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Automated Indentation Mapping of Vocal Fold Structure and Cover Properties across Species

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Abstract

Objectives/Hypothesis—Various animal models have been employed to investigate vocal fold (VF) and phonatory function. However, biomechanical testing techniques to characterize vocal fold structural properties vary and have not compared critical properties across species. We adapted a non-destructive, automated indentation mapping technique to simultaneously quantify VF structural properties (VF cover layer and intact VF) in commonly used species based on the hypothesis that VF biomechanical properties are largely preserved across species.

Study Design—Ex vivo

Methods—Canine, leporine, and swine larynges (n=4 each) were sagittally bisected, measured, and subjected to normal indentation mapping (indentation at 0.3mm; 1.2mm/s) with a 2mm spherical indenter to quantify normal force along the VF cover layer, structural stiffness, and displacement at 0.8mN; 2-D maps of the free VF edge through the conus elasticus were created for these characterizations.

Results—Structural stiffness was 7.79gf/mm (0.15–74.55) for leporine, 2.48gf/mm (0.20–41.75) for canine, and 1.45gf (0.56–4.56) for swine. For each species, the lowest values were along the free VF edge (mean $\pm$ SD; leporine: 0.40 $\pm$ 0.21gf/mm, canine: 1.14 $\pm$ 0.49gf/mm, swine: 0.89 $\pm$ 0.28gf/mm). Similar results were obtained for the cover layer normal force at 0.3mm. On the free VF edge, mean (SD) displacement at 0.08gf was 0.14mm (0.05) in leporine, 0.11mm (0.03) in canine, and 0.10mm (0.02) in swine.

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Conclusions—Automated indentation mapping yielded reproducible biomechanical property measurement of the VF cover and intact VF. Divergent VF structural properties across canine, swine, and leporine species were observed.

Level of Evidence—N/A

Keywords

larynx; vocal fold; mechanical testing; indentation; voice; structural stiffness

INTRODUCTION

Biomechanical testing methods to compare physiologically relevant mechanical properties of laryngeal tissues are limited. Unique laryngeal geometry and pliability of vocal fold tissues create a challenging environment for biomechanical testing approaches.[1–3] Indentation biomechanical testing techniques show promise in both animal models and engineered vocal fold products to objectively assess material stiffness and elasticity.[4–8] Comparison between excised human and canine vocal fold cover layers identified similar stiffness and elastic properties measured via Young’s modulus.[5] These preliminary studies provided useful biomechanical information, but are time-consuming and traditionally require significant tissue dissection or disruption. Tissue alteration and time make them less appealing for widespread adoption as biomechanical assessments comparing laryngeal tissue properties.

With the increased need to objectively assess vocal fold and laryngeal tissue after injury, novel therapies, or even engineered vocal fold substitutes or stem-cell based products, more efficient, accurate, and reliable biomechanical measurements are needed. Optimization of indentation techniques are an attractive approach to achieve this goal, as indentation measures displacement and resisting forces perpendicular to the plane of the vocal fold, thereby reproducing its physiologic motion during phonation. Conversely, rheologic and force elongation measurements, earlier pioneered biomechanical tests, do not provide perpendicular displacement measurement of the vocal fold structure. Previous work from our lab illustrated the potential for micrometer scale measurements of intact vocal fold elastic and stiffness properties through dynamic nanomechanical analysis.[7, 8] Results were limited by the length of time necessary to complete the study and the complexity of adequately securing the specimen in the testing system exactly perpendicular to the indenter tip during testing. Between each test position on each sample, the specimen requires manual repositioning. This nuanced set-up introduced the potential for measurement variability, seen in the wide range of elastic and stiffness values collected during these initial studies. This manual setup also limited data resolution, as it reduced the number of testing locations on a given sample.

To overcome limitations of traditional microindentation testing for biologic tissues, we employed an indentation system capable of collecting indentation mapping data across irregularly shaped surfaces to improve existing mechanical testing approaches for laryngeal tissues. This technique successfully measured mechanical properties of irregular geometric surfaces such as articular cartilage.[9, 10] For the purpose of this investigation and to collect

valuable normative data, we chose to compare intact, excised hemilarynges from three common animal laryngeal models: swine, leporine, and canine.

METHODS

Tissue Preparation and Setup

Four swine, four canine, and four leporine flash-frozen (-80°C) larynges were bisected in the sagittal plane maintaining an intact anterior commissure on one side for mechanical testing of the intact hemilarynx. The larynges were kept frozen at -20°C until testing and thawed at $4-8^{\circ}\text{C}$ for period of at least 1 hour before being removed for testing. To avoid mechanical property changes from refreezing, specimens were thawed only once. Vocal fold length, from anterior commissure to vocal process, was measured for each specimen as reference. To secure the larynges in position during testing, a sample holder was created for samples of each species (swine, leporine, and canine). The sample holder was made from Plaster of Paris fitted to a frozen specimen protected by plastic wrap and then coated with a protective water-based varnish after drying. Samples were fixed to the holder using cyanoacrylate adhesive and tested in phosphate buffered saline (PBS) to maintain moisture covered with approximately 5mm of solution above the specimen (Figure 1B–D). Samples equilibrated for 15 minutes in PBS prior to initiating testing.

Automated Indentation Mapping

Three mechanical properties were assessed: normal force and structural stiffness at a given indentation and displacement distance at a chosen force. A Biomomentum Mach-1 v500css (Laval, Quebec, Canada) mechanical tester with a 150g uniaxial load cell was used to collect automated indentation mapping on the non-planar laryngeal surfaces bathed in PBS (Figure 1A). On each specimen, a testing grid was projected and numbered so testing across the hemilarynx proceeded in a random order, preventing confounding data collection from any nearby local temporary deformation effects (Figure 1E). Calibrating indentations for each specimen were made on blue putty affixed to the specimen holder prior to each specimen's testing. For 0.3mm amplitude tests, a 2mm spherical indenter tip was employed in all species as well as in a 0.5mm second mapping in leporine larynx specimens. A 5mm indenter tip was used for a 1.5mm amplitude second mapping in swine and canine specimens to account for the increased travel of the indenter tip to prevent tip bending, damage, or inaccurate data collection.

Data Analysis

For the shallower 0.3mm indentation, at each position of each specimen, the normal force at 0.3mm and the normal displacement at 0.08gf (785 μN) were determined from normal force vs. normal position curve (Figure 2). Structural stiffness at 0.3mm was calculated by dividing the normal force at 0.300mm by indentation amplitude (0.3mm). The 0.08gf load was selected as the load that maximized normal positions from the normal load vs. normal position curve and were not located in either of the load peaks caused by the inertia effect, corresponding to the first 0.06mm and last 0.06mm of the curve (Figure 2).

For the deeper indentations, 0.5mm in the leporine specimens and 1.5mm in the canine and swine specimens, normal forces, structural stiffness, and displacement amplitudes were calculated in a similar fashion to the shallow indentation. For displacement amplitude in the 0.5mm leporine specimens, 0.2gf load was selected for the displacement at a specified load based on the same criteria for the 0.08gf of the 0.3mm indentation; the first 0.12mm and last 0.12mm of the curve were considered part of the inertia effect. Similarly, in the 1.5mm canine and swine specimens, 0.7gf load was chosen for displacement at a specified load based on the same criteria; the first 0.27mm and last 0.2mm of the load vs. displacement curve were considered part of the inertia effect.

Data were collected individually at each point on each specimen for both a shallow and deeper indentation (0.3 and 0.5mm in the leporine and 0.3 and 1.5mm in the swine and canine) and recorded in individual data files. Mean, data range at each indentation location, and standard deviation (SD) of these values were collected as well as those for each indentation and each characterization parameter using LabVIEW (National Instruments, Austin, TX). For visualization of results, heat maps were created and superimposed on photo documented specimens. These heat maps were constructed based on average values of each biomechanical test parameter, with lower limits set to blue and upper limits set to red based on the average, SD, and data range.

RESULTS

Mean leporine vocal fold length measured 7.2 ± 0.9 mm compared to 14.8 ± 1.0 mm in canines and 15.0 ± 0.6 mm in swine. Mean normal force (mean (min-max) gf) at 0.300mm was higher in leporine specimens at 2.34gf (0.05–22.37) compared to canine at 0.74gf (0.26–12.52) and swine at 0.43gf (0.17–1.37). Similar results were obtained for structural stiffness. Leporine specimen stiffness was 7.79gf/mm (0.15–74.55), with canine specimens lower at 2.48gf/mm (0.20–41.75) and swine lower still at 1.45gf/mm (0.56–4.56).

Across all three species, normal forces and the structural stiffness measured along the free edge of the vocal fold were lowest within each specimen. This pattern persisted for both the shallow and deeper indentation amplitudes. Displacement at a set force, 0.08gf, was larger along the vocal fold compared to regions inferior and on the anterior and posterior edge of the vocal fold in canines and leporine specimens, with less difference found in the larger swine specimens. Mean displacement at 0.08gf was 0.14mm (0.06–0.35) in leporine, 0.11mm (0.06–0.36) in canine and 0.10mm (0.018–0.180) in swine specimens.

Heat maps for average values illustrate differences in normal force, structural stiffness, and displacement at a set force for both shallow and deep indentation parameters (Figures 3 and 4). Variations between locations within the larynx from the vocal fold moving inferiorly to the trachea can be visualized within and between species. Mean and range values listed below each averaged heat map reveal similar trends when comparing the shallow indentation targeting the vocal fold cover and the deeper indentation aimed at including the composite vocal fold structure.

DISCUSSION

The results of this study illustrate the utility and value of employing this microindentation technique to measure stiffness and displacement forces in vocal folds of widely varying sizes and geometry. At only approximately half the size of the canine and swine larynges, indentation mapping was successful even on the leporine vocal folds. Small standard deviations across varying positions support the reproducibility piloted in this study, particularly with small differences between specimens of the same species. These results compare favorably to prior work from our group on intact hemilaryngeal vocal folds and to work by others on vocal fold cover measurements.[6, 8]

However, limitations in study design and data collection warrant further discussion. Size limitations within the leporine larynx, both anterior-posterior diameter as well as tissue thickness, prevented data collection at the larger 1.5mm indentation amplitude with the larger 5mm indenter tip. Review of normal force and normal displacement revealed an initial precipitous increase in load magnitude approximately 0.21gf within the first 0.05mm followed by an equally precipitous drop in magnitude in subsequent equipment analysis to reflect acceleration inertia of the testing machine. As this effect occurred outside the data region of interest, it did not impact data analysis. One important consideration in this testing, illustrated in prior work by Chhetri et al, includes the geometrical implications on indentation parameters.[6] As distance between the indenter and the bottom of the specimen is smaller in smaller specimens (such as leporine compared to canine and swine), larger pressures were generated under the indenter in smaller objects (Figure 5) which would also affect structural stiffness, calculated from the normal force. Therefore, both normal force and structural stiffness were expected to be larger, in this condition, for smaller objects. We attempted to minimize this effect in the current study by choosing small indentation depths and indenter tips.

Despite these limitations, this study suggests that indentation mapping with this system can provide reliable measurement of the excised, intact hemilaryngeal vocal fold structure. The average indentation map collected from a control specimen or group serve as a reference map to compare treatment effects on mechanical properties of the vocal fold and larynx or properties of equivalent sized engineered vocal fold products. Implementation of this indentation mapping technique with a control specimen of the same species obviates variations measured between species in this study.

Biomechanical property comparison among species requires additional evaluation. Chhetri and Ravizadeh found similar Young's modulus values for excised human and canine vocal fold covers, compared to decreasing stiffness values in the larger, canine and swine larynges included in the current study. A number of etiologies likely underlie this difference. Although the depth of indentation for measurements representing the vocal fold cover layer at 0.3mm, it is possible the intact hemilarynx continues to provide a baseline stretch to the vocal fold that results in increased stiffness. Increased displacement identified at a set force along the free edge of the vocal fold supports the stretched theory for the attached vocal fold. Increased stiffness and normal force values collected during deeper indentations, 1.5mm on the swine and canine larynges and 0.5mm on the leporine larynges, support

collection of data reflective of the intact vocal fold structure, as the indentation depth extended through the vocal fold depth into deeper vocal fold tissue. The ability to collect data on both the vocal fold cover layer and deeper, composite vocal fold structures, is unique to this study not previously described elsewhere. The overall utility of this microindentation approach can be useful in the measurement of vocal properties across a variety of geometries and provide reproducible data on normal force, structural stiffness, and tissue displacement.

CONCLUSION

Variations exist in biomechanical properties between species and likely relate both to anatomical size and tissue properties. The repeatable, high fidelity data acquisition in this laryngeal testing platform provided a reliable foundation for intervention and treatment testing within leporine, canine, and swine laryngeal models.

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Figure 1.

A.) Swine larynx specimen in six-screw positioning template covered with PBS solution in Mach-1 mechanical tester with 2mm spherical indenter tip. B.) Leporine larynx affixed to specimen holder with calibration putty in place. C.) Swine larynx affixed to specimen holder. D.) Canine Larynx affixed to specimen holder. E.) Indentation mapping grid superimposed on canine hemilarynx fixed to specimen hold illustrating random order and area of indentations.

Figure 2. Sample normal force (gf) verses normal position (mm) graph. Black arrowhead identifies 0.08gf where displacement measures are determined. Black arrow pointing to 0.3mm location where force information is collected.

Figure 3. Heat maps illustrating a composite representation of each species by averaging normal force (gf), structural stiffness (gf/mm), and displacement at 0.08gf at each indentation mapping position (illustrated in the top row) for the 0.3mm indentation depth. Heat map reference values labeled along the left for each row of data. *Indicates that values above this are also colored red.

Figure 4.

Heat maps illustrating a composite representation of each species by averaging normal force (gf), structural stiffness (gf/mm), and displacement at 0.08gf at each indentation mapping position (illustrated in the top row) at the 0.5mm leporine and 1.5mm canine and swine specimens. Heat map reference values labeled along the left for the leporine data and in between the first and second column for canine and swine data. *Indicates that values above this are also colored red.

Figure 5.

Diagram of two specimens with same geometry, but different sizes. The black circle corresponds to a spherical indenter, the white line on the indenter indicating the same indentation amplitude for both specimens. Dashed brackets illustrate the thickness of the specimen under the indenter.