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A mixed-effects model approach for the statistical analysis of vocal fold viscoelastic shear properties

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Abstract

A mixed-effects model approach was introduced in this study for the statistical analysis of rheological data of vocal fold tissues, in order to account for the data correlation caused by multiple measurements of each tissue sample across the test frequency range. Such data correlation had often been overlooked in previous studies in the past decades. The viscoelastic shear properties of the vocal fold lamina propria of two commonly used laryngeal research animal species (i.e. rabbit, porcine) were measured by a linear, controlled-strain simple-shear rheometer. Along with published canine and human rheological data, the vocal fold viscoelastic shear moduli of these animal species were compared to those of human over a frequency range of 1 to 250Hz using the mixed-effects models. Our results indicated that tissues of the rabbit, canine and porcine vocal fold lamina propria were significantly stiffer and more viscous than those of human. Mixed-effects models were shown to be able to more accurately analyze rheological data generated from repeated measurements.

Keywords

Vocal fold; Viscoelastic shear properties; Rheology; Mixed-effects model; Statistical analysis; Animal model

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1. Introduction

To date, many types of animal models have been utilized for the studies of vocal fold injury, scarring and their treatments. In order to select the most appropriate animal model, their laryngeal anatomical structures, the biomechanical properties of the vocal folds and their extracellular matrix (ECM) compositions, as well as phonatory characteristics (e.g. fundamental frequencies, vibratory amplitudes, aerodynamic pressures and flows) are among the important factors to be considered (Bless and Welham, 2010; Li et al., 2016). Some of these important characteristics of the larynx of various animal species have been intensively studied and compared with those of human (Alipour and Jaiswal, 2008; Garrett et al., 2000; Hahn et al., 2006a; Hahn et al., 2005, 2006b; Hunter and Titze, 2005; Jiang et al., 2001; Kim et al., 2004; Titze et al., 2016). However, despite being a major contributor to the determination of some key parameters of phonation (Chan and Titze, 1999, 2006; Zhang et al., 2006), only very few reports have statistically analyzed the differences in viscoelastic shear properties or rheological properties (Chan and Titze, 1999) between the human vocal fold lamina propria and those of animals (Kimura et al., 2011; Teller et al., 2012).

Viscoelastic shear properties of vocal fold mucosal tissues are directly related to phonation threshold pressure, phonation threshold flow, fundamental frequency, and the amplitude of oscillation (Chan and Titze, 1999, 2000, 2006; Zhang et al., 2007). Due to their defining role in self-sustained vocal fold oscillation during phonation, viscoelastic shear properties were often the only kind of biomechanical properties being assessed in vocal fold wound healing, scarring treatment and tissue engineering animal studies (Hansen and Thibeault, 2006; Kutty and Webb, 2009) and have been found to be frequency dependent in human and animal vocal folds (Chan, 2004; Chan and Rodriguez, 2008; Chan and Titze, 1999; Kimura et al., 2011). In order to capture the dependence of the viscoelastic shear moduli on frequency, several regression fitting methods such as linear least-squares regression (Chan and Rodriguez, 2008; Kimura et al., 2010, 2011) and log-transformed linear regression (Klemuk and Titze, 2004) had been utilized in the literature to parametrize the data. However, cautions shall be used when it comes to statistically comparing viscoelastic shear moduli of two or more groups across a range of frequencies because the regression analysis assumes that each observation is to be independent of another (Zar, 1998a). In most rheological tests, a vocal fold or biomaterial sample is being measured multiple times at different frequencies. Such repeated measurements on one sample suggest that the observations are not independent. The correlation among observations from the same subject (sample) should be taken into consideration during the corresponding statistical analysis. Unfortunately, this issue had never been fully investigated in previous studies. In this study, we introduced the mixed-effects model or mixed model approach (Harville, 1977; Laird and Ware, 1982) which takes such repeated measurements into consideration during the statistical analysis of data sets on viscoelastic shear properties.

In recent years, our group has reported rheometric measurements on biomaterials as well as vocal fold tissue samples from human and different animal species at phonatory frequencies using a simple-shear rheometer system (Chan and Rodriguez, 2008; Kimura et al., 2010, 2011). In the current study, viscoelastic shear properties of the rabbit and porcine vocal fold lamina propria were measured at frequencies between 1 and 250 Hz. Together with

published canine data (Kimura et al., 2011), the vocal fold viscoelastic shear properties of three animal species was compared to published human data (Chan and Rodriguez, 2008) using the aforementioned mixed-effects model approach. Furthermore, analysis of variance (ANOVA) as well as Akaike Information Criterion (AIC) were used to compare between the different statistical models. The primary objective of this study was to introduce a mixed-effects model approach for the analysis of vocal fold rheological data. The other objective was to provide insights on the similarities and differences in vocal fold viscoelastic shear properties between human and commonly used animal species in laryngeal research.

2. Materials and methods

2.1 Vocal fold tissue specimens

Five larynges were harvested from adult female New Zealand White rabbits weighted between 2.7 and 4 kg immediately postmortem. None of the rabbits received intubation or manipulation on the larynges and none of them were sacrificed specifically for this study. The euthanasia protocol was approved by the Institutional Animal Care and Use Committee of UT Southwestern Medical Center, and was in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication #85-23 Rev. 1985), and the Animal Welfare Act (7 U.S.C. et seq.). Four larynges from 6 to 9 months adult farm pigs were procured from a local abattoir. The larynges were excised immediately postmortem following slaughtering of the animals. The animal euthanasia protocol was approved by the U.S. Department of Agriculture Meat and Poultry Inspection Program.

All larynges included in this study were examined by a laryngologist and appeared normal. Vocal fold specimens consisting of the epithelium and the lamina propria were dissected from the larynges under magnified vision following the standard protocol (Choi et al., 2012; Kimura et al., 2011; Krishna et al., 2010). In brief, a small incision was first made on the superior surface of the vocal fold epithelium by a surgical blade. The lamina propria was then meticulously dissected off the underlying thyroarytenoid muscle. The dissected lamina propria specimens were kept hydrated in phosphate buffered saline solution at room temperature until rheometric measurements.

The previously reported canine vocal fold rheological data to be included in the study were obtained from six 2-year-old male dogs with an average weight of 59.1 lb (Kimura et al., 2011). Additionally, published rheological data of human vocal fold cover samples were also included for statistical analysis and comparison. Human data were gathered from seven subjects aged between 53 and 88 years old with no history of smoking, laryngeal disease and pathology (Chan and Rodriguez, 2008).

2.2 Rheometric instrumentation and measurements

Measurements of viscoelastic shear properties of tissue specimens were conducted with a custom-built linear, simple-shear rheometer system (Model ELF 3200, ElectroForce Systems Group, Bose Corporation, Eden Prairie, MN) as described in previous publications (Chan and Rodriguez, 2008; Kimura et al., 2010, 2011). In brief, the specimen was placed between

two parallel, rectangular acrylic tissue plates and subjected to a translational simple shear. The upper tissue plate was attached to the shaft of a linear motor through an actuator, applying a translational displacement to the specimen at prescribed magnitude and frequency with displacement feedback control. The resulting shear force at the lower plate was detected by a piezoelectric quartz force transducer (Model 209C12, PCB Piezotronics, Depew, NY). All mechanical testing was performed in a transparent acrylic environmental chamber in which the temperature was maintained at around 37°C, with a high relative humidity. The distance between the tissue plates, or gap size of the rheometer was set to be 0.3 mm for rabbit specimens or 0.5 mm for porcine specimens to ensure a complete contact between the specimen and the tissue plates, with the area of contact smaller than the overlapping area of the tissue plates. A digital camera was mounted directly above the chamber to photo the specimen mounted between the tissue plates, in order to determine the area of contact between the specimen and the upper plate. The rheometer was controlled by the WINTEST software (Bose Corporation, Eden Prairie, MN), which recorded the displacement signal output and force signal output, to calculate the viscoelastic shear moduli of the specimen as functions of frequency, according to the theory of linear viscoelasticity. (Chan and Rodriguez, 2008; Chan and Titze, 1999)

For each specimen, a strain-sweep test was first performed with the test frequency set at 100 Hz while strain amplitude increased from 1% up to 100%, in order to identify the linear region where viscoelastic shear properties were independent of the strain amplitude (Chan and Titze, 1999). Frequency-sweep experiment was then performed over a frequency range of 1–250 Hz in the small strain linear region of viscoelasticity (Chan and Rodriguez, 2008; Chan and Titze, 1999). Only the left vocal fold from each animal was used because of the observed mechanical symmetry in shear properties between mucosal tissues of contralateral vocal folds (Chan and Titze, 1999).

2.3 Repeatability test of rheometric measurements

A repeatability test can reflect the effect of the rheological testing procedure itself on shear properties of the tissue samples, as well as to identify the experimental error (Chan and Titze, 1999). Given that all of the samples in this study went through a strain sweep test followed by a frequency sweep test, each vocal fold specimen was actually measured twice at exactly the same set of oscillatory shear deformation condition (i.e. at a frequency of 100 Hz; and at a strain of 2% for all samples, except for one rabbit sample for which 3% strain was applied). In addition, tissue samples were subjected to repeated shear deformation between these two “duplicated” data points. To identify the possible effects of the rheometric test on the vocal fold tissue shear properties, paired Student’s *t* test was used to compare G' and η' between these two duplicated data points for all samples. The level of significance (α) was set at 0.05.

2.4 Data transformation and normality test

Conducting a parametric statistical test on non-normally distributed data may lead to spurious conclusion, especially if the deviation from normality is severe (Zar, 1998b). Transformation of the data from their original form to a different form can sometimes improve the normality and eventually increase the validity of the associated statistical

analyses (Zar, 1998b). Among different types of transformations, logarithmic transformation is one of the most popular approaches used to adjust skewed data to approximately conform to normality (Zar, 1998a, 1998b). However, not all variables that do not follow a normal distribution are lognormal (Zar, 1998b). Therefore, the normality of the elastic shear modulus G' , dynamic viscosity η' and their log-transformed data were assessed using the Shapiro-Wilk test (Shapiro and Wilk, 1965). The null hypothesis was that the data followed normal distribution. A small p-value would reject the null hypothesis and would indicate departure from normality. Histograms of the rheological data and their corresponding log-transformed data were plotted for visual illustration.

2.5. Mixed-effects models

To develop a mixed-effects model to compare the viscoelastic shear moduli of the vocal fold lamina propria between species, species and logarithmic transformed frequency represented the two obvious independent variables and fixed effects. The random effect in this model was the sample effect, which theoretically followed normal distribution with a mean of zero and an unknown variance (Harville, 1977). Therefore, the statistical model could be stated as:

$$\log \text{ Measurement}_{ijk} = \beta_0 + \beta_{1j} \cdot \text{species}_j + \beta_2 \cdot \log \text{ frequency}_k + u_i + e_{ijk} \quad (1)$$

where β_0 is the intercept, β_{1j} and β_2 are regression coefficients for the fixed effects, u_i is the random effect, and e_{ijk} is the error term.

However, as demonstrated by the rheological results in this study as well as in previous reports (Kimura et al., 2011; Teller et al., 2012), differences in the viscoelastic shear moduli between species could vary at different frequencies, especially for the elastic shear modulus G' (Figure 2). Such fluctuations in differences across testing frequencies were also observed between treatment groups in studies involving manipulating or operating on the vocal folds (Bartlett et al., 2013; Krishna et al., 2010; Woo et al., 2016). To account for the differences caused by both species and test frequency, an interaction term of species and log-transformed frequency was introduced here as an additional independent variable. The mixed-effect model was therefore revised as follows:

$$\log \text{ Measurement}_{ijk} = \beta_0 + \beta_{1j} \cdot \text{species}_j + \beta_2 \cdot \log \text{ frequency}_k + \beta_{3j} \cdot \text{species}_j \cdot \log \text{ frequency}_k + u_i + e_{ijk} \quad (2)$$

where β_0 is the intercept, β_{1j} , β_2 and β_{3j} are regression coefficients for the two fixed effects and their interaction term, u_i is the random effect, and e_{ijk} is the error term.

Given that model Eq. (1) was nested in model Eq. (2), ANOVA was performed to calculate the p-value of the additional interaction term in Eq. (2) using G' and η' data sets respectively. If the p-value was smaller than the conventionally used alpha of 0.05, an interaction effect represented by the interaction term was highly likely to exist.

Akaike information criterion (AIC) (Akaike, 1973) was also utilized to compare the model denoted by Eq. (2) with the model in Eq. (1). The AIC statistic is defined as twice the number of model parameters minus twice the model log-likelihood. The model with a smaller AIC is thought to have a better quality as it can achieve a good balance between the number of model parameters and the variance explained by the model (Akaike, 1973). Two commonly used variance structures in mixed models: compound symmetry and first order autoregressive structure, were also compared by AIC. G' and η' data sets of human and all three animals were used to calculate the AIC.

Finally, the null hypothesis that the viscoelastic shear properties of the vocal fold lamina propria between human and rabbit, porcine or canine were not significantly different was tested with the proper mixed-effects model. The level of significance (α) was set at 0.05.

All statistical analysis was done with the lme4 (version 1.1.12) and multcomp (version 1.4.6) packages in the R software (version 3.1.1, Wien, Austria) (Bretz et al., 2011).

3. Results

3.1 Identifying the linear region

The results of the strain sweep experiments of the rabbit and porcine vocal fold are shown in Figure 1, where the elastic shear modulus G' was plotted as a function of shear strain amplitude at a frequency of 100 Hz. G' remained nearly constant in a small-strain, linear region of viscoelasticity, with a strain amplitude of around 2%–5% for rabbits and 2%–10% for porcine. This region of linear viscoelasticity was similar to that for human (3% to 7%) as reported previously (Chan and Rodriguez, 2008; Chan and Titze, 1999).

3.2. Viscoelastic shear properties of rabbit and porcine vocal fold lamina propria

Once the linear region was identified, viscoelastic shear properties of rabbit and porcine vocal fold samples were measured under a small strain (2% to 3%) to ensure stress-strain linearity (Chan and Titze, 1999). The simple-shear rheometer system used in the study was able to obtain valid rheological data at frequencies of up to 250 Hz, which is within the physiologic range of human phonation (Chan and Rodriguez, 2008). Rabbit and porcine data were plotted in Figure 2 (G') and Figure 3 (η') along with published canine (Kimura et al., 2011) and human data (Chan and Rodriguez, 2008).

Our rabbit rheologic data were in good agreement with those reported in several previous studies (Dahlqvist et al., 2004; Hertegard et al., 2003; Svensson et al., 2011). For the porcine model, there have been only two studies reporting its vocal fold rheometric data at phonatory frequencies: Teller and colleagues measured viscoelastic shear properties of porcine vocal folds from different age groups (Teller et al., 2012); and 12 to 14 weeks old farm pigs were included in Krishna and colleagues' study (Krishna et al., 2010). Given that the viscoelastic shear properties of porcine vocal fold were shown to be age dependent (Teller et al., 2012), our porcine data were compared to those from the same age group (6- to 9-month-old adult). It was found that our results of elastic shear modulus G' as well as loss tangent/damping ratio ζ (data not shown) were very similar to those reported by Teller and colleagues (Teller et al., 2012), despite the very different testing methodologies utilized. As demonstrated in

Figures 2, trends of rabbit and porcine elastic shear modulus G' were quite different from those of canine and human: they remained nearly constant at lower frequencies, and started to rise significantly once the frequency was beyond about 100 Hz. A similar phenomenon was also observed in rabbit (Svensson et al., 2011; Thibault et al., 2002) as well as in porcine vocal folds (Krishna et al., 2010) by several different groups. On the other hand, all four species showed similar trends in η' .

3.3 Repeatability test of rheometric measurements

The p-values for two-tailed t tests for G' and η' of rabbit vocal folds and for G' and η' of porcine vocal folds were 0.3118, 0.1373, 0.1485 and 0.9742, respectively. Therefore, none of the viscoelastic shear moduli changed significantly even after repeated, large-amplitude rheometric measurements (up to 100% in strain at 100 Hz). This finding was consistent with that reported by Chan and Titze using human vocal fold samples (Chan and Titze, 1999).

3.4. Logarithmic transformation of the data and normality test

As revealed in the histograms (subfigures A, C, E, G in Figure 4 and Figure 5), G' and η' of human and all three animals tended to skew to the left. After being logarithmic transformed, the data appeared to be more normally distributed as demonstrated in subfigures B, D, F, H in Fig. 4 and Fig. 5. Shapiro-Wilk test results (Table 1) also showed that p-values of the log-transformed data were at least 100 times higher than those of the untransformed measurements. These findings confirmed that the log-transformation can greatly increase the normality of the rheological data, so as to fulfill the model assumption required for the subsequent statistical analysis procedure.

3.5 Statistical model selection

Results of ANOVA revealed that for the elastic shear modulus G' , p-value of the interaction term was 0.0078. It indicated that the interaction effect was highly likely to exist for G' , so it was necessary to include the interaction term of species and logarithmic transformed frequency as done in Eq. (2). On the other hand, p-value of the interaction term calculated with the η' data of three animal species and human was 0.6983, suggesting that the interaction effect of species and log frequency was insignificant for η' in this study.

G' data sets and η' data sets of all four species were also used for AIC calculation of the models. AIC comparison result indicated that for G' (Table 2), a mixed-effects model with the interaction term provided a more reasonable estimation of the species difference, as compared to a mixed-effects model without the interaction term [Eq. (1)]. However, when comparing η' between animal and human vocal folds, the model without the interaction term [Eq. (1)] was sufficient (Table 3). For η' , introducing the interaction term led to a slightly higher AIC score due to the increase in complexity of the model, without significantly increasing the goodness of fit as shown in the ANOVA results. Moreover, the first order autoregressive variance structure was shown to be preferable when compared to the compound symmetric structure, according to the Akaike information criterion.

3.6. Statistical comparisons of viscoelastic shear properties between human and animal vocal fold lamina propria

According to the model selection results, comparison of the elastic shear modulus (G') was conducted using the mixed-effects model in Eq. (2) while dynamic viscosity (η') data sets were compared using the model described by Eq. (1). First order autoregressive variance structure was chosen for both G' and η' . Table 4 shows pairwise comparison results of G' and η' between the three animal species and the human vocal folds. All p-values were much lower than 0.05, indicating that there were significant difference in viscoelastic shear properties of the vocal fold lamina propria between human and all three animal species. The mixed-effects model statistical analysis was able to demonstrate significant differences in G' between the human vocal fold cover and the canine vocal fold lamina propria from published data, which the original report failed to using only linear least-squares regression analysis (Kimura et al., 2011). Analysis of the rheological data also showed that G' of the canine vocal fold lamina propria was closer to that of human than rabbit and porcine, while its η' values were the highest among all species tested. The porcine vocal fold lamina propria demonstrated the exact opposite rheological properties, for being the stiffest and the least viscous among the three animal species.

4. Discussion

Regression analysis has been a common tool in vocal fold tissue mechanics research to parametrize the data in order to quantitatively capture the dependence of the viscoelastic shear moduli on frequency (Chan and Rodriguez, 2008; Kimura et al., 2010, 2011; Klemuk and Titze, 2004; Svensson et al., 2011). However, regression models in statistics often assume that values of Y (viscoelastic shear moduli in this case) are independent of each other (Zar, 1998a). Such an assumption is violated if a sample is measured multiple times during rheometric testing, even if the test is conducted at different frequencies. To account for the correlation among observations from the same subject (specimen) at different frequencies, a mixed-effects model approach was introduced in this study. The term “mixed-effects model” refers to the use of both fixed and random effects in the same analysis. In this study, sample effects represented random effects while species and log-transformed frequency were the fixed effects. The statistical models presented in this study can also be adopted to analyze the rheological data of phonosurgical biomaterials or other viscoelastic soft tissues such as liver (Kiss et al., 2004; Murakami et al., 2015; Nightingale et al., 2015), bladder (Barnes et al., 2015) and brain (Clayton et al., 2011).

In order to fulfill the assumption of a mixed-effects model (Harville, 1977; Laird and Ware, 1982), log-transformation of the viscoelastic shear moduli was used to increase the normality of the data. Log-log scale has been widely used to report vocal fold viscoelastic properties after being introduced in 1999 by Chan and Titze (Chan and Titze, 1999). As a standard in rheology, it ensures that decades of changes in shear properties and in frequency to be clearly shown on the same graph (Chan and Titze, 1999). In this study, we demonstrated for the first time that log-transformation are also able to greatly increase the distribution normality of G' and η' , so as to subsequently ensure the validity of parametric

statistical analyses (Zar, 1998b). Hence, logarithmic transformation is found to be crucial with regard to statistical analysis of vocal fold viscoelastic properties.

In several reports, Dr. Susan Thibeault and colleagues used log frequency and treatment group as independent variables to analyze the rheometric data through a repeated measures analysis of covariance (Cobell et al., 2006; Thibeault et al., 2002; Woo et al., 2016). In this study, both Akaike information criterion and ANOVA analysis demonstrated the importance of including an interaction term treatment (or species in this study)*log frequency in the mixed-effects model for the statistical analysis of G' . Results also showed that Eq. (1), which was the linear mixed-effects model without the interaction term, was more suitable when comparing η' . First order auto regressive variance structure between measurements at different frequencies was chosen for its better interpretation of the experimental data according to the AIC results. This study demonstrated that the mixed-effects model can be used to analyze the data acquired by repeated measurements of samples at different frequencies during rheometric tests. Furthermore, different mixed-effects models can be used for the statistical analysis of different viscoelastic shear moduli.

For decades, animal models of the larynx have been used for experimentation and studies of laryngeal physiology, biomechanics, phonatory function, and treatments for voice disorders. Animal work is especially critical for the study of complex vocal fold pathologies such as scarring and their prevention and treatment strategies, because it provides a means to systematically study the biomechanical and biochemical changes of the vocal fold lamina propria (Bless and Welham, 2010; Kutty and Webb, 2009; Li et al., 2016). Rabbit, porcine, and canine have been commonly used in such studies (Kutty and Webb, 2009; Li et al., 2016). Among them, the rabbit has been perhaps the most widely used animal model (Bless and Welham, 2010; Mau et al., 2014; Svensson et al., 2011; Thibeault et al., 2002). The canine larynx, besides being adopted for vocal fold scar treatment studies (Hirano et al., 2004; Karajanagi et al., 2011; Suehiro et al., 2010), is also a popular model for other aspects of phonatory research due to its gross morphologic, geometric and anatomic similarities to those of the human larynx (Jiang et al., 2001; Kimura et al., 2011). Meanwhile, the porcine model has been gaining more attention for vocal fold scarring studies in recent years, because the pig larynx is very similar in size, structure and geometry to those of human (Rousseau et al., 2004b; Stevens et al., 2015; Woo et al., 2016; Woodson, 2012).

Vocal fold viscoelastic shear properties have been measured in numerous animal species, including in the tiger and lion (Klemuk et al., 2011). However, to our best knowledge, there has been no report comparing viscoelastic shear properties of the normal vocal fold lamina propria between human and animals at phonatory frequencies using the proper statistical analysis method. In the current study, statistical analysis utilizing the mixed-effects model approach revealed that tissues in the rabbit, canine and porcine vocal fold lamina propria were significantly stiffer and more viscous than those of human. Moreover, among the three animal species, the porcine vocal fold appeared to be the stiffest but also the least viscous, while the canine vocal fold was the least stiff but also the most viscous at most testing frequencies. Such species-specific rheological properties were consistent with results from comparative studies on human and animal vocal fold extracellular matrix components and constructs. Histological data demonstrated that the layered structures of the canine, rabbit

and porcine vocal fold lamina propria are very different from that of human: all three animal species show essentially a two-layer structure defined by the distributions of collagen and elastin fibers (Kurita et al., 1983). The layered structure of the canine vocal fold has been found to be unique as having a superficial layer that is dense with elastic and collagenous fibers, while its deep portion of the lamina propria is less dense in fibrous components (Hahn et al., 2006a; Hahn et al., 2006b; Kurita et al., 1983). In contrast, the vocal fold superficial layers of porcine and rabbit only contain a small amount of collagen type I, whereas collagenous fibers are abundant in their deep layers (Hahn et al., 2006b; Mau et al., 2014). Elastin, another important fibrous protein in vocal fold, distributes rather evenly throughout the porcine and rabbit vocal fold lamina propria (Hahn et al., 2006a; Mau et al., 2014) instead of concentrating varyingly by tissue depth such as in the human vocal folds (Hahn et al., 2006a) (Gray et al., 2000). The distribution of interstitial proteins throughout the lamina propria is also different among species (Hahn et al., 2006a; Hahn et al., 2005). For example, hyaluronic acid, which plays an important role in vocal fold biomechanical properties (Chan et al., 2001), is abundant in the middle portion of both canine and human vocal folds while it is concentrated in the vocal fold superficial layer in rabbit and porcine (Hahn et al., 2006a; Mau et al., 2014). Besides the discrepancy in distribution patterns, marked interspecies differences in protein levels have also been reported. Hahn and colleagues showed that the mean collagen level in porcine was significantly higher than that in human but the collagen levels in canine and human were statistically similar (Hahn et al., 2006b). This finding indicated that the porcine vocal fold would be much stiffer than the human and canine vocal fold, thus being in good agreement with the observations of this study. The same group also reported that the HA levels in porcine and canine were more than double and three times of that in human, respectively (Hahn et al., 2006a). Given that an increasing concentration of HA would lead to higher tissue viscosity until a plateau is reached (Gray et al., 1999), the differences in the vocal fold HA levels were remarkably consistent with our rheological results, as human vocal folds being the least viscous and canine vocal fold being the most viscous. To our best knowledge, comparative quantification of ECM proteins between rabbit and other species' vocal fold lamina propria has not been studied comprehensively. However, similar ECM protein distributions in the vocal fold lamina propria between rabbit and porcine would likely contribute to the similarities in their viscoelastic shear properties. Analysis of the rheological data of difference species presented in this study indicated that the viscoelastic shear properties of vocal fold tissues are highly related to the species-specific content and distribution of ECM proteins.

Last but not least, it is important to point out that unlike human data which were generally consistent (Chan and Rodriguez, 2008; Chan and Titze, 1999; Kazemirad et al., 2014; Teller et al., 2012), published data on animal vocal fold viscoelastic shear properties varied tremendously (Borzacchiello et al., 2005; Choi et al., 2012; Cobell et al., 2006; Rousseau et al., 2004a; Rousseau et al., 2003; Svensson et al., 2011; Thibeault et al., 2002). For example, at 1 Hz the reported G' of normal rabbit vocal fold lamina propria ranged from approximately 70 Pa (Rousseau et al., 2004a) to as high as 30,000 Pa (Cobell et al., 2006). Their G'' fell anywhere between 70 Pa (Choi et al., 2012) to 5,000 Pa (Cobell et al., 2006), while η' has been reported to range between approximately 2 Pa.s and 500 Pa.s (Rousseau et al., 2004a). Comparing to rabbits, fewer data from dogs are available. Among them, intact

and scarred canine vocal fold viscoelastic data measured at 0.016–48 Hz by a controlled strain torsional rheometer were reported by Rousseau and colleagues (Rousseau et al., 2003), and data on 17 intact canine vocal folds measured at 0.016–50.33 Hz using the same rheometer were reported in another study (Chan, 2004). While η' in these two papers were in good agreement with the published canine data (Kimura et al., 2011) we used in this study, G' of canine vocal fold from these two reports were different from Kimura and colleagues' data by at least one order of magnitude. These discrepancies may be caused by inter-sample variability, system errors, testing conditions, and the age, size and weight of animals and so on. On the other hand, gender-related differences in vocal fold viscoelastic shear properties have not been found in animals so far. In this study, all the samples were tested on the same rheometer following standard dissection and testing procedures to eliminate as much variation as possible. Repeatability test also confirmed that the rheometric measurement presented in the study did not change vocal fold tissue shear properties. Still, given the small sample size, further studies are warranted.

5. Conclusions

In this study, we measured the viscoelastic shear properties of the vocal fold lamina propria of two of the most widely used animal models in laryngeal basic research (i.e. rabbit and porcine), at frequencies of up to the human phonatory range. Along with previously published canine data, the rheological data of three animal species' vocal fold lamina propria was compared to published human data using mixed-effects models. Being able to take observation correlation within each subject (specimen) into account, the mixed-effects model is an appropriate approach for statistical analysis when each sample is repeatedly measured during rheometric measurement. Another important finding of the study was that the process of log-transformation is able to increase the normality of the skewed vocal fold rheological data, which is crucial for statistical analysis.

Results of the mixed-effects model statistical analysis showed that the vocal fold lamina propria of all three animal species had significantly higher elastic shear modulus (G') and dynamic viscosity (η') than the published data of normal human vocal fold cover. This finding was remarkably consistent with published histological and biochemical data. This represented the first study comparing animal vocal fold viscoelastic shear properties with those of healthy human vocal folds at phonatory frequencies, using the proper statistical analysis method. Results of this study demonstrated that vocal fold rheological properties are species-specific, which can facilitate future laryngeal research studies, including comparative voice production studies, and animal model selections for vocal fold tissue engineering.

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Highlights

- Mixed-effects model approach for the analysis of vocal fold rheological data.
- Different mixed models can be used to analyze different viscoelastic shear moduli.
- Log-transformation can increase the normality of the vocal fold rheological data.
- Rabbit, dog and pig vocal folds are significantly stiffer than those of human.
- Rabbit, dog and pig vocal folds are significantly more viscous than those of human.

Fig. 1.

Elastic shear modulus G' of (A) rabbit and (B) porcine vocal fold lamina propria specimens as a function of shear strain (i.e., strain sweep; frequency = 100 Hz). Based on the data of G' the small-strain linear region of viscoelasticity can be identified with the strain amplitude up to around 2–5% for rabbit and 2–10% for porcine.

Fig. 2. Elastic shear modulus G' of human ($n = 7$), canine ($n = 6$), rabbit ($n = 5$) and porcine ($n = 4$) vocal fold lamina propria as a function of shear frequency (only upper error bars are shown for visual clarity on the plot).

Fig. 3. Dynamic viscosity (η') of human ($n = 7$), canine ($n = 6$), rabbit ($n = 5$) and porcine ($n = 4$) vocal fold lamina propria as a function of shear frequency (only upper error bars are shown for visual clarity on the plot).

Figure 4. Histogram of G' : (A) Human, (C) Canine, (E) Rabbit, and (G) Porcine. Histogram of $\log_{10} G'$: (B) Human (D) Canine, (F) Rabbit, and (H) Porcine.

Figure 5. Histogram of η' : (A) Human, (C) Canine, (E) Rabbit, and (G) Porcine. Histogram of $\log_{10} \eta'$: (B) Human (D) Canine, (F) Rabbit, and (H) Porcine.

Table 1

Shapiro-Wilk normality test of elastic shear modulus (G'), dynamic viscosity (η') and their log-transformed data $\log_{10} G'$ and $\log_{10} \eta'$ of human, canine, rabbit and porcine.

Species	p-value of G'	p-value of $\log_{10} G'$	p-value of η'	p-value of $\log_{10} \eta'$
Human	6.20×10^{-11}	3.30×10^{-1}	7.25×10^{-17}	1.42×10^{-1}
Canine	5.00×10^{-10}	5.83×10^{-2}	7.94×10^{-15}	6.09×10^{-5}
Rabbit	1.98×10^{-9}	2.88×10^{-3}	1.98×10^{-16}	1.64×10^{-4}
Porcine	4.36×10^{-3}	5.45×10^{-1}	2.08×10^{-15}	4.92×10^{-4}

Table 2.

Akaike information criterion (AIC) values of the statistical models for G' .

Model	Variance structure	
	First order autoregressive	Compound symmetric
With interaction term species*log frequency	127.27	178.87
Without interaction term	133.15	212.10

Table 3

Akaike information criterion (AIC) values of the statistical models for η' .

Model	Variance structure	
	First order autoregressive	Compound symmetric
With interaction term species*log frequency	169.41	273.06
Without interaction term	164.84	274.03

Table 4.

Pairwise comparisons of elastic shear modulus (G') and dynamic viscosity (η') among canine (n = 6) (Kimura et al., 2011), rabbit (n = 5), porcine (n=4) and human (n = 7) (Chan and Rodriguez, 2008) vocal fold lamina propria.

Pairwise comparisons of $\log_{10} G'$	p-value	Pairwise comparisons of $\log_{10} \eta'$	p-value
Canine vs. Human	6.38×10^{-9}	Canine vs. Human	5.92×10^{-19}
Rabbit vs. Human	2.39×10^{-19}	Rabbit vs. Human	5.03×10^{-11}
Porcine vs. Human	7.51×10^{-24}	Porcine vs. Human	3.12×10^{-9}