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Brief mechanical ventilation impacts airway cartilage properties in neonatal lambs

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Abstract

Ultrasound imaging allows *in vivo* assessment of tracheal kinetics and cartilage structure. To date, the impact of mechanical ventilation (MV) on extracellular matrix (ECM) in airway cartilage is unclear, but an indication of its functional and structural change may support the development of protective therapies. The objective of this study was to characterize changes in mechanical properties of the neonatal airway during MV with alterations in cartilage ECM. Trachea segments were isolated in a neonatal lamb model; ultrasound dimensions and pressure-volume relationships were measured on sham (no MV; n = 6) and MV (n = 7) airways for 4 h. Tracheal cross-sections were harvested at 4 h, tissues were fixed and stained, and Fourier transform infrared imaging spectroscopy (FT-IRIS) was performed. Over 4 h of MV, bulk modulus (28%) and elastic modulus (282%) increased. The MV tracheae showed higher collagen, proteoglycan content, and collagen integrity (new tissue formation); whereas no changes were seen in the controls. These data are clinically relevant in that airway properties can be correlated with MV and changes in cartilage extracellular matrix. Mechanical ventilation increases the *in vivo* dimensions of the trachea, and is associated with evidence of airway tissue remodeling. Injury to the neonatal airway from MV may have relevance for the development of tracheomalacia. We demonstrated active airway tissue remodeling during MV using a FT-IRIS technique which identifies changes in ECM.

Keywords

neonatal airway; elastic modulus; ultrasound; Fourier transform infrared imaging spectroscopy (FT-IRIS)

Introduction

Alterations in airway mechanical properties from ventilatory therapies such as continuous positive pressure airway pressure and mechanical ventilation (MV) have been implicated in

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airway disorders.¹⁻⁴ Although these treatments are often necessary and beneficial, positive airway pressure has also been associated with the pathogenesis of chronic lung and airway disease, particularly in the neonatal and pediatric population.⁵ Mechanical ventilation imposes physical forces such as pressure-induced stretch and high shear forces, which are not normally present in a developing airway.^{1-3,6,7} The physical forces inherent in positive pressure respiratory support alter the normal structure and function of the airway, often with detrimental clinical implications. For example, MV-induced barotrauma and volutrauma increase the dimensions (anterior-posterior diameter and tracheal volume) of the trachea, thus increasing dead space.^{5,8} Furthermore, although clinically advantageous for gas exchange, MV functionally alters airway mechanics, resulting in airway collapse and gas trapping.³ Taken together, the effects of MV present a pernicious challenge to clinicians trying to support infants with respiratory failure.

Previous studies have shown that real-time imaging assessment using ultrasound technology allows precise measurement of tracheal dimensions *in vivo*; thus, changes in mechanical properties can be monitored in real-time and compared to structural changes of the airway.^{5,8} Cartilage plays a major role in the structure of the airway. The resident chondrocytes are exposed to a normal degree of mechanical stimuli; however, under MV, abnormal pressures may be applied. To date, it is not clear whether mechanical stress during MV is damaging to the neonatal airway cartilage or, more importantly, if this stress has an impact on airway chondrocytes and extracellular matrix (ECM) expression. The effects of mechanical stress on articular cartilage have been extensively studied, as have changes in mechanical force on the chondrocyte biosynthesis of ECM and related cartilage genes⁹⁻¹¹; however, the effect of MV forces on neonatal airway cartilage has not.

We hypothesized that the anterior portion of the tracheal cartilage would undergo significant changes in mechanical stress during MV and that changes would occur concomitantly on chondrocytes and the cartilage ECM. In order to test this hypothesis, we examined mechanical properties, molecular, and morphological changes of ECM in airway cartilage during MV and in control (sham-operated) trachea. The changes in ECM were assessed using Fourier transform infrared imaging spectroscopy (FT-IRIS) and immunohistochemistry.

Fourier transform infrared imaging spectroscopy is a novel imaging methodology that has been utilized to examine various types of natural tissues including bone,¹²⁻¹⁷ cartilage,¹⁸⁻²³ and tissue-engineered, cartilage-like tissue and biomaterials.²⁴⁻²⁶ The specific molecular components in cartilage contribute to its infrared spectrum and these can be detected using FT-IRIS methodology. An FT-IRIS spectrometer, when coupled with a light microscope, enables one to identify intensity and distribution of tissue compositions. For cartilage, semi-quantified contents of collagen, proteoglycan, and collagen integrity (neo-collagen formation) have been demonstrated, as well as collagen fiber orientation using this technique.^{20,27} Thus, in the present study, collagen, proteoglycan, and collagen integrity (newly synthesized collagen) of trachea cartilage was assessed with respect to functional alterations in trachea dimensions and mechanical properties.

Materials and Methods

Tissue segment preparation and mechanical ventilation

The detailed methods of animal and experimental preparation of MV have been presented elsewhere.^{5,6,8,28} All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee, Department of Biomedical Research, Nemours/Alfred I. duPont Hospital for Children and were in accordance with National Institutes of Health guidelines. Thirteen neonatal lambs (147 day gestation) were anesthetized by injections (2 X

1 mL/kg) of an anesthesia mixture (ketamine: 23 mg/mL; azepromazine: 0.1 mg/mL; and xylazine: 0.05 mg/mL) (KAX). Prior to instrumentation, the animals were placed on a radiant warmer bed (Resuscitaire®; Hill-Rom Air-Shields, Hatboro, PA). The wool from the area of the lamb's neck covering the trachea was shaved and then completely removed by hair-removal cream.

As previously described,^{28,29} the double tracheotomy was performed to isolate the tracheal segment as shown in Figure 1. For venous access and arterial blood sampling, respectively, 5- and 8-Fr umbilical catheters were inserted into the external jugular vein and carotid artery through the lower surgical site. Subsequent anesthesia was maintained with intravenous infusions of KAX at 0.4 mL/kg/h or as needed. Maintenance fluid was provided by a continuous venous infusion of 5% dextrose solution at a rate of 6 mL/kg/h. Vital parameters, including arterial blood pressure, ECG, rectal temperature, and blood chemistry (Stat profile®; Nova Biomedical, Waltham, MA), were monitored throughout the study to confirm physiologic stability.

Pressure cycles delivered to the tracheal segments were set to mimic those used in the clinical setting; in this case, time-cycled, pressure-limited mechanical ventilation (Sechrist Neonatal ventilator, Sechrist Industries, Anaheim, CA) was delivered for 4 h at the following settings: peak inspiratory pressure = 35 cmH₂O, positive end expiratory pressure = 5 cmH₂O, system gas flow rate = 10 L/min, frequency = 40 cycles/min with inspiratory time = 0.30 s, and fraction of inspired oxygen = 0.21.

Tracheal segments were obtained from non-ventilated (sham; n = 6) or mechanically ventilated (MV; n = 7) neonatal lambs. Ultrasound dimensional measurements of the segments were made while static (0 PEEP; atmospheric pressure) and at pressure limits during dynamic ventilator cycling as noted above. Static ultrasound measurements were used to normalize pressure–volume data for resting volume, calculation of bulk modulus, stress–strain relationships, and the adapted elastic modulus associated with tangential wall stress. Temporal changes in bulk and elastic modulus demonstrate the time dependence of alterations in conducting airway mechanical properties *in vivo* during the course of MV.^{28,29}

Pressure–Volume relationships and bulk modulus

Airway pressure–volume relationships were measured as previously reported.^{5,8} Briefly, a saline-filled injection system was attached to the endotracheal tube of the isolated tracheal segment (Figure 1). The isolated tracheal segment was filled with normal saline, and the pressure within the saline-filled trachea was equilibrated with the ambient condition. The distal catheter was then connected to the pressure transducer (closed to the room), and 0.2-ml aliquots of saline were injected into the closed tracheal preparation in 15 sec intervals until a pressure of 80 cmH₂O was reached, and then the saline was withdrawn in the same manner.

The resultant pressure profiles associated with the step volume increments were recorded, and injected volume units were normalized to resting segment volume. Bulk modulus (K) was calculated from pressure–volume data using the formula in Equation 1. In this equation, Δp is the change in pressure, ΔV is the infused volume, and V is the resting volume. K is evaluated as a pressure quantity with units of kilopascals (kPa).

$$K=(\Delta p/\Delta V)V \quad \text{Equation 1}$$

Stress–Strain relationships and elastic modulus

At each time point in ventilated animals, the relationship between tangential wall stress and strain of the trachea was determined during dynamic ventilator pressure loading. The concept of the Young's modulus was adapted to the trachea as an elastic modulus (E) by making the assumption that the trachea behaves like a thin-walled cylinder. Thus, for a thin-walled cylinder, the wall stress is uniform throughout the thickness of the wall. This assumption is appropriate in that visual interpretation of the tracheal response to pressure pulses reveals most of the distortion to occur in the posterior wall, which is composed of the distensible trachealis muscle tissue. Equation 2 shows the calculation for E, where σ is the circumferential stress experienced by the cylinder walls and ϵ is the circumferential strain observed at the center of the cylinder.

$$\sigma = E \cdot \epsilon \quad \text{Equation 2}$$

Circumferential wall stress was determined using Equation (3), where p is the peak airway pressure achieved during the ventilator cycle, D_p is the airway diameter associated with p, and t_p is the corresponding wall thickness. The posterior, or muscle, wall thickness was used for t_p because this dimension varied over time and thus would indicate a susceptibility to the implied stress.

$$\sigma = (p \cdot D_p) / (2 \cdot t_p) \quad \text{Equation 3}$$

Circumferential strain is the ratio between the change in diameter and the resting diameter, as shown in Equation 4, where D_{static} is the diameter of the airway under static conditions (0 PEEP; atmospheric pressure).

$$\epsilon = (D_p - D_{\text{static}}) / D_{\text{static}} \quad \text{Equation 4}$$

Histology and immunohistochemistry

Tissue segments were fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were stained with safranin-O, anti-type II collagen antibodies. For type II collagen immunostaining, tissue sections were pretreated in chondroitinase-ABC (CABC), hyaluronidase (HASE), and protease-K to effectively retrieve antigen for 45 min at 37°C, 30 min at 25°C, and 30 min at 25°C, respectively. To reduce nonspecific background staining, hydrogen peroxide and ultra V Block solution (Thermo Scientific, Fremont, CA) were utilized, and 10% normal goat serum with 2.5% bovine serum albumin and 0.1% nonidet in phosphate buffer solution for 30 min were utilized to additionally block the background staining. After several washings, primary antibody for type II collagen (Chondrex, Redmond, WA; antibody dilution: 1/750) was applied, and the sections were incubated for 2 h in a humidified chamber at room temperature. Then, the detection system (Ultravision ONE HRP Polymer; Thermo Scientific) was used as described by the manufacturer. The substrate (AEC Single Solution; Thermo Scientific) was used and resulted in a brown-red staining where the anti-collagen antibody bound.

Fourier transform infrared imaging spectroscopy

To determine the biochemical or molecular characteristics of the cartilage, FT-IRIS was carried out using a Spectrum Spotlight 300 spectrometer (Perkin-Elmer, Waltham, MA) equipped with an optical microscope with an array detector. Five- μm thick sections from blocks prepared as described above were mounted onto barium fluoride windows. A region of interest (ROI) containing a hinged region that was anticipated to show significant changes

resulting from MV is shown in Figure 2. This area was selected and scanned at a spatial resolution of 25 μm and spectral resolution of 4 cm^{-1} . Based on our direct visual observations of wall motion (ultrasound images), the wall displacement is not uniform in response to applied pressure. Ultrasound images, as well as resulting histological sections of the tracheal wall, demonstrate that the majority of stretch and deformation is located at the posterior wall where the trachealis muscle connects the cartilage. Thus, it appeared that there is a midline region on the anterior wall of the trachea which acts as a hinge where the side-walls of the cartilage expand and relax in response to ventilator pressure. Based on the biomechanics of a thin-walled cylinder, we reasoned that this point, or hinge is a ROI where high stress would occur during inflation (compression on the outer wall and in-tension on the inner wall), as shown in Figure 2. Of course, during deflation, the opposite motion and stress occurs.

For these reasons, we focused our FT-IRIS studies on this small region of the cartilage to maximize the sensitivity of the analysis. Since this type of study had not been previously done, it was arbitrary how much of the cartilage ring we could analyze and maximize the sensitivity of the FT-IRIS approach. Therefore, as shown in Figure 2, we took an arc of the cartilage (800 to 1200 micrometers) in length along the midline anterior region of the trachea (ROI). The acquired data were analyzed by ISys software 5.0 (Malvern Instruments, Worcestershire, UK). Fourier transform infrared imaging spectroscopy parameters were measured based on the characteristics of the molecular bond (composition) and the number of molecules (intensity) at a specific frequency (wavenumber; cm^{-1}). In cartilage, collagen molecules were monitored using the integrated area of amide I absorbance arising from the C=O stretching vibration, centered at 1655 cm^{-1} , and proteoglycans were found in the 1125-920 cm^{-1} region attributing to sugar-ring vibrations. In addition, collagen integrity (new collagen formation) and collagen maturity (crosslink) were evaluated (see Figure 3).²⁶

Statistical analysis

Results are reported as mean $\pm$ standard deviation. Two-way ANOVA and post-hoc Student's *t* test were performed comparing groups within each time period ($P < 0.05$). All analyses were performed using SYSTAT 13 (SYSTAT Software, Chicago, IL).

Results

The isolated tracheal segments had a mean length of 59 ± 5 mm and no measured tracheal-segment dimension (internal diameter [ID], cross-sectional area [CSA], and segment volume [Vol]) was different between groups at baseline conditions.^{5,8} Furthermore, after 4 hrs, there were no differences in sham tracheal dimensions. However, as compared to the sham group, the MV group airway measurements increased over time (mean $\pm$ SEM; ID, 8.4 ± 0.2 mm to 9.6 ± 0.3 mm, $P < 0.01$; CSA, 55.7 ± 3.2 mm^2 to 73.1 ± 4.6 mm^2 , $P < 0.05$; and Vol, 3.45 ± 0.33 cm^3 to 4.37 ± 0.44 cm^3 ; $P < 0.01$). Representative histology for tracheal segment cross-sections harvested from sham and MV group immediately following the 4 hrs of treatment are shown in Figure 3. As shown, there was a thinner and stretched, pseudostratified, ciliated columnar epithelium wall resulting from ventilation pressure. In addition, as shown, radial expansion of the entire tracheal cartilage and muscle was observed.

As previously reported,^{5,8} the greatest deformation in trachea dimension is associated with the posterior region where the cartilaginous tips are spread apart. The ROI hinged area (Figure 2B) is a high stress point where tension (outer region of trachea) and compression (inner region of trachea) were simultaneously applied during MV. In this regard, this region was assessed between groups in ECM compositional intensity and distribution using histology shown in Figure 3. As demonstrated, the MV group showed an increase in airway diameter and damage on the epithelial layer compared to sham group (Figure 3A and 3B).

Furthermore, the MV group showed more type II collagen and the cartilage wall was thinner compared to sham group.

In parallel with previously reported functional results (bulk and elastic modulus; Table 1),^{5,8} FT-IRIS analysis demonstrated differences in intensity and distribution of ECM in the tracheal cartilage between the sham and MV groups, as shown in representative images (Figure 4). The collagen and proteoglycan content in the MV group was two times higher than in the sham group. In addition, fewer differences in distribution were observed in the sham group (Figure 4A and 4C). Based on the pixel intensity of tracheal sections from both groups, quantitative FT-IRIS results demonstrated that the MV neonatal trachea group showed significantly higher collagen ($P < 0.05$), proteoglycan ($P < 0.05$) content, and greater signals for collagen integrity (new tissue formation) ($P < 0.05$) as compared to the sham group (Figure 5).

Discussion

In this study, we demonstrated that mechanical ventilation changes neonatal tracheal morphology and cartilage composition. Airway dimensions in the ventilated group were remarkably increased along with tracheal cartilage, pseudostratified ciliated columnar epithelium, and muscle. These morphology findings are consistent with those from previous developmental sheep studies in which preterm lambs received MV for short durations.^{1,6}

After MV, analysis of tracheal internal diameter revealed a significant interaction between group and time ($P < 0.01$) where the internal diameter of MV animals increased over time (14%; $P < 0.05$) but did not change in the sham group. In addition, it has previously been shown that cartilage wall thickness was not different as a function of either time or group.^{5,8}

Fourier transform infrared imaging spectroscopy demonstrated differences between sham and MV groups with respect to intensity and distribution of ECM, while fewer differences were demonstrated by histological evaluation. Thus, FT-IRIS provided a more sensitive method for tissue comparison. Along these lines, FT-IRIS has been utilized to examine various types of natural tissues including bone,^{12–17} cartilage,^{18–23} and tissue-engineered cartilage-like tissue and biomaterials.^{24–26} As shown herein, the coupling of a Fourier transform infrared spectrometer and an optical microscope afforded us the ability to study the relative amount, molecular nature, distribution, and orientation of the components of tissues at a spatial resolution of 6.25–25 μm . For cartilage, it has been shown that FT-IRIS can provide a semi-quantified assessment of the distribution and amount of collagen, proteoglycan, and collagen integrity (new-collagen formation) as well as collagen fiber orientation.^{20,27} For collagen integrity, the 1338 cm^{-1} absorbance is from collagen CH2 side chain vibrations, and this absorbance band has been shown to decrease in intensity as collagen denatures.³⁰ For example, the integrated area ratio of 1338 band (1356–1326 cm^{-1})/amide II band (1590–1500 cm^{-1}) is reduced in human osteoarthritic tissues compared to normal tissues.²²

Results from this specialized imaging approach showed that the MV group demonstrated remarkable increases in collagen, proteoglycan content, and evidence of new collagen formation as compared to the sham group. Collectively, these findings demonstrate that MV increased the level of constitutive ECM components of the neonatal tracheal cartilage, thus suggesting both airway damage and a concomitant repair process. Furthermore, these results indicate a potential change in ECM biosynthesis as a result of MV. It is noteworthy that these biochemical alterations are concomitant with functional alterations in bulk and elastic modulus^{5,8} and that these findings are consistent with previously reported airway structure-function alterations following MV.^{1–3,6,7}

In summary, the current study supports our hypothesis that there is a significant increase in collagen and proteoglycan content as a result of MV, which may parallel the repair response to mechanical stress in neonatal cartilage. The experimental model and outcomes presented here serve as a plausible platform to evaluate various “protective” therapies and alternatives to conventional MV with the overriding goal being to maintain or restore airway function and biochemical preservation during MV. Furthermore, based on previous developmental airway studies,³¹ we know that it is possible to assess function-structure from generation 0–4. Thus, using the FT-IRIS approach, it should be possible to evaluate the impact of mechanical ventilation on cartilage in the more compliant distal airways as a function of time, level of pressure exposure, and mode of ventilation. In this regard, the more distal airways may be a more sensitive bioassay of cartilage alterations in response to mechanical stress.

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Abbreviations

CSA	cross-sectional area
ECM	extracellular matrix
FT-IRIS	Fourier transform infrared imaging spectroscopy
ID	internal diameter
KAX	ketamine, azepromazine, xylazene mixture
MV	mechanical ventilation
ROI	region of interest
Vol	segment volume

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Figure 1.

Schematic of the surgical preparation. A lower endotracheal tube (ETT) was placed into the distal trachea to allow for spontaneous breathing. Arterial and venous catheters provided access for anesthesia maintenance fluid, arterial sample, and pressure monitoring. The isolated tracheal segment was instrumented with an upper ETT and a lower catheter for flush and airway pressure transducer.

Figure 2.

(A) Schematic of trachea (cross section) and (B) region of interest (ROI) for Fourier transform infrared imaging spectroscopy (FT-IRIS). Compression and tension are simultaneously applied to the hinged region (inner: solid arrow and outer: dotted arrow) to the indicated directions according to the mechanical ventilation.

Figure 3. Histological evaluation of tracheal cartilage. Region of interest of tracheal cartilage. Safranin-O (A and B) and immunohistochemistry of type II collagen (C and D) for sham and mechanical ventilation (MV) (10x magnification, Scale bar = 100 μ m). Each representative image was shown at the same magnification and represents the notable changes in airway diameter in the MV versus sham group. Figure (A and B) shows proteoglycan content by safranin-O staining. Mechanical ventilation induced tension and compression to the trachea, and the forces applied to the hinged area resulted in a decrease of tracheal thickness (B, D) and more abundant type II collagen (D).

Figure 4.

Fourier transform infrared imaging spectroscopy analysis of tracheal cartilage for collagen (A–B) and proteoglycan (PG) (C–D) content. The color map demonstrates intensity and distribution of compositional contents of tracheal cartilage. The scale bar indicates that red was the highest and blue was the lowest in intensity; collagen content showed higher distribution near the edge, whereas PG demonstrated higher distribution near the center of the ventilated sample (B, D). The distribution of pixel intensity of color map images was demonstrated by histogram, and both collagen and PG contents in the ventilated group were notably higher than in the sham group. Left column indicates sham, and right column indicates mechanical ventilation (MV).

Figure 5. Quantitative Fourier transform infrared imaging spectroscopy (FT-IRIS) analysis of tracheal segment (sham and mechanical ventilation [MV]); (A) collagen, (B) proteoglycan (PG), (C) collagen integrity; and (D) collagen crosslink (* $P < 0.05$; $n = 4$ /group).

Table 1

Alterations in functional properties as a function of time and ventilation

Time	Elastic Modulus (% BL)		Bulk Modulus (% BL)	
	Control	MV	Control	MV
Baseline (BL)	100	100	100	100
1 hr	93.2 ± 3.7	138.8 ± 29.8	93.3 ± 3.7	113.4 ± 2.8
2 hr	95.4 ± 10.9	169.6 ± 21.6 ^a	95.5 ± 10.9	115.6 ± 3.9
3 hr	98.7 ± 5.6	204.9 ± 30.4 ^a	98.7 ± 5.6	118.0 ± 9.3
4 hr	102.8 ± 8.2	281.8 ± 58.7 ^a	102.8 ± 8.2	130.9 ± 11.5 ^a

Changes in tracheal stress–strain relationships associated with 4 hrs of mechanical ventilation (MV) or control (sham). Elastic modulus increased over time in animals exposed to mechanical ventilator pressures ($P < 0.05$) but did not increase in control animals. Elastic and bulk modulus increased compared to control animals as noted,

^a $P < 0.01$. n = 6 (Control; sham) and 7 (MV). (Adapted from Miller et al., See ref [5,8])