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Viscoelastic properties of human aryepiglottic fold and ventricular fold tissues at phonatory frequencies

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Abstract

Objectives—The purpose of this study was to quantify the viscoelastic shear properties of the human ventricular fold (or false vocal fold) mucosa and aryepiglottic fold mucosa at frequencies of phonation.

Methods—Linear viscoelastic shear properties of the mucosa of false vocal fold and aryepiglottic fold specimens from 7 cadaveric subjects were determined as functions of frequency (5–250Hz), and compared to those of the true vocal fold cover. Measurements of elastic shear modulus (G') and dynamic viscosity (η') were made with a controlled-strain simple-shear rheometer. Linear least-squares regression was conducted to curve-fit $\log G'$ and $\log \eta'$ versus $\log$ frequency, and statistical analysis was performed with one-way ANOVA.

Results—All specimens showed similar frequency dependence of the viscoelastic functions G' and η' , with G' gradually increasing with frequency and η' monotonically decreasing with frequency. The magnitudes of G' and η' of the false fold mucosa were generally higher than those of the aryepiglottic fold mucosa and true vocal fold cover, but there were no significant differences in G' and η' among the false fold, aryepiglottic fold and true vocal fold.

Conclusion—The false vocal fold and aryepiglottic fold mucosa showed similar frequency dependence and a similar range of tissue viscoelastic behavior as the true vocal fold. These preliminary findings suggested that such tissues could become candidates for the replacement of the true vocal fold lamina propria in patients with significant tissue loss and deficiencies, for those requiring laryngeal reconstruction following partial laryngectomy or airway reconstruction.

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Keywords

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Introduction

The viscoelastic properties of human vocal fold tissues have been examined in a number of previous studies, as the viscoelastic behavior of the true vocal fold critically dictates the acoustics, biomechanics, and energy transfer mechanisms of phonation.¹⁻⁷ However, to our knowledge there has been virtually no report on the viscoelastic properties of human supraglottal structures, including the aryepiglottic fold and the ventricular fold (false vocal fold), although the elastic properties of the false vocal fold have been examined previously.^{8, 9} It has long been clinically observed that certain patients with voice disorders, including those with muscle tension dysphonia and unilateral vocal fold paralysis, may demonstrate supraglottal compression including false vocal fold adduction, possibly as a compensatory mechanism.¹⁰⁻¹³ Ventricular phonation has often been reported, in which the false vocal folds are set into airflow-induced self-sustained oscillation similar to that of the true vocal folds, with the propagation of a surface mucosal wave.¹⁴⁻¹⁶ Ventricular fold and aryepiglottic fold vibration has also been observed in many styles of Mongolian “throat singing”, or overtone singing.¹⁷⁻¹⁹ YouTube videos posted for illustrating the laryngopharyngeal behavior of freestyle beatboxing apparently demonstrated that vibration of the ventricular fold and the aryepiglottic fold is part of the repertoire of this unique vocal performing art.²⁰ Anatomical studies of muscles in the ventricular folds and their neural control supported the possible active role of the false vocal folds during normal swallowing and phonation. Young et al. demonstrated that selective neuromuscular control of ventricular fold adduction is possible, in association with ventricularis muscle contraction.²¹ Moon and Alipour also suggested that contraction of the ventricularis muscle could result in ventricular fold medialization, which would facilitate flow-induced self-sustained oscillation with the propagation of a “mucosal wave” on the surface tissue layer.²² As such mucosal wave is a shear wave, the viscoelastic shear properties of the aryepiglottic fold mucosa and the false vocal fold mucosa become important parameters for determining the biomechanics of their oscillation.

Surgical procedures involving false vocal fold flaps have also been performed for the functional reconstruction of new laryngeal tissues following different kinds of partial laryngectomy for patients with laryngeal cancer.^{23, 24} Supraglottal structures like the false vocal fold mucosa and the aryepiglottic fold mucosa could become a source of vibrating replacement tissue following extensive tissue loss, as in certain cases of partial laryngectomy or airway reconstruction.^{15, 16} In order to better understand the feasibility of using these supraglottal structures as potential tissue replacement candidates from a biomechanical perspective, i.e., being capable of vibration and sound production, the viscoelastic shear properties of the false vocal fold mucosa and the aryepiglottic fold mucosa have to be quantified. These biomechanical measurements would also facilitate computer modeling and simulation studies that could help predict the functional voice outcome of phonosurgical procedures involving any supraglottal structures, in the same principle as surgical procedures

involving the true vocal folds, as illustrated in the National Center for Voice and Speech Phonosurgery Optimizer-Simulator.²⁵

The purpose of this study was therefore to measure the viscoelastic shear properties of the mucosa of the human false vocal fold and aryepiglottic fold. Linear viscoelastic shear properties of supraglottal specimens excised from human cadaveric larynges were quantified with a simple-shear rheometer, as a function of frequency.² This rheometer has been validated by our research group for quantifying the viscoelastic properties of a variety of tissue specimens and material samples at frequencies of phonation (up to 250Hz), including the human vocal fold cover,² canine vocal fold lamina propria and muscle,²⁶ injectable phonosurgical biomaterials,²⁷ and extracellular matrix scaffolds for laryngeal tissue engineering.^{28, 29}

Materials and Methods

Tissue specimens

Tissue specimens were obtained from 7 human larynges (3 male and 4 female) excised within 11 hours after death. Table 1 shows some descriptive information for the cadaveric subjects. All subjects happened to be white, but race was not one of the specimen selection criteria. The subjects were nonsmokers with no history of any head and neck disease, laryngeal disorders, or trauma, and physical examination of the larynges by a laryngologist also showed no abnormalities. The tissue procurement protocol and the experimental protocol were approved by the Institutional Review Board of the University of Texas Southwestern Medical Center. Rheometric testing was performed immediately after specimen procurement, without the need of tissue freezing. A previous study showed that viscoelastic shear properties did not change significantly following up to 24 hours of postmortem storage in saline solution.³⁰

The mucosal (surface layer) tissues of the aryepiglottic fold and ventricular fold (false vocal fold) were dissected from the excised larynges. Similar to the true vocal fold, the major vibratory tissue layer of the false fold is the lamina propria or mucosa, which is primarily an extracellular matrix (ECM) made of various fibrous proteins and interstitial proteins, as well as seromucous or seromucinous glands.⁸ The major vibratory layer of the aryepiglottic fold is also assumed logically to be the mucosa for the purpose of the present investigation, for biomechanical measurements. Under this premise, the mucosal tissues of the aryepiglottic fold and false vocal fold were carefully isolated from the muscle tissues, including the aryepiglottic muscle, thyroepiglottic muscle, and ventricularis muscle, using blunt phonosurgical instruments. The epithelium of the aryepiglottic fold and false vocal fold remained attached intact with the dissected specimens, serving as a natural boundary of attachment to the rheometer during mechanical testing, i.e., with the epithelium in complete contact with the lower plate of the rheometer (Figure 1), similar to the tissue mounting process described in our previous studies.^{1, 2} In particular, being aware of the anisotropic nature of the true vocal fold lamina propria, all three tissue specimens types were not assumed to be isotropic. All specimens were mounted between the tissue plates of the rheometer in an orientation such that the direction of simple shear deformation of the specimen (translational shear of the tissue plates) would correspond to the direction of

expiratory airflow in an intact larynx, in an effort to simulate any “mucosal wave” propagation and any normal force that could be induced by the expiratory airflow. With this approach, the viscoelastic shear properties obtained with the rheometric measurements would be in a direction that is relevant for flow-induced tissue oscillation and phonation.

In order to allow for a quantitative comparison in tissue viscoelastic properties between the supraglottal structures and the true vocal folds, true vocal fold cover specimens were also dissected from the same set of 7 excised larynges in the same way as described previously,^{1, 2} for rheometric measurements of their viscoelastic shear properties.

Rheometric instrumentation

A controlled-strain, linear, simple-shear rheometer system (Bose Corporation, ElectroForce Systems Group, Eden Prairie, MN) was used for viscoelastic characterization of the specimens.² As illustrated in Figure 1, a specimen was subjected to translational, sinusoidal simple shear deformation between two parallel, rectangular acrylic tissue plates. The upper plate was attached to the shaft of a linear motor (actuator), applying a translational displacement (x) to the specimen at a specified magnitude and frequency. The shear force (F) resulting from the viscoelastic response of the specimen was detected by a precision PCB piezoelectric force transducer (Model 209C12, PCB Piezotronics, Depew, NY) attached to the lower plate. A normal load transducer was also mounted to measure the compressive force between the specimen and the tissue plates, to ensure there was no slippage in between them under a specified gap size (d) between the two plates. The gap size (d) of 0.3 mm was found to ensure complete contact between the specimen and the tissue plates without slippage. Mechanical testing was performed in a transparent acrylic environmental chamber under temperature and humidity control. A digital camera was mounted directly above the chamber, allowing for photos of the mounted specimen to be taken. Scaled images of the specimen were examined with the Image J software (National Institutes of Health, Bethesda, MD), in order to determine the area of contact (A) between the specimen and the upper plate.

The rheometer was controlled by the WINTEST software (Bose Corporation, Eden Prairie, MN). Data collection was performed on the displacement transducer output and the piezoelectric force transducer output, for calculating the amplitudes of the two signals and the phase shift between them. As detailed in our previous studies establishing the link between the theory of linear viscoelasticity and the empirical determination of viscoelastic functions with a rheometer,^{1, 2} the elastic and viscous shear moduli G' and G'' are related to the strain amplitude, the stress amplitude, and the phase shift between stress and strain. With these data and with the gap size (d) and the area of contact (A) known, the elastic shear modulus (G') and dynamic viscosity (η') of the specimen can be determined as functions of strain amplitude or frequency according to the theory of linear viscoelasticity.²

Mechanical testing protocol

A testing protocol following those of our previous studies was conducted.^{1, 2} A strain sweep was first performed to identify the region of linear viscoelasticity, which involved oscillatory shear deformation of the specimen at a constant frequency (100 Hz), over a range of strain

amplitude (0.4 % – 100 % shear strain). The elastic shear modulus G' was determined as a function of strain amplitude. A frequency sweep was then performed, with oscillatory shear deformation of the specimen at a small-strain amplitude (1.0% to 2.0% strain) within the linear viscoelastic region, over a frequency range of 5 – 250 Hz, including phonatory frequencies. The temperature of the environmental chamber was maintained at 37°C $\pm$ 0.1°C, with a relative humidity of close to 100 % to prevent dehydration of the specimen.

Results

The results of the strain sweep tests for a representative subject (subject 6, a 65-year-old male) are shown in Figure 2, with the elastic shear modulus (G') of the aryepiglottic fold, false vocal fold, and true vocal fold mucosal tissue specimens plotted as a function of strain amplitude, ranging from 0.004 to 1.0 (corresponding to a shear strain range of 0.4% to 100%). It can be seen that G' for all three specimen types remained relatively constant in the small-strain linear viscoelastic region (up to about 4% strain), before gradually decreasing with strain amplitude. The results for the other six subjects showed the linear viscoelastic region in a similar range. Hence, these data confirmed that oscillatory shear deformation of the specimens in the range of 1.0% to 2.0% shear strain for the frequency sweep experiments was indeed within the region of linear viscoelasticity, for which the assumption of stress-strain linearity was valid.^{1, 2}

Figure 3 shows the elastic shear modulus (G') of the aryepiglottic fold, false vocal fold, and true vocal fold mucosal tissue specimens as a function of frequency on a log-log scale, which is a rheological standard. Figure 4 shows the dynamic viscosity (η') of the tissue specimens on a log-log scale. The data points represent mean values of the viscoelastic functions, and the error bars represent standard deviation values. Only the upper error bars are shown for visual clarity in the graphs. A similar dependence of the viscoelastic functions on frequency was observed for all three tissue specimen types, with G' gradually increasing with frequency, and η' decreasing monotonically with frequency. This dependence of both G' and η' on frequency can be described by the power law relationship, as in Chan and Rodriguez:²

$$G' = a f^b \text{ or } \log G' = \log a + b \log f \quad (1)$$

$$\eta' = a f^b \text{ or } \log \eta' = \log a + b \log f \quad (2)$$

where a and b are the coefficients of the parametric curve-fitting, f is frequency in Hz. Linear least-squares regression analysis was conducted to curve-fit all data to Equations (1) and (2). In the log-log plots of G' or η' versus frequency, Equations (1) and (2) become simple linear equations in the slope-intercept form. Hence, in the equations the parametric coefficient a represents the magnitude of the viscoelastic function (y intercept of the best-fit curve), whereas the parametric coefficient b indicates the frequency dependence of the viscoelastic function (slope of the best-fit curve). Table 2 shows the results of the curve-

fitting, with the coefficients a and b , and the coefficient of determination R^2 as an indication of the goodness of fit. It can be seen that both G' and η' were well described by the power law (Equations 1 and 2) for all specimens, with high values of R^2 . ANOVA tests of the coefficient a among the three tissue specimen types allowed one to examine any potential differences in the magnitude of G' and η' , whereas ANOVA tests of the coefficient b among the different tissue specimen types could reveal potential differences in the frequency dependence of G' and η' .

As shown in the plots, the false vocal fold mucosa appeared to have higher magnitudes of G' (Figure 3) and η' (Figure 4) than those of the aryepiglottic fold mucosa and the true vocal fold cover. Since G' is an indication of tissue stiffness and η' is an indication of tissue viscosity or damping, the false fold mucosa seemed to be somewhat stiffer and more viscous than both the aryepiglottic fold mucosa and the true vocal fold cover (Figures 3 and 4). Overall, the true vocal fold cover and the aryepiglottic fold mucosa showed comparable magnitudes in G' and η' at most frequencies, i.e., the aryepiglottic fold mucosa seemed to be the closer rheological match to the true vocal fold cover in comparison with the false fold mucosa. Statistically, one-way ANOVA tests were conducted to analyze the differences in the linear regression coefficients a and b among the false fold mucosa, the aryepiglottic fold mucosa and the true vocal fold cover. Results revealed that both of the coefficients a and b were not significantly different among the three types of specimens tested (Table 3). In other words, the viscoelastic functions of the mucosal tissues of the true vocal fold, the false vocal fold and the aryepiglottic fold did not show any statistically significant differences in their magnitudes and in their frequency dependence, despite the seemingly higher G' and η' of the false fold mucosa.

Discussion

Using a controlled-strain, linear, simple-shear rheometer system, this study attempted to measure the linear viscoelastic properties of the mucosal tissue layer of the aryepiglottic fold, the ventricular fold (false vocal fold), and the true vocal fold under oscillatory shear deformation in vitro. To our knowledge this represented a first attempt at quantifying the viscoelastic shear properties of human supraglottal structures at frequencies of phonation (100–250 Hz), which would be important for describing the biomechanics of airflow-induced self-sustained oscillation of the false vocal fold mucosa and the aryepiglottic fold mucosa, much like the biomechanics of oscillation of the true vocal fold lamina propria. As ventricular fold phonation and aryepiglottic fold vibration have often been observed for cases of various vocal pathologies, Mongolian singing, beatboxing, and even normal speech,¹⁰⁻²⁰ the false vocal fold mucosa and the aryepiglottic fold mucosa could become a source of replacement tissue potentially capable of phonation following partial laryngectomy or airway reconstruction.^{15, 16, 23, 24} To this end, data on the viscoelastic shear properties of the false vocal fold mucosa and the aryepiglottic fold mucosa should help dictate whether they could be effective functional tissue replacement from a biomechanical perspective.

Table 2 shows that linear least-squares regression analysis based on the power law relationship (Equations 1 and 2) can effectively parametrize the dependence of elastic shear modulus (G') and dynamic viscosity (η') on frequency for the false vocal fold mucosa, the

aryepiglottic fold mucosa, and the true vocal fold cover, as shown by the high values of the coefficient of determination (R^2), indicating a high goodness of curve fitting. The elastic shear modulus G' quantifies the elasticity or energy storage in a viscoelastic tissue, and it indicates the tissue stiffness under small-amplitude oscillatory shear deformation. The dynamic viscosity η' quantifies the viscous energy loss behavior of the tissue, as it is related to the viscous shear modulus G'' , with $G'' = 2 \pi f \eta'$. It reflects the resistance of the tissue to small-amplitude oscillatory flow. Graphical plots of G' and η' showed that the false vocal fold mucosa seemed to have somewhat higher magnitudes of G' (Figure 3) and η' (Figure 4) than the aryepiglottic fold mucosa and the true vocal fold cover at most frequencies, whereas the aryepiglottic fold mucosa seemed to have comparable magnitudes in G' and η' as the true vocal fold cover.

Statistical analysis (ANOVA) results indicated that the linear least-squares regression coefficients a and b (parametric coefficients in Equations 1 and 2) were not significantly different among the false vocal fold mucosa, the aryepiglottic fold mucosa, and the true vocal fold cover (Table 3). Despite the apparently higher magnitudes of G' (Figure 3) and η' (Figure 4) observed for the false fold mucosa in comparison with the aryepiglottic fold mucosa and the true vocal fold cover, none of the differences reached statistical significance. This finding supported the biomechanical feasibility of using both the false fold mucosa and the aryepiglottic fold mucosa as functional replacement tissues for laryngeal or airway reconstruction, potentially capable of phonation following tissue reconstruction.

One limitation of the current study was that the rheometric testing procedure *in vitro* did not directly take into account the important normal forces applied by the intraglottal airflow onto the different tissue types *in vivo*, despite our efforts to simulate the direction of such normal forces during the simple shear deformation. Also, the results of the present study should be regarded as only preliminary, given the limited number of specimens that were tested. Further studies involving a larger number of specimens could potentially reveal certain significant differences in viscoelastic shear properties among the three types of tissue specimens. In particular, even though the slopes and the y-intercepts of the best-fit regression curves for the different tissue types were not found to be significantly different from one another, the different tissue types were not necessarily indistinguishable in viscoelastic shear properties, because there existed the possibility of a Type II error with the ANOVA test, especially with our small sample size. Perhaps the apparently higher magnitudes of G' (Figure 3) and η' (Figure 4) observed for the false fold mucosa could become statistically significant in future studies with more specimens, which would imply that only the aryepiglottic fold mucosa could be a proper rheological match of the true vocal fold lamina propria for the purpose of functional tissue reconstruction.

Conclusion

This study reported the linear viscoelastic shear properties of the aryepiglottic fold mucosa and the false vocal fold mucosa in comparison with the true vocal fold lamina propria, over a frequency range of 5 – 250 Hz. Results showed that all three types of specimens showed a similar frequency dependence of the elastic shear modulus G' and the dynamic viscosity η' . Although G' and η' of the false vocal fold mucosa were generally higher than those of the

aryepiglottic fold mucosa and the true vocal fold cover, none of the differences reached statistical significance. These preliminary data suggested that from a biomechanical viewpoint, both the aryepiglottic fold mucosa and the false vocal fold mucosa could be considered as tissue replacement candidates for the true vocal fold lamina propria during laryngeal reconstruction, in cases of partial laryngectomy or airway reconstruction. Further studies involving measurements of additional specimens with more statistical power could help determine if one of these two supraglottal structures would be a better rheological match of the true vocal fold cover, for the purpose of facilitating phonation.

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Figure 1.

Principle of the controlled-strain, linear, simple-shear rheometer as described in Chan and Rodriguez.² Linear, translational sinusoidal shear deformation at a particular frequency was applied to the tissue specimen through the upper plate (with displacement x), resulting in a shear force response F detected by the lower plate. Viscoelastic functions (elastic shear modulus G' and dynamic viscosity η') of the tissue specimen can then be calculated given the gap size d and the area of contact A between the specimen and the tissue plates.²

Figure 2.

Elastic shear modulus (G') of the true vocal fold cover, the false vocal fold mucosa and the aryepiglottic fold mucosa of Subject 6 (65-year-old male) as a function of shear strain, in order to identify the region of linear viscoelasticity (0.4% – 4.0% shear strain). The strain sweep test was performed at a frequency of 100 Hz.

Figure 3.

Elastic shear modulus (G') of the true vocal fold cover ($n = 7$), the false vocal fold mucosa ($n = 7$) and the aryepiglottic fold mucosa ($n = 7$) as a function of frequency. Only the upper error bars are shown for clarity (mean + standard deviation).

Figure 4.

Dynamic viscosity (η') of the true vocal fold cover (n = 7), the false vocal fold mucosa (n = 7) and the aryepiglottic fold mucosa (n = 7) as a function of frequency. Only the upper error bars are shown for clarity (mean + standard deviation).

Table 1

Cadaveric human subjects characteristics.

Subject	Age	Gender	Race	Number of hours postmortem ^a
1	64	Female	Caucasian	7
2	75	Female	Caucasian	5
3	67	Female	Caucasian	6
4	29	Female	Caucasian	7
5	75	Male	Caucasian	11
6	65	Male	Caucasian	9
7	88	Male	Caucasian	9

^aThis represents the time elapsed from death of the subject to the beginning of the rheometric experimentation.

Table 2

Results of linear least-squares regressions for the parametric description of elastic shear modulus (G') and dynamic viscosity (η') of the true vocal fold cover ($n = 7$), the false vocal fold mucosa ($n = 7$) and the aryepiglottic fold mucosa ($n = 7$) according to Equations (1) and (2). R^2 is the coefficient of determination indicating the goodness of curve fitting.

$G' = a f^b$	a (Pa.s)	b	R^2
True vocal fold ($n = 7$)	57.789	0.888	0.813
False vocal fold ($n = 7$)	49.260	1.080	0.770
Aryepiglottic fold ($n = 7$)	20.784	1.216	0.823
$\eta' = a f^b$	a (Pa.s ²)	b	R^2
True vocal fold ($n = 7$)	201.522	-0.846	0.908
False vocal fold ($n = 7$)	200.317	-0.665	0.958
Aryepiglottic fold ($n = 7$)	156.903	-0.670	0.964

Table 3

Results of one-way ANOVA for the parametric curve-fitting coefficients a and b for elastic shear modulus (G') and dynamic viscosity (η') among the true vocal fold cover ($n = 7$), the false vocal fold mucosa ($n = 7$) and the aryepiglottic fold mucosa ($n = 7$).

Viscoelastic function	Parametric coefficient	$F (2,18)$	p value
G'	a	0.433	0.655
	b	0.953	0.411
η'	a	0.651	0.533
	b	2.227	0.137