temperature and water content of the airway surface liquid (3). The rate that mucus is moved by the cilia, the mucus transport velocity (MTV), depends on the hydration and viscosity of the mucus (4, 5), these being determined by the mucin and water content of the airway surface liquid. Compromised mucociliary transport is found in lung diseases such as cystic fibrosis, chronic obstructive pulmonary disease (COPD), and asthma (6) and leads to an accumulation of mucus that elicits coughing (7). Recent advances in the knowledge of cilia and mucus biology (6, 8, 9) highlight the importance of well-controlled interactions between cilia and the airway surface liquid for effective mucociliary transport. These interactions have been studied using a variety of models (10, 11) and in vitro experiments using frog palates (12–14), ferret (15), sheep and bovine tracheas (16, 17), and in vivo experiments with pig tracheas (18), mice airways (19, 20), and the human nasal cavity (21). Because often only a single component of the transport mechanism was investigated, such as cilia beat frequency (CBF) (22–24) or MTV (19–21, 25), causal relationships between components could not be inferred. Also, environmental physiological conditions during the experiments were not controlled (18, 26, 27), although both temperature and humidity of inspired air are known to play an important role in mucociliary transport (28–31).

Currently it is known that mucociliary transport depends primarily on the CBF, the cilia density, mucus viscosity, and the airway humidity and temperature (29, 32–36). In experimental models, a direct linear relationship between CBF and temperature has been demonstrated (37–39). A similar relationship has been observed between humidity and CBF, most probably because of the effects of hydration on mucus production and mucus properties (29, 40–42). A direct effect of modifying the inspired air on mucociliary transport has
also been shown in human studies (43, 44). Humid air maintains the water content of the airway surface liquid and plays an important role in optimizing mucociliary clearance (29, 42). Humidification of inspired air is used in the treatment of a variety of respiratory conditions (45). Heat and moisture exchangers (HME), used in bypassed Airways, vary in performance depending on the model and breathing pattern (46) and can maintain absolute humidities between 13.2 to 31.9 mg/L (47). The use of HMEs have been shown to reduce pulmonary complications when the upper respiratory tract is bypassed in laryngectomized patients (48). Additionally, humidification during nasal high-flow therapy has been shown to improve mucociliary clearance (44) and reduce exacerbations days in COPD patients (49). The thermodynamic changes during ventilation may influence cilia activity, as well as the production of mucus and its properties. Further experiments, performed over a relatively longer time period, are needed so that any changes in mucociliary transport, induced by altered air temperature and humidity can manifest and be measured (50).

Indirect methods of measuring mucociliary clearance with tracers such as radioactive aerosols (51), fluorescent beads (52), and tantalum disks (53) may disrupt the depth and physical properties of the airway surface liquid and thereby reduce the reliability of measurements (6, 54). Emerging techniques using synchrotron radiation sources to non-invasively study the mucociliary transport mechanisms look promising (55) but also have limitations, and access to these faculties can be difficult.

To overcome many of the aforementioned issues associated with studying mucociliary transport, the authors developed an in vivo video-microscopy method using reflected visible light through a surgically implanted optical window in the lamb trachea to simultaneously measure the CBF and MTV. To prevent dehydration of the airway surface liquid during the lengthy experiments, the lambs were ventilated with air that was heated to body temperature and fully saturated with water vapor under ambient pressure (BTPS). Naturally occurring particles were traced in the mucus to determine MTV, and CBF was measured on the underlying beating cilia. To ascertain the relationship between CBF and MTV, we compared CBF and MTV results obtained by video-microscopy with infrared macro-imaging (IR-MI) using high-speed cameras. IR-MI was also used to measure the surface temperature of the tracheal epithelium during ventilation with air heated and humidified to conditions created with a pass-over humidifier—i.e., fully humidified at body temperature, and to conditions that mimic those typically produced by an HME—i.e., with reduced temperature and humidity, or without humidification. The purpose of the study was to determine the relationship between MTV and CBF in the tracheal epithelium in vivo over prolonged periods of time and to determine the effect of different temperatures and humidities of the inspired air on the mucosal surface temperature, CBF, and MTV, during ventilation.

# MATERIALS AND METHODS

In Vivo Lamb Model

The study was approved by Monash University Animal Ethics Committee. Eleven new-born male lambs of age 20 ± 3 days and weight 8.2 ± 0.8 kg were used in the study. The lambs were sedated with ketamine hydrochloride (5 mg/kg; Ketamil, ILEUM Veterinary Products) and anesthetized with α-chloralose (80 mg/kg starting bolus followed by continuous infusion at 20 mg/kg/h), using a jugular vein catheter, before being placed in dorsal recumbency for surgery to implant the optical window into the trachea (Fig. 1). The animals were instrumented with carotid artery catheters to measure systemic blood pressure and heart rate to monitor depth of anesthesia. SaO₂, ventilatory parameters (PEEP 16.0 ± 0.4 cmH₂O; Vt 10.2 ± 0.6 mL/kg; respiratory rate 28 ± 3/min and flow 8.7 ± 1.0 L/min), expired end tidal CO₂, and inspired gas O₂ were closely monitored. Body core temperature was monitored with a rectal probe, and the data provided the input temperature for a water bath (HE-4 Heating Circulator, Julabo, Germany) that circulated heated water through the mats underneath the animal to prevent hypothermia during sedation. When the lamb was stable and breathing spontaneously, an optical window (diameter 25 mm) of fused silica with visible/near infrared broadband anti-reflective coating (VIS-NIR λ/10, Edmund Optics, Singapore) or a calcium fluoride window was framed in custom-made plastic housing. The optical window was surgically implanted into the anterior wall of the trachea in the middle of the neck to provide an inspection area (7.5 mm × 20 mm) for visible light video-microscopy and IR-MI, respectively (Fig. 2). Hemostasis was achieved with electrocautery and the window housing was sealed to the tracheal wall with purse string sutures and elastic bands. After ensuring the optical window was airtight along its edges, the trachea was dissected below the larynx and a custom-made short endotracheal tube was attached to the end of the trachea. A custom-made dew-point humidity probe was inserted into the endotracheal tube to control dew-point of the inspired gas to provide feedback to a modified heated respiratory humidifier (MR730, Fisher & Paykel Healthcare, New Zealand). The dew-point humidity probe consisted of relative humidity and temperature sensors (HM70, Vaisala, Finland) in a temperature-controlled housing. The air temperature in the trachea was measured with two fast-response thermocouples placed in the center of the trachea on either side of the optical window and was used to control heating in the inspiratory limb of the breathing circuit. All data were recorded using an ADC converter PowerLab and Lab Chart software (ADInstruments, New Zealand). At the end of the experiments, the lambs were euthanized with Letharb (150 mg/kg body weight, Virbac Pty Ltd, Australia).

**Muccilary Transport Imaging**

Visible light video-microscopy recordings were made using a video microscope (VMU 378505, Mitutoyo, Japan) with a long (37.5 mm) working-distance lens (M Plan ApoNIR, 5×, f200, depth of field 14 μm; Mitutoyo, Japan) and inline coaxial illumination, and high-speed CCD monochrome digital camera (Lm085, Lumenera, Canada) was used to record 30-s video images of mucociliary transport through the silica optical window at 149 frames per second over an area of 750 μm × 275 μm on the epithelium. Infrared thermography macro-imaging (IR-MI) recordings were made in addition to the video-microscopy recordings in the five lambs with a mid-wave infrared camera (SC7600BB InSb...
640x512, FLIR) and a close-up lens (L0905, FLIR) with a working distance of 300 mm, depth of field of 300 μm and field of view of 9.6 × 7.7 mm. The infrared camera was calibrated by the manufacturer and the emissivity of the tissue was set to 1.0. Recordings at 100 frames per second were processed through Altair software (FLIR) for surface temperature measurements and to generate video files. The plastic housing of the optical window allowed micro-adjustment in three-axes to focus the microscope onto the tracheal epithelial surface and it was also rigidly attached to the side-rails to reduce movement of the tissue. The trachea was stabilized through the rigid connection between the optical window and the side-rail of the surgical table to reduce the effects of lung and heart movements. Three 30-s videos were recorded every 15 min over the 4-h period from randomly chosen spots within the optical window.

**Temperature and Humidity Setting**

The effects of different inspired air settings on the surface temperature and mucociliary transport of the tracheal mucosa were studied with video-microscopy and IR-MI recordings made in five lambs following 30 minutes of ventilation with air heated to body temperature and fully saturated with water, termed body temperature pressure saturated (BTPS).

**Figure 1.** Temperature control system for in vivo measurements of mucociliary transport in the trachea. A temperature-controlled heated mat reduced changes in body temperature during the 4-hour experiment. Body core temperature was used as an input to control dew-point and gas temperature of the inspired air at the entrance to the trachea during mechanical ventilation. A warm air blower prevented fogging of the exposed optical window.

**Figure 2.** Sealed optical window in the trachea (left and center) and a screenshot (right) from video-microscopy recording and infrared macro-imaging (right, bottom) showing the field of view used for mucus transport velocity measurements. The dashed square represents the region used in cilia beat frequency measurements.
Inspired air was then altered in three lambs such that temperature was reduced to 35°C and humidity to a dew point of 30°C; in two of the lambs dry air (i.e., without a humidification) was delivered by the ventilator chamber at 33°C.

Image Analysis

From every video-microscopy recording (Supplemental Video S1) CBF was determined with sliding Fast Fourier transforms (56) (Supplemental Video S2) and MTV was determined by tracing natural particles across the field of view. Similarly, paired data of the CBF and MTV were determined from every IR-MI recording (Supplemental Video S3) in the same way (Supplemental Video S4). The mucus displacement, induced by the beating cilia, was calculated as a function of MTV, converted to μm/s, and then divided by the CBF to measure the distance the mucus layer traveled for each cycle of the beating cilia.

Data Analysis

Excel (Microsoft) was used to analyze data sets. Statistical analysis was performed with GraphPad Prism (GraphPad (V 8.3.0)). The CBF and MTV were compared using one-way ANOVAs, where a P < 0.05 was regarded as statistically significant and is indicated by an * beside the value. The MTV and CBF data from both the video-microscopy and IR-MI were normally distributed with P-values > 0.05 from the D’Agostino-Pearson test. Results are presented as the mean ± standard deviation, unless stated otherwise.

Scanning Electron Microscopy

The tracheal tissue removed to create the viewing window and the tissue from the area subsequently imaged by video-microscopy were examined by scanning electron microscopy (SEM) to assess the epithelium and the length of its cilia. All animals underwent a post-mortem examination.

Latent and Sensible Heat Transfer Estimates

Thermodynamic equations were used to determine the amount of heat transferred between the mucosal surface and the air within the trachea (57). Specifically, the equation for sensible \( q_s \) and latent \( q_l \) heat transfer in a continuous system and the thermal properties of water per breath was used:

\[
q_s = \dot{m}c_p(T_{exp} - T_{ins}) \quad \text{and} \quad q_l = \lambda \dot{m}p^{-1}(\Psi_{exp} - \Psi_{ins})
\]

Where \( \dot{m} \) is the mass flow rate of inspired air (kg/s) and \( c_p \) is the specific heat capacity of air (J/kg°C). In the trachea, the air that enters the tube is inspired at \( T_{ins} \) (°C) and as that air flows along inside the tube, its bulk temperature increases as heat is transferred from the trachea wall before it is expired at \( T_{exp} \) (°C). For latent heat transfer, \( q_l \) is the total amount of latent heat exchanged between the trachea wall and the air (Watts), \( \lambda \) is the latent heat of vaporization of water (J/g), \( p \) is the density of air (kg/m³), and \( \psi_{exp} \) is the absolute humidity of the expired air (g/m³) at \( T_{exp} \), and \( \psi_{ins} \) is the absolute humidity of inspired air (g/m³) at \( T_{ins} \). Additional details are presented in Supplemental Data.

Using the total heat transfer, determined by the equations above, an approximation of the change in airway surface liquid height was determined using the latent heat of vaporization of the air and an assumed cylindrical geometry of the trachea.

RESULTS

BTPS conditions in the airways, adjusted to body temperature and kept close to 100% relative humidity, were maintained throughout the experiments on six lambs to minimize fluctuations between inspired and expired air temperatures. During inspection of the video-microscopy recordings, heterogeneity of the mucus layer was evident by the observed patch-work of lighter and darker areas corresponding to mucus with lesser and greater opacity, respectively. These areas moved across the recorded field, in which the cilia were beating underneath them (Supplemental Video S1, top frame marked HL01). In one lamb a visually thick and opaque mucus layer moved slowly across the recorded field (Supplemental Video S1, bottom frame marked SL01). The thickness and opacity of the mucus layer was visually determined from the video-microscopy recordings and was not measured further in this study. MTV results were determined by tracing an average of 54 particles per video-microscopy recording (84 video files in total) and 761 particles per lamb. The traced particles tended to move at an angle of +1−2° around a horizontal plane toward the left of the field of view in the cranial direction) with an average distance traveled of 197 μm in the healthy lambs and 32 μm traveled in the lamb with visually thick and opaque mucus (Supplemental Fig. S1).

In five lambs, measured with video-microscopy under BTPS conditions over 4 hours, mean MTV was 11.0 ± 3.9 mm/min with a wide range of values (Median:10.2, Min:0.1, Max:26.1, LQ:6.7, UQ:12.6) (Fig. 3) but in the lamb with the slow-moving mucus (SL01 in Table 1 and Supplemental Video S2), mean MTV was 1.0 ± 0.7 mm/min, with a maximum value of 2.5 mm/min. Post-mortem examination of this lamb revealed lobar pneumonia, which was not detected.
before the experiment. Individual data on the CBF and MTV over time for each lamb are presented in Supplemental Fig. S2. Analysis of the video-microscopy recordings revealed similar (P > 0.05) CBFs among the five lambs, with a mean value of 13.9 ± 1.6 Hz (Table 1) (Median:13.7, Min:10.3, Max:19.6, LQ:12.7, UQ:14.7). The mean CBF from the sick animal was not statistically different from CBF measured in the other five lambs.

No correlation was found between CBF and its corresponding MTV in the lambs inspiring air at BTPS (R² 0.04) (Fig. 3). There was also no correlation between body core temperature, CBF, and MTV in these lambs (R² 0.001 and R² 0.02, respectively) (Supplemental Fig. S3). Mucus displacement was calculated to combine the simultaneous CBF and MTV measurements. When ranked paired measurements of the CBF and mucus displacement were plotted in ascending order (Fig. 4), the mucus displacement increased from 0 to 34.2 μm/beat whereas the CBF varied randomly between 10 and 20 Hz; mucus displacement was significantly slower in the sick lamb (Fig. 4, open circles) and did not exceed 3 μm/beat.

The SEM of the tracheal epithelium from the five healthy lambs showed that the cilia were normal in appearance, covered the entire surface and were ~6 μm long (Fig. 5, A-E). In the sick lamb the cilia were noticeably stunted (Fig. 5F) (all SEM images from this animal are presented in Supplemental Fig. S4). There were no differences between the SEM images taken at the beginning of the experiment from the removed part of the tracheal epithelium and the tissue sampled after the experiment from the area under the optical window 4 hours later.

In the second series of experiments the IR-MI thermography recordings showed MTV and CBF (Fig. 6) and the mucosal surface temperature (Supplemental Video S3) with a change in temperature during inspiration and expiration (Fig. 7 and Table 2). There was a strong correlation between the IR-MI and video-microscopy measurements of MTV (R² 0.81), however, CBF measurements by IR-MI and video-microscopy were weakly correlated. Because CBF did not change significantly (P > 0.05) throughout the experiments, or between lambs, measurements made by either IR-MI or video-microscopy gave similar estimates, between 11 and 15 Hz. Therefore, IR-MI and video-microscopy measurements were pooled for determining the relationships between MTV and CBF and inspired air temperature and humidity (Fig. 6).

Surface temperature measurement revealed a strong positive correlation between the mucosal surface temperature and air temperatures in the trachea (R² 0.81). When BTPS air was inspired, the surface temperature was higher and more like the air temperature inside the trachea (Fig. 7, left) and there was little intra-breath fluctuation in the mucosal surface temperature (37.3 ± 0.5°C during inspiration versus 37.6 ± 0.6°C during expiration). However, fluctuations were seen to develop when the colder and dryer air was introduced, causing the surface temperature to drop briefly to 32.0 ± 3.3°C during inspiration and return rapidly to 32.9 ± 2.8°C during expiration. Although the inspired air was set at a constant temperature when lambs were ventilated with dry air, the tracheal air temperature fluctuated, being highest at the end of expiration (peak carbon dioxide percentage (37.6 ± 0.7°C) and lowest at end inspiration (32.7 ± 1.1°C); similarly, the absolute humidity in the trachea was lowest at the end of inspiration (0.5 ± 1.8 mg/L) and highest at the end of expiration (31.0 ± 3.3 mg/L) (Fig. 7, right). CBF and MTV measurements were constant under BTPS conditions (15.4 ± 0.2 Hz and 19.1 ± 1.0 mm/min, respectively). When dry air was introduced, MTV declined within seconds (from 17.5 ± 0.8 to 12.9 ± 1.6 mm/min) (Fig. 7, center) and stabilized at 3.3 ± 0.6 mm/min after 30 minutes of exposure whereas CBF dropped slightly when initially exposed to dry air but then remained relatively unchanged at 14.2 ± 0.9 Hz thereafter (Fig. 7, right).

Table 1. Mean mucus transport velocity, cilia beat frequency and mucus displacement in the five healthy lambs (HL) and the sick lamb (SL) over the 4-hour experiment

| Sample ID | Mucus Transport Velocity (mm/Min) | Mucus Transport Velocity (μm/s) | Cilia Beat Frequency, Hz | Mucus Displacement, μm/Beat |
|-----------|----------------------------------|---------------------------------|--------------------------|-----------------------------|
| HL01      | 9.8 ± 3.0                        | 164.0 ± 49.8                    | 14.1 ± 0.8               | 11.6 ± 3.4                  |
| HL02      | 11.2 ± 2.8                       | 186.7 ± 45.9                    | 13.0 ± 1.2               | 14.4 ± 3.6                  |
| HL03      | 13.5 ± 4.2                       | 224.7 ± 69.7                    | 14.3 ± 1.7               | 16.3 ± 6.0                  |
| HL04      | 9.1 ± 3.6                        | 151.3 ± 59.5                    | 13.5 ± 2.4               | 11.3 ± 4.3                  |
| HL05      | 11.2 ± 5.8                       | 186.7 ± 96.3                    | 14.6 ± 1.7               | 13.1 ± 7.4                  |
| SL01      | 1.0 ± 0.7                        | 16.7 ± 11.5                     | 13.6 ± 1.1               | 1.2 ± 0.9                   |

Data are presented as means with standard deviations. * Values that are statistically significantly different (P < 0.05).
Results from all experiments are summarized in Table 2, where similar changes to the above were seen in the lambs with inspired air with reduced temperature and humidity.

MTV was also poorly correlated with CBF measurement, with both IR-MI ($R^2 0.24$) and video-microscopy ($R^2 0.28$). The MTV was more dependent on the mucosal surface temperature (MTV $R^2 0.45$), tracheal air temperature (MTV $R^2 0.24$), and absolute humidity in the trachea (MTV $R^2 0.28$) than the CBF was ($R^2 0.19$, $R^2 0.14$ and $R^2 0.21$, respectively). When the mucosal surface temperature and humidity of the air in the trachea were reduced, MTV was significantly lower ($P < 0.05$) but there was no significant effect on CBF ($P > 0.05$) (Fig. 6).

Sensible and latent heat transfers by convection were calculated between the surface and the air inside the trachea (assumed length 100 mm and diameter 15 mm) using measurements presented in Table 2 (Fig. 8 and Supplemental Data). Under BTPS conditions, the total heat transfer was 1.4 W with a 27% contribution from sensible heat. When air with reduced temperature and humidity was inspired, the total heat transfer increased to 10 W and the contribution from sensible heat decreased to 13%, and, when inspired air was colder and dryer, the total heat transfer increased to 33 W whereas the sensible heat transfer contribution fell to just 7%.

**DISCUSSION**

In this study we present a technique that, by measuring both CBF and MTV simultaneously in vivo when lambs are ventilated with different air conditions, allows investigation of the relationship between CBF and MTV. The key finding from the first series of experiments was the variability in MTV and constant CBF during ventilation with BTPS air over 4 hours. In the lamb with pneumonia, a greatly reduced MTV was measured, while CBF remained like that found in the healthy lambs. The change in MTV was likely to be related to a change in the properties of the mucus or the airway surface liquid. In the second series of experiments, in which animals were ventilated with dry or not fully humidified air, substantial reductions in MTV were measured whereas CBF was not as affected, suggesting the efficiency of mucociliary transport is not related to the frequency at which the cilia beat. When inspired air was heated to BTPS, heat and mass (water) exchange was prevented between the mucosal surface and the air, thus creating optimal conditions for mucociliary transport. Ventilation parameters are known to have an effect on mucociliary function (58) and were held constant for all experiments. The intravenous anesthetic was selected to avoid affecting mucociliary transport (37); and high-speed cameras with 149 and
100-Hz frame rates were used for the recordings to avoid the introduction of aliasing during measurements. The measurement locations in the tracheas were selected to provide easy access during surgery and to permit comparisons with those from previous in vitro studies in the lamb trachea (16, 28, 29).

In the first series of experiments the data from the sick animal were not excluded from the study as they provide

**Figure 7.** Example of real-time infrared thermography measurements showing a stable mucosal surface temperature (red) and the tracheal air temperature (gray), absolute humidity (AH) (blue), and carbon dioxide (CO₂) from sensors near the implanted tracheal window in a lamb breathing inspired air at 38°C and 100% relative humidity (left). Recordings were also made as the temperature and relative humidity of the inspired air was decreased to 31°C and 0% (middle) where fluctuations in the surface temperature and air temperature become prominent and 30 minutes later when parameters had stabilized (right) but were still fluctuating. Body temperature (black dashed line) was maintained throughout and measured rectally. Mucus transport velocity (MTV) (black circle) and cilia beat frequency (CBF) (gray square) were measured at 5-minute interval using the infrared macro-imaging recordings showing a response to the change in air conditions.

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**Table 2. Mucociliary transport (MTV and CBF), temperature and humidity in lamb tracheas measured in vivo through an optical window**

| Inspired Air Conditions: | Btps | Reduced Temp. and RH | Dry |
|-------------------------|------|----------------------|-----|
| MTV (IR-MI), mm/min     | 14.6±4.1 | 8.2±2.8 | 3.8±2.8 |
| MTV (Vis-VM), mm/min    | 10.5±3.4 | 7.2±2.6 | 2.5±1.1 |
| Overall average MTV, mm/min | 12.6±3.8 | 7.7±2.7 | 3.2±2.0 |
| CBF (IR-MI), Hz         | 14.7±0.8 | 14.5±1.2 | 13.5±1.2 |
| CBF (Vis-VM), Hz        | 13.8±1.5 | 12.1±3.3 | 10.9±1.4 |
| Overall average CBF, Hz | 14.3±1.2 | 13.3±2.3 | 12.2±1.3 |
| Air temperature in trachea - inspiration | 37.9±0.5°C | 35.2±1.4°C | 32.7±1.1°C |
| Mucosal surface temperature - inspiration | 37.3±0.5°C | 35.0±4.1°C | 32.0±3.3°C |
| Absolute humidity in trachea - inspiration, mg/L | 45.0±0.7 | 30.6±90 | 0.5±1.8 |
| Relative humidity in trachea - inspiration | 96.9±0.6% | 75.4±13.9% | 2.6±2.5% |
| Dew point in trachea – inspiration | 37.5±2.2°C | 29.9±4.4°C | -26.0±14.1°C |
| Air temperature in trachea - expiration | 37.9±0.5°C | 35.2±4.1°C | 32.7±1.1°C |
| Air temperature in trachea - expiration | 38.7±0.7°C | 38.0±9.9°C | 37.6±0.7°C |
| Mucosal surface temperature - expiration | 37.6±0.5°C | 35.5±3.9°C | 32.9±2.8°C |
| Absolute humidity in trachea - expiration, mg/L | 46.0±0.7 | 39.9±2.3 | 31.0±3.3 |
| Relative humidity in trachea - expiration | 83.1±1.5% | 86.1±0.8% | 68.2±4.0% |
| Dew point in trachea – expiration | 35.3±0.9°C | 35.3±0.9°C | 30.7±16°C |

Mucociliary transport (mucus transport velocity (MTV) and cilia beat frequency (CBF)), temperature and humidity in lamb tracheas measured through an optical window using infrared macro-imaging (IR-MI) and visible-light video-microscopy (Vis-VM) with different inspired air temperatures and humidities (body temperature and fully saturated with water vapor (BTPS) (38°C and 100% relative humidity (RH)). Reduced temperature and humidity (35°C and 80% RH); and Dry (33°C and 0% RH). Measurements are presented as the mean ± standard deviation.
additional information on the relationship between MTV and CBF in disease. There was no correlation between CBF and MTV. MTV varied considerably in five healthy animals (up to 26.1 mm/min) and was substantially faster than in the animal with pneumonia, where it did not exceed 2.4 mm/min. This suggests that beating cilia, which propel mucus along the airway epithelium, do not change their frequency in the physiological conditions studied here in vivo. Further, mucus displacement per cilia beat substantially exceeded double the length of the cilia in most measurements, which suggests the mucus layer gains momentum during cilia-mucus interactions and keeps sliding during the recovery stroke of the cilia. The results raise questions about the interpretation of in vitro and ex vivo mucociliary transport studies in which cilia function alone is used as a proxy for mucociliary transport (43, 59).

The observed CBFs were like those previously measured in mammals, 2 to 15 Hz (18, 22), using a variety of other techniques. Despite efforts to maintain isothermal conditions, body core temperature varied between 36.2°C and 41.1°C. Body temperature was not correlated with either CBF or MTV (Supplemental Fig. S3). It has been reported that CBF can vary considerably with temperature (34, 37, 38). These variations can be related to the effects of temperature on biochemical processes in the epithelial cells, such as those providing energy for the cilia, and to the effects of temperature on the mechanical properties of cilia and the viscosity of the airway surface liquid (38, 60). MTV and CBF measurements were relatively consistent, with standard deviations varying between 6 and 18% (Table 1), unlike in some published experiments performed under ambient conditions, in which standard deviations were 20–37% (50, 60, 61). These discrepancies may indicate the importance of maintaining a thermodynamic balance when evaluating ciliary function.

The MTVs in the tracheas of the healthy lambs were higher than those reported elsewhere (18, 19, 21, 25, 41, 62), although there was considerable variation in individual readings over the 4 hours of each experiment where BTPS air was used for ventilation. For example, the MTV of lamb number HL01 (Supplemental Fig. S2) varied between 16 and 5 mm/min over a 15-min period. This may have been because of variability in the naturally occurring particles used to determine MTV, as their size and chemical structure have been shown to have a major influence on their clearance from the airway (63, 64). Specifically, finer particles (aerodynamic diameter ≤ 2.5 µm) are more difficult to clear (63, 65). Unfortunately, determining particle size with video-microscopy is not easy: it is difficult to determine leading and trailing edges, and to consider the effects of rotation of irregular shapes. Further studies are indicated to compare the reliability of measuring MTV with uniform-shaped highly-visible tracer particles compared to natural particles. Additionally, it should be noted that the MTV measurements presented here do not represent movements of mucus at different depths or across multiple planes relative to the surface layer.

The heterogeneity of the mucus that was observed with video-microscopy might also have contributed to the variability in the MTV results and our finding that the CBF was not related to the MTV (Fig. 3), the latter being contrary to a correlation reported between CBF and MTV in an in vitro study (33). Gerber et al. in 1997 (66) found that the CBF was only weakly correlated with MTV and suggested this was because of mucus being heterogeneous across the trachea and thus not forming a homogenous blanket over the upper airway. The heterogeneity in the opacity of the mucus that was observed in the video-microscopy recordings might be because of differences in the depth of the mucus layer resulting from undulations in the underlying epithelium. It might also be because of variations in viscoelastic properties of the airway surface liquid because of local variations in mucin production by airway cells (6, 67). Because the area analyzed in the trachea was relatively large (0.2 mm²), it is assumed that a mixture of ciliated and secretory cells made up the underlying epithelium. Further investigations into the physical properties of mucus may help to explain the reported mechanism of improved clearance of airway mucus with osmotic agents such as hypertonic saline (68) and mannitol (69), these improvements occurring consistently in the disease setting, especially in cystic fibrosis where airway mucus is likely dehydrated, but not in healthy lungs where mucociliary clearance is reduced (70). Likewise, further study may clarify the effect of humidified inspiratory gases on airways with mucosa dysfunction (42) and reports of improved mucociliary transport in clinical settings (43, 44).

Mucus displacement per cilia beat (µm/beat) varied greatly throughout the experiments, reaching maximum values of 34.2 µm/beat. Considering that the maximum distance the tip of an average 6 µm cilium (71) can travel between the initiation and completion of the effective stroke is, at most, 12 µm, it is noted that ca. 50% of the mucus displacement data recorded here would exceed double the length of a

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**Figure 8.** Sensible (gray) and latent (light gray) heat transferred by convection between the mucosal surface and the air inside the trachea when inspired air was conditioned to: body temperature and fully saturated with water (BTPS) (38°C and 100% relative humidity (RH)); Reduced Temp. and RH (35°C and 80% RH); and Dry (33°C and 0% RH). Heat transfers were calculated from values presented in Table 2. The percentages represent the latent heat contribution to the total heat transferred. Heat transfer by convection from the tracheal mucosa increases with dry air and is predominately latent heat transfer.

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[FIGURE 8]
cilium. Considering the kinematics of an individual cilium versus collective cilia motion, which creates metachronal waves, the simple comparison above is unrealistic; however, it does highlight how fast the mucus can travel relative to the maximum theoretical displacement an individual cilium can induce. Mathematical models have predicted the maximum velocity of the cilia tip to be 200–370 \( \mu \text{m/s} \), translating to 14–26 \( \mu \text{m/beat} \) (72–74). Some of the values recorded here also exceed this maximum theoretical displacement. Although we did not study the mechanism of mucociliary transport, it appears likely that the mucus layer slides over the periciliary layer between effective strokes of cilia; and, during physiological conditions CBF does not affect MTV.

It was noted that one of the lambs, which appeared normal before the experiments, had markedly abnormal MTVs yet typical CBFs. SEM of the epithelium from the sick animal showed that cilia were truncated and thus it appears likely that, although beating with a normal frequency, they would have propelled mucus with a substantially lower velocity. Although the cause of the pathological changes could not be determined, it is known that a variety of respiratory pathogens alter the planar arrangement of the respiratory epithelium, damage the cilia, and alter mucus composition and the cilia-gens alter the planar arrangement of the respiratory epithelium. From the second series of experiments, we were able to make contactless measurements of the mucosal surface temperature before the airway mucus, along with entrapped particles, is swallowed. The change in surface area, from the lower to upper airway, and the rate at which mucus moves up from the distal airway, appears to have no major implications on the MTV measured in the trachea regarding a change in mucus to airway surface volume. Absorption of water through the lower bronchi through active ion transport mechanisms appears important for maintaining the airway surface liquid volume (35, 78–80). Delayed mucociliary clearance or hypersecretion in distal airways can be seen in the trachea even in the absence of abnormalities in the tracheal epithelium. Using the trachea to study mucociliary transport in vivo offers an important advantage over in vitro models, which rely only on the local production of mucus and might not fully represent the actual physiological environment of the airways.

From the second series of experiments, we were able to make contactless measurements of the mucosal surface temperature in vivo, data which previously have been collected using thermocouples (48, 81, 82) and thermistors (83, 84). Unfortunately, when thermocouples and thermistors contact the mucosal surface, they may produce erroneous measurements by stimulating the cough reflex, by creating turbulence in the trachea, and by measuring wet-bulb temperatures owing to the sensor being in direct contact with the wet surface. IR-MI mitigates the above-mentioned issues but requires an optical window in the trachea. A further advantage of using IR-MI, in addition to measuring the surface temperature, is that it can also simultaneously measure CBF and MTV over a large surface (74 mm²).

With IR-MI, we found that the difference between the tracheal air temperature and the mucosal surface temperature was only minor during ventilation with BTPS-conditioned air. The difference became greater as the temperature and humidity of the inspired air decreased and pronounced fluctuations were seen in the mucosal surface temperature during the breathing cycle. These temperature fluctuations suggest a thermodynamic imbalance on the mucosal surface because there is a greater effect of evaporative cooling during inspiration and of condensation during expiration when inspired air is not conditioned to BTPS. Under such conditions, the transfer of heat and water from the surface into the air follows the second law of thermodynamics, resulting in a change in the physical properties of the airway surface liquid. In this study we see the change in MTV is more affected by decreases in mucosal surface temperature and humidity of air in the trachea than was CBF. Specifically, CBF was not significantly different when inspired air was at BTPS compared to when the air had reduced temperature and humidity. Optimal mucociliary transport occurs when inspired air is conditioned at BTPS (42) and the reduced fluctuations of the mucosal surface temperature that we observed between inspiration and expiration when the lamb was breathing BTPS air are consistent with this, which indicates a thermodynamic balance on the airway surface and an absence of evaporation from the airway surface liquid. Inspiration of colder and dryer air may trigger mucin hypersecretion (85). Mucins are the major proteins in mucus, imparting most of its hydrogel properties. The mucin granules are secreted from goblet cells in the epithelium and when these secretory glands are in a condensed state they absorb water (up to 600 fold mass/mass) to reach an expanded hydrated state, thereby coating the epithelium surface (86). Under BTPS conditions, over a prolonged period it was shown that MTV in the trachea varied and this could be linked to various hydration states of the mucus depending on the local variations in mucin concentrations and proximity to secretory cells. As air humidity decreases, the water available for mucin hydration becomes limited. With lower hydration and decreased temperature, mucus becomes more concentrated and viscous (4), making it more difficult for the cilia to propel the mucus, thereby lowering MTV. This was evident from our results, as inspired air with no or reduced humidity resulted in substantial decreases in MTV. Temperature receptors in the epithelium will stimulate mucosal hypersecretion (85), resulting in increased levels of poorly hydrated, hyper-concentrated mucus produced to protect the underlying cells which are known to be sensitive to changes to temperature (60) and desiccation. It appears that the hypersecretion of mucin acts as a protective mechanism that reduces evaporation of water and protects the epithelium.

Even though MTV decreased with cooler and dryer air, CBF was not significantly affected by these changes. Previously, CBF was found to be responsive to changes in air temperature and humidity when measured in vitro (28); however, the experimental setup had unidirectional air flow which caused rapid evaporation and cooling of the mucosal surface, and the heat and moisture was not recovered during expiration. The IR-MI measurements in vivo demonstrate heat and moisture recovery during expiration is sufficient to prevent a decrease in cilia function and maintain mucus.
movement. It is known that, to beat, cilia require ATP produced by enzymes that are temperature dependent (41, 60, 87, 88). No significant changes were found in CBF, suggesting that body temperature was maintained in the ciliated epithelium and that metabolic activity in the cells remained mostly unaffected by the changes in the tracheal air and the surface temperatures.

The heat transfer by convection calculations, using the mucosal surface temperature and tracheal air temperature and humidity, showed heat losses were mainly related to latent heat transfer and that, as the inspired air became cooler and dryer, the latent heat transfer comprised 92% of the total heat lost from the mucosa to the air. The viscous mucus layer associated with cold and dry inspired air would therefore act to minimize further evaporation and preserve conditions in the ciliated epithelium. Evaporation comes at an energy cost, where a change from normal physiological conditions to air temperature and humidity decreased substantially caused a total heat transfer increase of 96% (calculated over an assumed 15 mm diameter and 100 mm length of the trachea). From the calculated heat transfers by convection, a change in airway surface liquid height over a portion of the trachea could also be estimated. When cold dry air was inspired, the airway surface liquid height would change by 0.67 μm/s (assuming the air was the only source of water for the airway surface liquid and the change in height occurs over the geometry assumed above, details can be found in Supplemental Data). This rate was substantially greater than that determined from heat transfers under BTPS inspired air conditions, where a change in airway surface liquid height was approximated as 0.023 μm/s, an amount that could be replenished by the capillary bed (89).

In summary, the simultaneous measurements of the CBF and MTV, using video-microscopy through the optical tracheal window in mechanically ventilated lambs with BTPS air revealed a constant CBF 13.9 ± 1.6 Hz and a variable MTV, the latter of which reached 26.1 mm/min and resulted in a maximum displacement of the mucus layer of 34.2 μm/beat. Variability in the MTV over the 4-h experiment, where inspired air was heated and humidified at BTPS conditions, could be attributed to the heterogeneity of the mucus layer, which affects its physical properties and the data suggest that CBF measured alone may not be sufficient for assessing the effectiveness of mucus clearance. Additionally, use of IRMI enables measurement of CBF and MTV, as well as mucosal surface temperature in vivo. Air humidified to BTPS prevents evaporation from the airway surface liquid and maintains a thermodynamic balance in the airway, seen as minimal fluctuations in the mucosal surface temperature during ventilation that created optimal conditions for mucociliary transport. Further, latent heat transfer, related to the evaporation of water, appears to be a key contributor to the change in mucosal surface temperature and when inspired air is dry or not fully humidified, mucociliary transport slows, as evidenced by a reduction in MTV without an effect on CBF, suggesting a change in the physical properties of the mucus layer caused by heat and mass transfer because of convection. The methods presented herein, using in vivo video-microscopy over a prolonged period, could be applied to studies of the effect of various interventions on mucus clearance.

### SUPPLEMENTAL DATA

Supplemental Video S1: https://doi.org/10.6084/m9.figshare.13064042.
Supplemental Video S2: https://doi.org/10.6084/m9.figshare.13064045.
Supplemental Video S3: https://doi.org/10.6084/m9.figshare.13206239.
Supplemental Video S4: https://doi.org/10.6084/m9.figshare.13206251.
Supplemental Data (Sensible and Latent Heat Transfer): https://doi.org/10.6084/m9.figshare.13206050.
Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.13205996
Supplemental Fig. S2: https://doi.org/10.6084/m9.figshare.13063994.
Supplemental Fig. S3: https://doi.org/10.6084/m9.figshare.13064036.
Supplemental Fig. S4: https://doi.org/10.6084/m9.figshare.13206002.

### ACKNOWLEDGMENTS

Andy Ramsden, Graham Murray, Geraldine Keogh, David Liu, and Nikolai Fazoulline are acknowledged for contributions to the study.

### GRANTS

This study was supported and funded by Fisher & Paykel Healthcare.

### DISCLOSURES

S. J. Kelly and S. Tatkov are employees of Fisher & Paykel Healthcare Limited. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

### AUTHOR CONTRIBUTIONS

P.B. and S.T. conceived and designed research; V.B., E.M.S., P.B. and S.T. performed experiments; S.J.K. and S.T. analyzed data; S.J.K., V.B., E.M.S., P.B. and S.T. edited and revised manuscript; S.J.K., V.B., E.M.S., P.B. and S.T. approved final version of manuscript.

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