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Functional assessment of the ex vivo vocal folds through biomechanical testing: A review

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Abstract

The human vocal folds are complex structures made up of distinct layers that vary in cellular and extracellular composition. The mechanical properties of vocal fold tissue are fundamental to the study of both the acoustics and biomechanics of voice production. To date, quantitative methods have been applied to characterize the vocal fold tissue in both normal and pathologic conditions. This review describes, summarizes, and discusses the most commonly employed methods for vocal fold biomechanical testing. Force-elongation, torsional parallel plate rheometry, simple-shear parallel plate rheometry, linear skin rheometry, and indentation are the most frequently employed biomechanical tests for vocal fold tissues and each provide material properties data that can be used to compare native tissue versus diseased for treated tissue. Force-elongation testing is clinically useful, as it allows for functional unit testing, while rheometry provides physiologically relevant shear data, and nanoindentation permits micrometer scale testing across different areas of the vocal fold as well as whole organ testing. Thoughtful selection of the testing technique during experimental design to evaluate a hypothesis is important to optimizing biomechanical testing of vocal fold tissues.

Keywords

voice; biomechanics; nanoindentation; phonation; elastic modulus; mechanical stress; vocal folds; shear strength

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1. INTRODUCTION

As with synthetic materials, properties of biologic materials stem, in large part, from their mechanical characteristics. Understanding mechanical properties of biological materials allows for the creation of more accurate and useful biomechanical models. The human voice results from the interaction of numerous tissues, muscles, and nerves from the trachea to the oral cavity, of which the vocal folds (VF) play a central role (Figure 1). Air passing between the apposed VFs generates vibration and subsequent vocalization. The vocal folds themselves consist of distinct layers, each with separate mechanical properties that allow for smooth, coordinated motion during vocalization to produce the precision required for speech and singing (Figure 2). These layers are combined into the cover (epithelium and superficial lamina propria) and body (thyroarytenoid muscle) for analytical purposes, as the cover and body can move independently. These layers, moving independently under forces created by airflow through the glottis, create a wave-like flow of the VF cover that can be seen on videostroboscopy and is referred to as the mucosal wave. The propagation of the mucosal wave from the subglottis through the vocal folds is fundamental to the production of vocalization and relies on the varying properties of the vocalis muscle, ligament, lamina propria, and epithelium. The vocal folds are lengthened, shortened, raised, and lowered by the intrinsic laryngeal muscles to subtly modulate glottal airflow and create variations in timbre and pitch. Disruption of this delicate mechanical orientation from trauma, neoplasm, or inflammation can alter biomechanical tissue properties and voice.

The vast majority of the vocal fold cover is comprised of extracellular matrix [1]. The extracellular matrix proteins of the VF lamina propria consist of fibrous and interstitial proteins, in addition to lipids and carbohydrates. Many authors have posited that the fibrous proteins, collagen and elastin, dictate the tensile stress-strain characteristics of the vocal folds, whereas interstitial proteins such as proteoglycans and glycoproteins determine the viscoelastic shear properties by affecting fluid content and thickness of the lamina propria layers [1-3].

In historically-significant work, Hirano et al described the superficial lamina propria as loosely organized with few collagen or elastin fibers, the intermediate layer with increased number of elastin fibers, and the deep layer with increased collagen fibers [4]. Gray et al further elucidated that elastin was present throughout all three layers of the lamina propria, but elastin fibers are noted at an increased concentration within the vocal ligament [1]. Both elastin and collagen fibers are oriented longitudinally in the intermediate and deep layers of the lamina propria to ideally withstand the stress of the intrinsic laryngeal muscles [1, 5].

The mechanical properties of VF are fundamental to the study of both the acoustics and biomechanics of voice production. Significant resources have been dedicated to quantifying the precise biomechanical properties of these tissues to use in design and development of improved synthetic and biologic materials, ultimately for clinical application. However, the specific techniques employed for quantification of the *ex vivo* biomechanical properties of the VFs are not standardized. This lack of standardization is problematic, as many laryngology and voice science laboratories are investigating novel, translational therapeutics to alter wound healing and develop a regenerative model or frank replacement of the lamina

propria. Traditional histological outcomes do not adequately capture vocal fold dynamics, and more advanced assessment of the vocal folds is warranted. To provide a foundation for future investigation, the current literature on techniques and results of *ex vivo* laryngeal experimentation were reviewed.

2. MATERIALS AND METHODS

A database search to include Medline/Pubmed, Embase, and Web of Science was undertaken to compile relevant articles. Both a keyword search and MeSH analysis were employed to identify pertinent literature between January 1st, 1965 and November 30th, 2015. Keywords included vocal fold biomechanics, vocal fold and stress, strain, properties, *in vivo* testing, *in vitro* testing, *ex vivo* testing, elasticity, biomechanical testing, rheometry, indentation and nanonindentation, and mechanical testing. Relevant texts on biomechanical testing and materials science were searched for additional references for inclusion. Literature was then compiled and categorized for inclusion in the current manuscript.

3. BIOMECHANICAL TESTING OVERVIEW

The biomechanical properties of VFs have been measured *in vivo*, *in vitro*, and *ex vivo*. *In vivo* data are currently impractical and difficult to obtain due to inherent access and anatomical limitations. Early work in the 1990s by Tran, Berke, and Tanaka attempted to obtain in-vivo data [6-9]. Tanaka and Hirano designed a small tube that attached to the operative channel of a fiberoptic laryngoscope to measure stiffness via suction pressure applied to the VF [9]. Berke, Tran, and colleagues modified a force gauge to function through a laryngoscope and record *in vivo* vocal fold elasticity [6-8]. More recently, Hsiao and colleagues attempted to derive Young's modulus of the VF using color Doppler imaging, string vibration estimations, and known vocal fold properties [10]. To date, no simple, accurate mechanism exists to measure vocal fold biomechanics *in vivo*.

Kniesburges et al reviewed *in vitro* studies of vocal fold biomechanical properties, subdividing studies into static models, externally-driven models, and self-oscillating models [11]. Static models appear ideal for aerodynamic studies and glottal airflow measurements. Externally-driven models permit for detailed analysis of flow fields and vocal fold motion. Self-oscillating, synthetic vocal fold replica models permit the analysis of aerodynamics, acoustics, and structural tissue motion [11]. The self-oscillating models are currently the closest approximation to *in-vivo* functional assessment. However, studies of *ex vivo* tissues provide the most accurate information on biomechanical tissue properties.

Ex vivo tissue studies allow for adaptation of available mechanical testing techniques to characterize the VFs. These techniques can be conducted on both animal and human tissue. Typically, animal models are employed to obtain initial methodological insight, with canine and pig typically selected for gross morphologic and anatomic homology to humans, providing close approximation with a few key discrepancies. The canine vocal fold has a two-layer structure consisting of a cover and body, and no vocal ligament [12]. In the canine, a thin band of elastin fibers is deep to the basement membrane zone in the superficial layer of the vocal fold, and the vocal fold cover is thicker [13]. The human vocal fold, conversely,

has higher elastin content in the deeper layers of the lamina propria [13]. Pig larynges may be more ideal *ex vivo* models due to increased homology to human vocal fold cover thickness, structure, and overall stiffness [14]. The collagen, elastin, and hyaluronic acid content varies subtly between human and pigs [15]. Testing extends to human samples as well [16, 17]. Tissue preservation is generally not an issue; Chan et al described that the elastic shear modulus and dynamic viscosity do not change significantly following a 24-hour storage interval at room temperature or quick-freeze [18].

Ex vivo biomechanical testing can be broken down into five general approaches: force-elongation, torsional parallel plate rheometry, simple-shear parallel plate rheometry, linear skin rheometry, and indentation. Each of these testing techniques, summarized in Table 1 and have distinct advantages and disadvantages that allow for determination of various vocal fold biomechanical properties.

3.1 Force-elongation

An early technique to assess the biomechanical properties of the vocal folds involved quantification of force-elongation, typically conducted via an ergometer (Figure 3A). The experiments were conducted using fresh or early post-mortem canine vocal folds [19]. By applying frequency-specific sinusoidal signals to the ergometer, specimens were stretched and released, and the displacement of the ergometer arm and the force exerted by the tissue was measured. The force and displacement signals were calibrated and converted to stress and strain using the sample's cross-sectional area and length. The average cross-sectional area (A) is obtained using the sample mass (m) (without cartilage), *in situ* length (L) and density (ρ) at the end of the experiment according to $A = m/(\rho L)$ to calculate stress (σ) and strain (ϵ) [20, 21]. In general, the stress-strain curves produced by the force-elongation techniques demonstrated non-linear elastic behavior of the tissue, especially as elongation increases. When examining the stress-strain curve, the slope (inclination) represents the stiffness of the specimen, while hysteresis, the difference in the stress-strain curve between when the stress is increasing versus decreasing, represents viscosity of the specimen [22]. The non-linearity of the stress-strain curve is likely a result of the collagen content in the tissue, in particular the three dimensional collagen architecture in an accordion-like orientation [23]. For small strains (<0.15), the stress-strain relationship can be assumed to be linear, with the slope of the curve being Young's modulus. Young's modulus (E), is the elastic tensile strength of the material, calculated as the ratio of stress to strain ($E = \sigma/\epsilon$) and recorded in units of pressure (pascals, psi, etc). With low-strain, it is suspected that elastin is the primary load-bearing component within the tissue. Upon further displacement, initially unstretched collagen fibers begin to bear load [20]. Plotted over a traditional stress strain curve, Young's modulus can be extracted from the initial linear stress-strain relationship. Low-strain portions of stretch-strain curves behave linearly, and the formula above can be employed to estimate Young's modulus. Alternatively, high-strain regions are exponential and fitted using the model $\sigma = A(e^{B\epsilon} - 1)$, where A and B are determined by nonlinear least squares [24]. Alipour and Titze reported an average low strain Young's modulus of 21kPa for canine vocal fold body (muscle) and a value of 42kPa for the cover as measured along the longitudinal axis of the VF.

The longitudinal elastic properties of the human vocal ligament were also quantified using stress-strain measurements with subsequent mathematical modeling [21]. Min et al obtained human vocal ligaments from surgery and autopsy and subjected them to a dual-servo ergometer. The mean Young's modulus for the low-strain region (<15%) was 33.1+/-10.4KPa. At approximately 25% strain and 40% strain, Young's moduli were calculated to be 135KPa and 600KPa respectively [21]. The authors demonstrated the typical non-linearity and hysteresis previously described in vocal fold tissue experimentation. However, they demonstrated that these qualities were most pronounced in the vocal ligament when compared to other vocal fold tissue components.

A summary of the reported Young's Moduli of ex vivo specimens using force-elongation experimentation are presented in Table 2.

3.2 Rheology

Rheology is the study of matter flow applied to substances with complex structures. Rheology extends the disciplines of elasticity and fluid mechanics to materials with mechanical behavior that cannot be described with classical theories. Parallel plate and linear skin rheometry, described below, have evolved as popular methods to determine the viscoelastic properties of vocal fold tissues.

3.2.1 Torsional Parallel Plate Rheometry—A parallel-plate ("plate-on-plate") testing consists of a stationary lower plate and a rotating upper plate, with the gap size between the plates adjusted to fit the size of the specimen (Figure 3B). The sample is subjected to a sinusoidal torque from the upper plate, driven by a drag cup motor with a defined torque range. A transducer monitors resulting angular displacement and angular velocity of the upper plate as a function of time. Sinusoidal varying shear stress, shear strain, and strain rate are calculated from the torque and measured angular velocity, based on conversion constants of the testing system geometry in use. The shear stress and strain functions allow shear amplitudes and phase shifts to be measured. From this information, elastic shear modulus, dynamic viscosity, and damping ratio can be quantified [25].

For viscoelastic material, the general linear constitutive equation relating shear stress with shear strain and strain rate is $\tau = \mu\gamma + \eta\dot{\gamma}$ where τ is shear stress, γ is shear strain, $\dot{\gamma}$ is strain rate, μ is elastic shear modulus or storage shear modulus, and η is dynamic viscosity. Steady-state oscillation conditions will be reached for small-amplitude oscillations, for which the sinusoidal shear strain (input) results in sinusoidal shear stress (output) at the same frequency of oscillation. Resulting shear stress amplitude is proportional to the strain amplitude, and a phase lag independent of stress or strain amplitude. The same linear theory applies when sinusoidal shear stress is applied, resulting in a sinusoidal shear strain.

Shear strain and shear stress are represented as $\gamma^* = \gamma_o e^{i\omega t}$ and $\tau^* = \tau_o e^{i(\omega t + \delta)}$, where γ^* is complex shear strain, γ_o is shear strain amplitude, τ^* is complex shear stress, τ_o is shear stress amplitude, ω is angular frequency of oscillation, t is time, and δ is the phase shift of shear stress relative to shear strain. The ratio of the shear amplitudes and the phase shift, δ , are related to the elastic shear modulus μ and the dynamic viscosity η , which are functions of the frequency of oscillation and thermodynamic variables such as temperature and

pressure. The complex shear modulus, also known as the dynamic shear modulus or modulus of rigidity and calculated as $\mu^* = \mu + i\omega\eta$, can be defined as the ratio of complex shear stress to complex shear strain. In some rheology literature, G is used to represent shear modulus, where $G^* = \mu^*$, $G' = \mu$, and $G'' = \omega\eta$.

Application of sinusoidal shear strain to viscoelastic material, described by the linear constitutive equation results in a sinusoidal shear stress at the same frequency of oscillation (ω), represented as $\tau_o = |\mu^*| \gamma_o$. The elastic shear modulus μ and the dynamic viscosity η can be expressed in terms of shear amplitudes and the phase shift, which are measurable, by oscillatory shear deformation. The damping ratio ζ is defined as the tangent of the phase shift δ , or the ratio of the viscous shear modulus to the elastic shear modulus. A material is purely elastic if $\zeta=0$, (when $\eta=0$), purely viscous if $\zeta=\infty$ (when $\mu'=0$), and viscoelastic if ζ lies somewhere in between ($0^\circ < \delta < 90^\circ$). In the context of oscillation, a system is under-damped at $\zeta < 1.0$, critically damped at $\zeta = 1.0$, and over-damped at $\zeta > 1.0$. These phenomena can be characterized with a parametric model from experimental data.

These shear properties are important factors in selecting optimal biomaterials for tissue repair and have been evaluated in human vocal fold tissues [25]. The epithelium and superficial lamina propria from 15 excised human larynges (10 male and 5 female) were tested using a sinusoidal-oscillating, controlled-stress, parallel plate rheometer at varying frequencies from 0.01 to 15Hz to measure the complex shear moduli [25]. The elastic and viscous shear moduli and dampening ratios remained constant over a wide frequency spectrum. Conversely, the dynamic viscosity decreased monotonically with frequency, indicating shear thinning. Large inter-sample variability was observed postulated to originate, at least partially, from age and gender differences, as older and male samples were stiffer and more viscous when compared to younger and female subjects.

In subsequent studies, Chan and colleagues addressed the limitation that rheological data were obtained at a maximum of 15Hz, far below typical vocal fold vibratory frequencies of greater than 100Hz. The authors described methods to extrapolate low-frequency data to physiologically-relevant frequencies associated with phonation (100 to 1000 Hz) [26, 27]. In 2004, the authors modified their original protocol and introduced the use of controlled-strain torsional rheometry to collect measurements at higher frequencies [28].

Compared to controlled stress rheometry, controlled-strain torsional rheometry directly controls input strain, introducing significantly reduced system inertial errors, and allowing viscoelastic data to be obtained from 17 canine vocal fold mucosa specimens at frequencies up to 50Hz (0.016-50Hz) [28]. The higher frequency data were consistent with previous studies; the elastic shear modulus, viscous shear modulus, and damping ratio of the vocal fold mucosa were relatively constant across measured frequencies, whereas the dynamic viscosity decreased monotonically with frequency [28].

3.2.2 Simple-Shear Parallel Plate Rheometer—Simple-shear rheometry involves a parallel-plate configuration in which a linear, simple-shear deformation is applied to a tissue or material specimen by the upper plate with small-amplitude translational sinusoidal displacement. As a result of the viscoelastic response of the specimen, a harmonic shear

force is transmitted to the lower plate, which is separated from the upper plate by a small gap. A transducer detects this force. The contact area between the specimen and upper plate can be visualized through a transparent upper plate.

The displacement of the upper plate, x , can be represented as $x^* = x_o e^{i\omega t}$, where x_o is the amplitude of displacement, ω is the angular frequency, and t is time. The equation of motion for the system is $m(d^2x^*/dt^2) + c(dx^*/dt) + kx^* = F^*$, where m is mass, c is damping, k is stiffness of the system, and F^* is the resulting shear force in complex notation. For linear, small-amplitude shear, once steady state is reached, the applied sinusoidal displacement would result in a harmonic shear force at the same frequency, with a phase shift of δ according to the theory of linear viscoelasticity, such that $F^* = F_o e^{i(\omega t + \delta)}$ where F_o is the amplitude of F^* . For linear viscoelasticity, the phase shift, δ , is independent of the displacement amplitude and force amplitude (x_o and F_o).

In order to compute viscoelastic shear properties of a specimen, shear strain and shear stress of the specimen can be defined based on the displacement and force. Shear strain can be defined as the displacement, x , divided by the distance between the plates, or the gap size d ; $\gamma^* = \tan^{-1}(x^*/d) \approx x^*/d$ for $x_o \ll d$.

Shear stress is defined as $\tau^* = F^*/A$, where A is the area of the specimen experiencing the strain, as indicated by the area of contact between the specimen and the upper plate. Therefore, $\gamma^* = \gamma_o e^{i\omega t} = (x_o/d) e^{i\omega t}$ and $\tau^* = \tau_o e^{i(\omega t + \delta)} = (F_o/A) e^{i(\omega t + \delta)}$, where γ_o is the amplitude of γ^* and τ_o is the amplitude of τ^* . The corresponding linear constitutive equation relating shear stress to shear strain is $\tau^* = G^* \gamma^*$ where G^* is the complex shear modulus. G^* is composed of a real part and an imaginary part, with G' the elastic shear modulus and G'' the viscous shear modulus. For the linear theory of viscoelasticity, this can be written as $\tau^* = G' \gamma^* + [(G'' d \gamma^*)/(\omega dt)]$.

The elastic and viscous shear moduli, G' and G'' , are related to the strain amplitude and the stress amplitude (γ_o and τ_o) and phase shift, δ , and are expressed in terms of displacement amplitude and force amplitude. The dynamic viscosity η' is related to the viscous shear modulus G'' such that $\eta' = G''/\omega$. The damping ratio, ζ , is the ratio of viscous to elastic moduli, $\zeta = G''/G'$. Empirical data can be characterized with a parametric model, and the dependence of G' , G'' , and η' on frequency, f , described by a power law [29]. A summary of the least-square regressions for the parametric description of viscoelastic functions of VF tissues measured by parallel-plate rheometry is presented in Table 3.

Using linear, controlled-strain, simple-shear rheometry, Chan et al addressed the inherent limitations of torsional rheometry and investigated vocal fold viscoelastic properties at phonatory frequencies (100–200Hz for male and 200–300Hz for female speech) [29]. The vocal fold cover (epithelium and superficial lamina propria) from seven human larynges (5 cadaveric and 2 post-laryngectomy) were evaluated and the relationship of elastic shear modulus to viscous shear modulus changed with increasing frequency. Up to approximately 50Hz, the elastic shear modulus, G' , was lower than the viscous shear modulus G'' . Around 50Hz, cross-over occurred, and G' and G'' appeared to converge and cross each other at higher frequencies, indicating that at frequencies below 50 Hz, the tissue's viscoelastic

response was dominated by viscous properties. At frequencies above 50 Hz, elastic properties dominated. The dynamic viscosity η' decreased monotonically with frequency, reflecting shear thinning also observed in prior torsional rheometry studies. The damping ratio ζ of the seven specimens generally decreased with frequency, with ζ above 1.0 at frequencies less than 50Hz and below 1.0 at frequencies above 50Hz. This finding suggests over-damping of the vocal fold cover at low frequencies (<50Hz) where the viscous shear modulus is higher than the elastic shear modulus and underdamping at higher frequencies (>50Hz) consistent with human phonatory frequencies.

To evaluate differences in viscoelastic properties at various anatomic locations along the vocal fold, Kimura et al studied six canine larynges with a linear, controlled-strain, simple-shear rheometer [30]. Deformations were created along anterior, middle, and posterior portions of the vocal fold cover at frequencies ranging from 1Hz to 250Hz. The rheometric properties of the thyroarytenoid muscle were also studied, as it may become part of the vibratory tissue during large-amplitude vibration for high-intensity phonation [31]. Kimura et al demonstrated the elastic shear modulus gradually increased with frequency and the dynamic viscosity decreased monotonically with frequency identically across all regions of the vocal fold [30]. Variations were noted in different layers of the vocal fold, with the canine vocalis muscle significantly more stiff and viscous when compared to the canine vocal fold cover and human vocal fold cover. Additionally, the canine vocal fold cover was significantly more viscous than the human vocal fold cover, a finding likely secondary to canine vocal folds having 3-4 times more hyaluronic acid in the lamina propria leading to increased tissue viscosity [32]. Because the vocalis muscle is stiffer and more viscous than the vocal fold cover during vibration at phonatory frequencies, the threshold for vocal fold vibration involving the vocalis muscle is high [30].

3.2.3 Linear Skin Rheometer—A Linear Skin Rheometer (LSR) is an electromechanical instrument originally designed to measure the viscoelastic properties of the stratum corneum of skin. The probe is attached to a tissue using a needle mounted at right angles to the primary axis of the rod. The rod is capable of rotating through a full circle, which allows the needle to be inserted at any angle into the tissue. Adapting this technique to measure the viscoelastic properties of the vocal fold, the needle probe is inserted onto the vocal fold epithelium so that the direction of motion is perpendicular to a line drawn between the vocal process and anterior commissure.

A force-controlled miniature direct-current servo, gearing, and lead screw, drive the LSR probe. The force exerted on the probe is measured directly by a calibrated load beam mounted vertically within the instrument casing. The needle probe is attached to the surface of the tissue under test creating a shear force. F_{max} , the peak force applied to the skin surface, X_{max} , the peak displacement occurring as a result of that force, and T , the phase shift between the two signals are recorded. The resultant force/displacement data forms an ellipse secondary to displacement lagging behind the applied force as a result of viscous tissue properties [33, 34].

The dynamic spring rate (DSR) refers to the strain response of an elastic material when cyclic loading is applied. The DSR of the tissue is F_{max}/X_{max} , expressed as grams force

per millimeter. High DSR values are equivalent to high shear modulus values and high stiffness test material. The shear modulus can be derived from the DSR if tissue properties and measurement geometry are known. The shear modulus, stress, σ , per unit strain ϵ , $G = \sigma/\epsilon$, with ϵ given by tangential displacement X_{max} per material thickness H , $\epsilon = X_{max}/H$. When inputting known values, G can be reduced to $G = DSR \times H/A$.

Unlike *in vitro* rheological methods, *in vivo* LSR cannot be used to determine absolute values for viscoelasticity parameters on a unit area or volume basis as the volume of tissue deformed cannot be determined. However, relative *in vivo* measurements are sensitive, repeatable, and have clinical applicability. *In vivo* measurements could help identify regions of abnormal pathology, document changes resulting from treatment, and provide other important information regarding vocal fold properties during clinical and/or surgical examination [33, 34].

Hess et al employed LSR to evaluate the elasticity of male vocal folds. The greatest stiffness was observed at the vocal process, followed by the anterior commissure when the larynx was bisected [33]. The group also examined pig larynges and found stiffness greatest at the vocal process followed by the anterior commissure. The vocal fold was least stiff when displacements were oriented perpendicular to the long axis of the specimen and stiffest when displacements were oriented along this axis. Only DSR was measured; shear modulus was not calculated [33].

Goodeyer et al investigated the range of normal shear modulus values for male and female human vocal folds, measuring these viscoelastic properties using a LSR via two methods, applying cylindrical shear stress transverse to the axis between the vocal process and anterior commissure and as an indentometer [35]. Using the first method, the shear modulus for male VFs ranged from 246-3,356Pa (mean=1,008; SD=380) and female VFs 286–3,332Pa (mean=1,237; SD=768). Using the indentometer method, the shear modulus for male VFs ranged from 552 to 2,741Pa (mean=1,000; SD=460) and females VFs 509–1,989Pa (mean=1,332; SD=428).

More recently, Goodeyer et al measured shear modulus of vocal folds from intact, non-dissected, hemilarynges [36]. Previously, the majority of published vocal fold viscoelasticity data were obtained from excised vocal fold covers, and the shear modulus ranged from 10Pa to 100Pa. Using a LSR with a modified suction attachment in place of a needle probe placed against the vocal fold epithelium, the force was applied in a transverse direction at the mid-membranous point between the vocal process and the anterior commissure. The authors found the shear modulus of the three female vocal folds ranged from 814 to 1232Pa and three male vocal folds ranged from 1021 to 1796Pa. These values were significantly greater than previous studies (10 to 100Pa) [36]. The increased values are expected, however, as the vocal fold was under tension and anchored in its anatomical orientation.

Dailey et al obtained rheometric properties using LSR in normal vocal folds and various pathologies [34]. In normal canine vocal folds, DSR was highest (most stiff) in anterior and posterior-most regions. Within the mid-membranous region of the VF, DSR was greatest near the free edge. In normal human vocal folds, the posterior aspect of the specimen had the

highest DSR. Shear modulus was not calculated. These trends may be explained by thinning of the superficial lamina propria and presence of the anterior and posterior maculae flavae, an area of dense extracellular matrix.

Rohlf et al described the relationship between shear elastic properties of the vocal folds with respect to the direction of the applied stress within the multiple layers of the vocal fold [17]. Fourteen intact human larynges were subjected to LSR; the specimens were mounted to correspond to an anatomical context similar to the Goodeyer et al study. The authors reported a statistically-significant increase in lamina propria stiffness when measured in the longitudinal compared to the transverse plane. With each removed layer, stiffness was increased compared to the intact vocal fold [17]. These conclusions were supported by histological findings of increased collagen density in deeper layers and parallel subepithelial alignment of fibers in sagittal sections. Additionally, Rohlf et al suggested an anisotropic nature of vocal folds given directionality-specific (longitudinal versus transverse) mechanical responses.

Recently, the shear moduli of the vocal fold cover during intrinsic laryngeal muscle contraction were measured. Chhetri et al employed a modified LSR both *in vivo* and *ex vivo* in canine and human larynges, respectively [37]. Data from *ex vivo* human larynx confirmed the shear modulus increased up to 1.6 times the baseline value with graded arytenoid adduction. Similarly, the shear modulus increased to 3.7 times baseline values with cricothyroid approximation. These results were consistent with those obtained from *in vivo* canine larynges and suggest cricothyroid muscle contraction generates a greater change in cover stiffness than the laryngeal adductors. However, the role of each adductor in cover stiffness remains unknown. A summary of shear modulus measured by LSR is presented in Table 4.

3.3 Indentation

Accurate stiffness measurements using stretching or rheometry are challenging, as these methods require a relatively large specimen relative to the small larynx to ensure accuracy. Indentation, in various forms, has been used to measure material properties for many years and is often used to measure material properties of small samples such as biologic tissues [38]. Initially, the concept of atomic force microscopy, where a small probe is attached, via an arm, to an atomic force microscope to measure forces in as small as single cells, was built upon for mechanical testing of biologic tissue samples. Currently, these measurements can be made on the nanometer scale, compared to earlier equipment permitting only macroindentation. Employing a small, rigid indenter to locally deform the sample surface, contact forces generated by the indenter displacements are recorded. Using the slope of the force-displacement curve, a Young's modulus is then calculated based on the Hertzian contact theory of elastic bodies [39].

Measuring viscoelastic properties with an indentation technique poses some challenges. For example, effects of indentation depth on stiffness are not directly addressed by Hertzian theory, but are known to influence accuracy of the estimated Young's modulus [40]. The multi-layer structure of the vocal fold also poses challenges for stiffness estimation given its multi-layered structure resulting in an effective modulus of the body and cover layers.

Haji and colleagues utilized a version of indentation to measure vocal fold properties in 1992 using an 1mm in diameter indenter [22]. Excised canine larynges were exposed to a force-detecting probe to obtain stress-strain data under various experimental conditions. The authors reported increased vocal fold stiffness with increasing tension, but stiffness decreased after stripping the vocal fold mucosa. The authors also simulated thyroarytenoid muscle contraction, vocal fold dehydration, and vocal fold edema.

More recently, Chhetri and colleagues examined vocal fold Young's modulus using the indentation technique in various anatomic conditions [38]. To calculate Young's modulus, loading-unloading cycles were recorded for each sample. The slope of the initial portion of the unloading force-indentation depth curve (dF/dh) was fitted to a fourth-order polynomial. The Young's modulus of the tested sample (E) was estimated, based on a Hertzian model for a cylindrical contact, as $E = [(1-\nu^2)/2R] (dF/dh)$, where ν is Poisson's ratio, and R is the radius of the cylindrical indenter. The authors found that Young's modulus was measured with reasonable accuracy when indentation depth was smaller than the indenter diameter, and both the indenter diameter and the indentation depth were much smaller than the material thickness. When testing the mid-membranous vocal fold tissue, measurements revealed location-dependent Young's moduli. The intact hemilarynx had the greatest Young's modulus at 8.6KPa (5.3–13.1KPa), followed by the isolated inferior medial surface cover at 7.5KPa (7–7.9KPa), the isolated medial surface cover at 4.8KPa (3.9–5.7KPa), the isolated superior surface cover at 2.9KPa (2.7–3.2KPa), and finally the isolated thyroarytenoid muscle at 2.0KPa (1.3–2.7KPa). Note, these values differ significantly from the values obtained from force-elongation experimentation [38].

3.4 Biomechanical Testing Techniques Summary

Force-elongation, rheometry in its many forms, and indentation each offer specific advantages to study the three-dimensional, anisotropic nature of vocal folds. As described in Table 1 and expanded in the corresponding sections of the text, each technique offers the opportunity to evaluate a particular property relative to vocal fold architecture.

Force-elongation permits measurement of the vocal fold functional unit attached to the arytenoid complex, a translational research advantage. However, testing is only feasible possible in plane with the vocal fold and not perpendicular to the vocal fold edge, where phonatory forces displace push the vocal folds laterally. With that in mind, force elongation testing would allow for testing of how alterations to vocal fold composition, such as changes in collagen or loss of tissue bulk related to with aging, affect forces required to create sufficient tension for phonation. Torsional or simple-shear rheometry recreates the physiologic effects of airflow over the vocal fold, causing the vocal fold to move independently and parallel to underlying tissue layers. Thus, torsional and simple-shear rheometry can assess how physiologically-relevant tissues act under dynamic phonatory forces. LSR has less direct clinical application, as it pulls tissue away from underlying tissue layers, opposing opposite clinically relevant motion. LSR does have the capacity to provide data on attractive vocal fold layer properties and could be useful in assessment of scar or other vocal fold injuries extending between layers that may cause adherence between layers. Nanoindentation complements other testing techniques, as it can assess both dynamic and

static properties of the vocal folds in a clinically relevant fashion, perpendicular to the vocal fold. Nanoindentation can measure local and whole organ properties on the micrometer scale, a feat none of the other mechanisms can achieve.

Each *ex-vivo* biomechanic technique provides unique application capacity depending on the relevant clinical hypothesis. In most cases, the information collected from different methods is not comparable, but rather complimentary, as the properties measured (Young's modulus vs shear modulus, etc) vary. Nanoindentation is most suited for testing both functional units and individual tissue layers. Force-elongation tests functional units, and rheology is best suited to individual tissue layers. The vocal fold tissue property of interest can be carefully matched to the most appropriate testing technique using this information to obtain meaningful data.

Although each of these mechanical testing techniques offers measurement advantages, there are important limitations to consider. Force-elongation measurements can only be taken "in plane" (i.e. along the longitudinal axis) with the vocal fold. This is relevant for muscle tension information but, in the anisotropic vocal fold, does not translate to accurate measurements perpendicular to the vocal fold, where airflow through the glottis to create phonation acts. Alternatively, rheometric measurements are limited by the directionality of their assessment ability. Rheometry, while ideal to assess shear along the airstream plane, is not able to create forces perpendicular to the vocal fold tissue. Indentation techniques do not measure shear forces and are not capable of measuring tension, instead testing compression and relaxation. Indentation techniques are ideal for perpendicular measurements but cannot assess shear or longitudinal functional units. An understanding of these limitations is crucial during mechanical testing technique selection during experimental design.

4. Clinical Implications

Understanding the mechanical properties of individual and whole organ structures within the larynx is paramount to advances in therapeutics, synthetic injectable materials, and wound healing. Current challenges in vocal fold surgery include augmenting atrophic, paretic, or paralyzed vocal folds to mimic, as best possible, the natural functioning vocal folds and better understanding the biomechanical tissue alterations that occur in vocal fold scarring that create dysphonia. The dynamic functions of the larynx rely on the interaction of multiple intrinsic muscles and tissues to create the human voice. Application of the discussed biomechanical techniques provides critical information on these tissues to allow for advances in translational medicine.

Vocal fold atrophy and malfunctioning or nonfunctional vocal fold motion results in incomplete closure of the VFs during phonation, referred to as glottal insufficiency. In this scenario, the vocal folds do not create the typical mucosal wave second to airflow or restrict airflow egress, resulting in a weak, breathy voice and inability to maintain prolonged phonation without vocal fatigue or the sensation of shortness of breath. In the case of VF motion disruption, the defect is initially purely mechanical immediately after injury to the recurrent laryngeal nerve innervating the muscles that create VF motion but ultimately results in muscle tissue atrophy that can alter local VF biomechanics. In the setting of glottal

insufficiency second to VF atrophy, global effects of aging and gender (collagen/elastin makeup, etc) create vocal folds of various tissue constituents, which would alter their biomechanical properties and, thus, function. Both of these diagnoses are corrected with vocal fold augmentation, where materials, synthetic polymers or autologous tissues, are placed deep to the vocal folds to enlarge them and permit sufficient glottal closure for phonation. To create ideal phonatory structures, tissue properties after augmentation should match, as best possible, the natural biomechanical properties of VF tissues.

Disruption of the mucosal wave, as occurs in vocal fold scarring (sulcus vocalis), results in dysphonia. This scar can result in tethering of varying layers of the vocal fold, thus altering the global mucosal wave. In this situation, properties of both the vocal fold cover and vocal ligament are critical to understand how the vocal fold will move under phonatory forces. Furthermore, the local properties of scar (collagen/elastin makeup, etc) vary from the native vocal fold tissue. Knowledge of scar tissue properties is crucial to predict vocal fold behavior under scar conditions and develop therapeutic approaches to managing this difficult clinical situation.

Vocal fold atrophy and scar are two of the clinical scenarios where VF biomechanical properties are crucial to developing treatment strategies for these conditions. Work in these areas, as outlined, allows for some translation between alterations in local mechanical properties and overall vocal fold function and clinical performance.

4.1 Biomechanical effects of intrinsic molecular structures within vocal fold

Using stress-strain curves, Gray et al demonstrated some of the biomechanical properties of the vocal ligament [1]. The vocal ligament stretches easily under low levels of stress in a linear fashion, which likely reflects elastin fibers within the ligament. At a certain point, though, stress required for further vocal fold lengthening significantly increased. Furthermore, vocal fold cover and vocal ligaments from male and female human larynges were examined histologically, identifying higher levels of collagen in males in vocal fold cover and vocal ligament compared to females [5]. With regard to elastin, male larynges contained significantly higher levels in the vocal fold cover than the vocal ligament. To quantify elastic properties, specimens were tested using a dual-mode servo-control lever arm system. Similar to the results of Gray et al, a non-linear stress-strain response was observed for all specimens including the vocal fold cover and ligament, when subjected to high tensile deformation. At the high tensile strength, the elastic modulus of the male vocal fold cover was about twice the female cover, and the male vocal ligament was 3-5 times stiffer than the female vocal ligament in the same range. Overall, the ligament was stiffer in males, although the opposite was observed for female specimens. The authors speculated that collagen and elastin might differentially contribute to the elasticity of each the cover and ligament. Regression analyses failed to confirm if relative densities of collagen and elastin in vocal ligament specimens predicted the elastic modulus and various levels of strain. This lack of correlation may be attributed to varying collagen and elastin fiber orientations in the specimens, and that other matrix proteins (i.e. fibronectin, hyaluronic acid, etc) were not accounted for [5].

Hyaluronic acid (HA) is a heavily-hydrated glycosaminoglycan found in the ECM that likely contributes substantively to tissue biomechanics. As it is hydrophilic, HA increases water and ion content of the ECM and in turn increases the total volume or space the molecule occupies. Therefore, HA can have significant effects on several biomechanical properties, notably tissue viscosity, tissue flow, tissue osmosis, and tissue dampening [3]. Chan et al evaluated the effect of HA on the human vocal fold cover [2]. Vocal fold covers from five human male cadavers were subjected to a sinusoidal-oscillating, controlled-stress, parallel plate rheometer up to 15Hz. The rheometric properties were determined before and after application of bovine testicular hyaluronidase to selectively removed HA from the ECM of the lamina propria. The authors found an average decrease of 35% in the elastic shear modulus after removal of HA across all frequencies (0.01-15Hz). Additionally, the dynamic viscosity increased by approximately 70% after HA removal. The authors suggested that ECM HA maintains a relatively low viscosity, leading to a relatively low threshold level of energy required for phonation. Furthermore, HA likely contributes to optimal tissue elasticity, or stiffness, that may be important for control of vocal fundamental frequency.

As hyaluronic acid content alters viscosity, recent investigation demonstrated that vocal fold hydration altered the biomechanical properties. Hemler et al and Haji et al evaluated tensile viscoelastic properties, finding that stiffness of the vocal fold increased with dehydration [22, 41]. Chan and Tayama studied the effects of hydration on shear viscoelastic properties of vocal fold covers in canine larynges [42]. Using the same controlled-stress, parallel plate rheometer with the same frequencies as above (0.01-15Hz), the elastic shear modulus G' and dynamic viscosity η' significantly increased with tissue dehydration across all frequencies. Rehydration decreased G' and η' to a much lesser degree, and after rehydration, these values were still 3-3.5 times higher than that of tissues *in situ*, implying that rehydration does not restore native tissue properties. The damping ratio also changed similarly to elastic shear modulus and dynamic viscosity with dehydration and rehydration.

4.2 Viscoelastic Properties of Scar

Histological changes associated with vocal fold scarring are numerous and have mainly been characterized in animal models. Approximately two months post vocal fold scar formation, the amount of collagen precursors increase and density of collagen and elastin decrease in comparison to normal vocal folds. In late stages of wound healing (>6 months), elastin is replenished to normal levels but found in disorganized, fragmented fibers. Collagen levels are higher than that of normal vocal fold tissues in the late stages of wound healing, and are found in thick, disorganized bundles [43-45]. This altered collagen and elastin content and orientation alters the local biomechanical tissue properties.

Glycoprotein levels within the extracellular matrix fluctuate as well. Fibronectin, an important adhesion molecule with properties that are chemotactic for fibroblast and inflammatory cells, is increased in the superficial lamina propria for up to six months after injury in canine models [46]. Decorin maintains collagen fibril organization and is decreased in scarred vocal fold tissue, possibly leading to disorganized collagen bundles in the late stages of wound healing [3, 45-47]. Fibromodulin is thought to inhibit TGF-beta regulated collagen synthesis, and this ECM molecule is decreased in the superficial lamina propria

during scar formation [48]. Hyaluronic acid decreases significantly in the immediate post scar period (<2 weeks), but then seems to normalize as early as two months after injury [46]. As above, decreased levels of HA in the ECM increases tissue viscosity and decreases stiffness [2].

Thibeault et al illustrated rheologic characteristics of vocal fold scarring in rabbits [45]. After a unilateral vocal fold injury was created and allowed to scar, a sinusoidal-oscillating, controlled-stress, parallel plate rheometer was used for tissues assessment, finding that elastic shear modulus G' and dynamic viscosity η' were greater in scarred vocal folds across tested frequencies. In general, it is well accepted that the scar and subsequent stiffness are the result of an increase in fibrosis or an increase in collagen. However, on histology, overall collagen density decreased and the bundles were disorganized. This observation led the authors to suggest that the increase in stiffness and viscosity does not result from an increase in collagen, but rather with the loss of the normal collagen architecture. Rousseau et al evaluated the rheometric properties of vocal fold scar in a canine model at late stages of wound repair [43, 44]. The specimens were subjected to controlled-strain rheometry at frequencies up to 48Hz. Elastic shear modulus G' and dynamic viscosity η' measurements increased in nearly all cases at six months. Increased G' and η' indicate increased stiffness and resistance to shear flow for scarred tissue samples during oscillatory shear deformation.

LSR was also used to study pathologic tissue in human, canine, and rat cadaveric larynges [34]. Scar was unilaterally induced in a rat via vocal fold stripping and allowed to heal. The mean DSR was significantly greater for the scarred specimen compared to the uninjured side. In the canine model, a deep and shallow sulcus vocalis (scar trough) were histologically confirmed. DSR values in the region of the deep sulcus vocalis were significantly greater than surrounding tissue, which was not the case in the superficial sulcus vocalis. Lastly, trichloroacetic acid topically applied and injected into a normal human vocal fold to demonstrate the influence of chemical stiffening on DSR, with the DSR increasing across all measured points. The calculated DSR in this scenario remained higher in the anterior and posterior regions than in the mid-membranous region.

4.3 Viscoelastic properties after injection

An area under active research is the change in viscoelastic properties after injection augmentation. Dahlqvist et al and Borzacchiello et al compared the mechanical properties of various hyaluronan based materials, cross-linked collagen, and Teflon in rabbit vocal folds six months after injection. Measurements were conducted with a strain-controlled parallel plate rheometer. Vocal folds injected with hyaluronic acid based materials showed the lowest dynamic viscosity η' values and were similar to non-injected control samples [49, 50]. Likewise, Choi et al found no significant difference in the elastic shear modulus G' and viscous shear modulus G'' in rabbit vocal folds injected with hyaluronan-based material versus non-injected controls [51]. Together, these studies demonstrate that HA substances may be appropriate for preserving or restoring vibratory capacity.

Hyaluronan has been further studied in scar models. Hertegard et al scarred rabbit vocal folds and eight weeks later injected either hyaluronan or saline into the scarred vocal folds [52]. Eleven weeks post-injection, the rabbits were sacrificed and viscoelastic measurements

were then conducted with an LSR. Histological analysis demonstrated increased lamina propria thickness in scarred and injected animals versus non-scarred rabbits. No differences in the thickness of lamina propria were observed based on type of injection (hyaluronan or saline). On rheological testing, no significant difference was observed with regard to relative Young's modulus in vocal folds injected with saline compared to hyaluronan.

The Hertegard group also investigated the viscoelastic and histologic properties after injection of mesenchymal stem cells in scarred rabbit vocal folds [53]. A torsional parallel plate rheometer was used to obtain viscoelastic measurements. The dynamic viscosity and elastic shear modulus of the stem cell treated group was significantly lower than an untreated scar control group. However, these values were still significantly greater than a non-scar group. Histologic analyses showed significantly more type I collagen in the untreated scar group compared to the stem cell-treated and non-scar groups. The mesenchymal stem cells remained viable in all treated samples, indicating that the injected cells are likely contributory to the improved biomechanical profile.

Similarly, Cedervall et al injected human embryonic stem cells into scarred rabbit vocal folds [54]. On histology, untreated scarred vocal folds had the thickest lamina propria and showed thick collagen bundles. The stem cell treated group had thinner lamina proprias and only a few had polyp formation. Furthermore, significantly decreased dynamic viscosity and elastic shear modulus (measured with a torsional parallel plate rheometer) in the stem cell treated cohort. However, comparisons of viscoelastic comparison between treatment and a non-scar control group were not performed.

4.4 Future Directions

Over the past few decades, an increasing body of literature on mechanical properties of vocal folds using force-elongation, rheometry, and, more recently, indentation, serves as a foundation for investigation of clinically-relevant conditions, including vocal fold atrophy, scar, wound healing, injectable biopolymers, autologous tissue transfer, and implantation of synthetic materials to determine optimal therapy. Combining these biomechanical testing techniques in *ex-vivo* experimental systems, such as whole organ nanoindentation and force-elongation studies of pathologically altered vocal fold tissues can most thoroughly provide clinically-relevant information to optimize treatments. Similarly, shear force data on atrophic vocal folds can be combined with nanoindentation results to create a multi-dimensional assessment of this clinical entity to best compare interventions in experimental systems. Thoughtfully designed experimental approaches building on the work already compiled and selecting the most appropriate testing technique to measure properties of interest will provide the most clinically-appropriate insight. To this end, multidisciplinary teams with expertise in materials science, mechanical engineering, and laryngology allow for the creation of ideal experimental systems that best measure desired properties. These efforts will allow for accurate assessment and selection of the most appropriate novel therapies to create artificial or stem-cell based vocal fold tissue.

5. FINAL REMARKS

Knowledge of the mechanical properties of vocal fold tissues is critical for medico-surgical management of vocal fold disorders. The effect of tissue properties on vocal fold oscillation must be considered in phonosurgery, and the introduction of implantable biomaterials into the vocal fold change the inherent mechanical properties and alter the mechanics of vocal fold oscillation. This phenomenon is particularly relevant when the vocal fold mucosa is directly involved in repair as the mucosa is the major vibratory portion of the vocal fold, especially in small-amplitude oscillations like phonation onset and offset [4, 31]. Shear properties of vocal fold tissues and implantable biomaterials must be elucidated because oscillation of the mucosa involves the propagation of a surface mucosal wave, which is a shear wave [25]. Furthermore, in augmentation procedures where materials are injected lateral to the vocal fold, viscoelastic properties of the thyroarytenoid muscle and vocal ligament should be considered. Moving forward, mechanical tissue properties will play an even larger role in assessing new therapies and better understanding laryngeal physiology.

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Highlights

- Development of novel, translational therapeutics to alter wound healing and develop regenerative or replacement for vocal fold tissues relies on quantifying vocal fold mechanical properties.
- Mechanical testing techniques for vocal fold tissues include: force-elongation testing, torsional parallel-plate rheometry, simple-shear parallel plate rheometry, linear skin rheometry, and nanoindentation.
- *In-vivo* mechanical testing is difficult and largely impractical, *in-vitro* models are best for aerodynamic studies, and *ex-vivo* mechanical testing techniques are most widely as they allow for adaptation of existing mechanical testing techniques.
- Nanoindentation is most suited for testing both functional units and individual tissue layers. Force-elongation tests functional units, and rheology is best suited to individual tissue layers.
- Biomechanical properties differ between normal vocal fold tissue and vocal fold scars. Similarly, properties differ when vocal folds are therapeutically augmented. Both facts support the important role of tissue biomechanics in therapeutics.
- Combination of testing techniques in experimental design could provide more clinically relevant information when testing new therapeutic interventions or pathologic conditions of the vocal folds. This is particularly important when considering maximizing the use of human and animal tissues for experimental use.

Figure 1.

Videolaryngoscopic view from above of fully abducted healthy vocal folds.

Figure 2.

Coronal sketch of the vocal tract from trachea to supraglottis (LEFT). Cross-sectional view of the vocal folds (RIGHT) depicting the various layers that comprise the vocal fold. SLP = superficial lamina propria, ILP = intermediate lamina propria, DLP = deep lamina propria, VM = vocalis muscle.

Figure 3.

A) Force-Elongation test system diagram, B) Torsional plate rheometer diagram. The rotating upper plate oscillates in a sinusoidal fashion.

Table 1
Biomechanical testing techniques for vocal fold tissue. Advantages and disadvantages listed in adjacent columns

Testing Technique	Advantages	Disadvantages
Force – Elongation	<ul style="list-style-type: none"> • Tests functional unit • Repeatable 	<ul style="list-style-type: none"> • Only tests in one direction (along vocal fold)
Torsional Parallel Plate Rheometry	<ul style="list-style-type: none"> • Excellent viscosity assessment. • Possible to test layers of vocal fold. 	<ul style="list-style-type: none"> • Less clear relationship to Young's modulus. • Small specimen region – cannot test functional unit
Simple-Shear Parallel Plate Rheometry	<ul style="list-style-type: none"> • Excellent viscosity assessment. • Possible to test layers of vocal fold. 	<ul style="list-style-type: none"> • Less clear relationship to Young's modulus. • Small specimen region – cannot test functional unit
Linear Skin Rheometry	<ul style="list-style-type: none"> • Tests functional unit. • Mechanical analysis of layer attachment 	<ul style="list-style-type: none"> • Complex setup.
Indentation (atomic force microscopy, macro- and nanoindentation)	<ul style="list-style-type: none"> • Microanalysis possible in functional unit. • Small sample required. • Functional unit possible. 	<ul style="list-style-type: none"> • Expensive equipment. • Type of tip used will likely impact data collected.

Table 2
Force-elongation biomechanical testing studies

Study	Technique	Specimen	Number of specimens	Minimum (kPa)	Maximum (kPa)	Mean (kPa)
Perlman 1984*	Force-displacement transducer	VCVM: in situ, immediately post elongation	7	NR	NR	34.7
Perlman 1984*	Force-displacement transducer	VCVM: dissected, immediately post elongation	6	NR	NR	14.7
Perlman 1984*	Force-displacement transducer	VCVM: in situ, 20 min. post relaxation	7	NR	NR	20.3
Perlman 1984*	Force-displacement transducer	VCVM: dissected, 20 min. post relaxation	7	NR	NR	94.6
Perlman 1984*	Force-displacement transducer	PMCV: in situ, immediately post elongation	4	NR	NR	22.6
Perlman 1984*	Force-displacement transducer	PMCV: dissected, immediately post elongation	4	NR	NR	18.9
Perlman 1984*	Force-displacement transducer	PMCV: in situ, 20 min. post relaxation	4	NR	NR	14.5
Perlman 1984*	Force-displacement transducer	PMCV: dissected, 20 min. post relaxation	4	NR	NR	92.9
Alipour 1991*	Dual-servo ergometer	Human vocalis muscle	10	NR	NR	20.7 ± 2.4
Alipour 1991*	Dual-servo ergometer	Human vocal fold cover	9	NR	NR	41.9 ± 7.1
Min 1995*	Dual-servo ergometer	Human vocal ligament (surgical specimen)	4	21.9	47.1	36.1 ± 10.6
Min 1995*	Dual-servo ergometer	Human vocal ligament (cadaveric specimen)	4	21.2	42.2	30.1 ± 10.7
Alipour 2011	Dual-servo ergometer	Pig superior vocal fold	6	13.8	26.1	19.2 ± 4.2
Alipour 2011	Dual-servo ergometer	Pig inferior vocal fold	6			16.3 ± 1.9

Study	Technique	Specimen	Number of specimens	Minimum (kPa)	Maximum (kPa)	Mean (kPa)
Alipour 2011	Dual-servo ergometer	Sheep vocal fold	4	6.7	19.1	11.7 ± 4.6
Alipour 2011	Dual-servo ergometer	Cow vocal fold	9	18.1	44.4	29.9 ± 8.2

NR = not reported. * = at strain <15%. VCVM = viable canine vocalis muscle. PMCVM = post-mortem canine vocalis muscle.

Table 3
Studies and results for biomechanical testing using parallel plate rheometry

Study	Specimen	Technique	Viscoelastic function	a	b	R^2
Chan 2008	Human VF cover	Simple-shear	G'	17.246 Pa.s	0.6320	0.9493
Kimura 2011	Canine VF cover	Simple-shear	G'	39.431 Pa.s	0.871	0.797
Kimura 2011	Canine middle cover	Simple-shear	G'	33.366 Pa.s	0.964	0.795
Kimura 2011	Canine anterior cover	Simple-shear	G'	55.997 Pa.s	0.785	0.815
Kimura 2011	Canine posterior cover	Simple-shear	G'	30.144 Pa.s	0.846	0.781
Kimura 2011	Canine vocalis muscle	Simple-shear	G'	233.533 Pa.s	0.540	0.743
Chan 2008	Human VF cover	Simple-shear	G''	58.488 Pa.s	0.3243	0.8363
Chan 1999	Human male VF cover	Torsional	η'	3.1440 Pa.s ²	-0.8755	0.999
Chan 1999	Human female VF cover	Torsional	η'	0.7038 Pa.s ²	-0.8345	0.996
Chan 2008	Human VF cover	Simple-shear	η'	10.509 Pa.s ²	-0.7391	0.9934
Kimura 2011	Canine VF cover	Simple-shear	η'	76.651 Pa.s ²	-0.593	0.749
Kimura 2011	Canine middle cover	Simple-shear	η'	99.636 Pa.s ²	-0.615	0.738
Kimura 2011	Canine posterior cover	Simple-shear	η'	60.646 Pa.s ²	-0.562	0.867
Kimura 2011	Canine vocalis muscle	Simple-shear	η'	253.311 Pa.s ²	-0.535	0.915

Table 4
Studies and results for biomechanical testing using linear skin rheometry. Shear modulus presented

Study	Model	Condition	Shear modulus (Pa)	Min (Pa)	Max (Pa)
Goodeyer 2006	Human male	Method 1	1,008 (mean)	246	3,536
Goodeyer 2006	Human male	Method 2	1,000 (mean)	552	2,741
Goodeyer 2006	Human female	Method 1	1,237 (mean)	286	3,332
Goodeyer 2006	Human female	Method 2	1,332 (mean)	509	1,989
Goodeyer 2009	Human	-	1,123* (mean)	814	1796
Chhetri 2009	Human	-	1,191.5* (mean)	1,076	1,307
Chhetri 2009	Human	CT approximation	4,786 (median)	NR	NR
Chhetri 2009	Human	Arytenoid adduction	1,723 (median)	NR	NR
Rohlf's 2013	Human	-	671 (median)	440	780
Rohlf's 2013	Human (LP+muscle)	-	769 (median)	490	1,090
Rohlf's 2013	Human (ligament+muscle)	-	859 (median)	510	1,260
Rohlf's 2013	Human (muscle)	-	849 (median)	310	1,500

*=calculated from data provided. CT=cricothyroid. NR=not reported.