Published in final edited form as:

J Voice. 2011 November ; 25(6): 657–662. doi:10.1016/j.jvoice.2010.06.005.

Ex vivo canine vocal fold lamina propria rehydration after varying dehydration levels

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Abstract

Objectives—To examine the recoverability of canine vocal fold lamina propria upon rehydration from varying dehydration levels.

Study Design—Open controlled experimental trial

Methods—The vocal fold lamina propria was excised en block using a scalpel from ten canine larynges, providing twenty tissue samples. The initial volume of each sample was measured. Ten samples were dehydrated to 30% by mass, and the other ten samples to 70%. Each sample was rehydrated in 0.9% saline until the mass stabilized. The liquid mass and volume fractions, liquid:solid mass and volume ratios, and the fractions of the original tissue masses and volumes were calculated.

Results—All calculated parameters were significantly different between 30% and 70% dehydration recovery, with all parameters lesser in the 70% dehydration treatment group. Half of the tissue samples subjected to 30% dehydration fully recovered to their original volumes, while only 1 of the 10 samples subjected to 70% dehydration fully recovered its volume.

Conclusions—The level of attainable rehydration recovery of vocal fold lamina propria tissue in an *ex vivo* setting depends on the level of dehydration. The results correspond with the biphasic theory and may be used to help model the biomechanical and physiological properties of vocal fold lamina propria tissue during rehydration.

Keywords

Vocal fold lamina propria; rehydration; dehydration; recovery; biphasic tissue; parameters

INTRODUCTION

It is widely accepted that hydration is a crucial factor for proper vocal function and performance. Singers are advised to hydrate before performing as increased humidification and hydration facilitate singing (1). Hydration is also used to alleviate vocal dryness, discomfort, and fatigue by increasing the fluid level in the body and the airway (2). Solomon et al examined the relationship between hydration and phonation threshold pressure (PTP),

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the minimum subglottal pressure required for phonation. Increased systemic hydration was induced by drinking water, which delayed the elevation of PTP (3). In human subjects, the lowest PTP resulted from a hydrated condition with high pitch, and the highest PTP resulted from a dry condition with low pitch (4). Jiang et al and Finkelhor et al examined the effect of rehydration on phonation in excised canine larynges, finding rehydration increased vocal efficiency and decreased PTP (5,6). Nasal breathing, which can increase the humidity of inspired air, reduces PTP compared to oral breathing, which superficially dehydrates the airway lumen (7). These findings support the theoretical relationship between PTP and hydration; since PTP is directly proportional to viscosity, which is inversely related to hydration, PTP must be inversely related to hydration (3).

The biomechanical mechanism for hydration mediating vocal function is that decreased viscosity decreases energy losses during vocal fold (VF) vibration, requiring less vocal effort to produce phonation (1,8,9). Since water is less viscous than VF tissue, it can influence VF vibration (1). Hemler et al passed air with high (100%) and low (0%) relative humidity over microdissected sheep VFs and found that the humid air resulted in a flaccid, less viscous VF cover than dry, dehydrated air (10). Chan and Tayama studied vocal fold lamina propria (VF LP) and found that VF LP stiffness and viscosity decreased upon rehydration with distilled water (2). The LP is a loose connection of mainly noncellular fibrous and nonfibrous structures in the second outermost layer of the VF. Most vocal lesions, such as nodules and polyps, originate in the lamina propria, which consists of both solid and liquid phases (11). The recently developed biphasic theory considers both the solid and liquid components in modeling VF stress relaxation behavior (12). The biphasic theory may provide a more accurate depiction of VF vibration than previous single-phase models that only consider the solid component (13,14,15,16,17). Our previous study supported the biphasic theory and presented preliminary values for the biphasic mass and volume fractions in canine VF LP (18).

Vocal hydration is also important clinically, as hydration treatments provide a therapeutic benefit to patients with nodules, polyps, or other lesions pertaining to disordered liquid balance (2,8). Methods for increasing vocal hydration experimentally and clinically include increased water consumption, inhalation of humidified air, nose breathing, lozenges, propylene glycol solutions, lemon juice, herbal teas, expectorants, mucolytics and comfrey (1,2,8,19).

Despite the experimental and clinical evidence supporting hydration's positive effects on vocal function and performance, there have been limited studies on the physiological impact of hydration on the solid and liquid VF structure. Tanner et al found that after VF surface dehydration in women, three different nebulized hydration treatments did not significantly enhance recovery of PTP to normal levels (20). In some of these studies, rheological properties do not fully recover following rehydration, implying that the dehydration was extensive enough to cause irreversible damage to the physical properties of the tissue. Without quantifying the specific level of dehydration achieved, it is impossible to determine to what extent laryngeal tissue can be dehydrated, without suffering irreparable effects. Ten excised canine larynges from dogs not killed for this study provided twenty VF LP tissue samples that were dehydrated to 30% or 70% by mass and rehydrated. The samples' masses and volumes were measured in order to quantify the recovery of the tissue upon rehydration.

MATERIALS AND METHODS

Tissue Sample Preparation

The following tissue preparation, volume measurement, and dehydration procedures were described and conducted in our previous study (18). Twenty tissue samples were obtained

from larynges harvested from ten healthy laboratory dogs not killed for this study. Canine larynges were excised and prepared according to the procedure described by Jiang and Titze (21). After excision, the larynges were inspected for disorders or trauma, placed in a 0.9% saline solution, and frozen. The 0.9% saline solution had a density (ρ_{sa}), of 1.004 g/mL. The larynges were thawed the day of each experiment in 0.9% saline according to the procedure described by Chan and Titze that minimizes post mortem changes in VF tissue (22). VFs were excised en block using a scalpel, with the anterior border 1 mm posterior to the anterior commissure, the posterior border 1 mm anterior to the arytenoid cartilage, the inferior border 5 mm inferior to the superior glottic edge, and the lateral border 5 mm lateral to the glottic edge (Figure 1). The thyroarytenoid muscle was then dissected away from the LP using a scalpel under a dissecting microscope. The epithelium was included in each tissue sample to preserve the entire VF LP. Following dissection, the tissue samples were placed in 0.9% saline and allowed to equilibrate for 10 minutes.

Volume Measurement

A liquid displacement small volume measurement apparatus was described and validated in our previous study (18). Using a syringe, a known volume of 0.9% saline solution was pushed into a vial containing the VF LP tissue sample. The tissue sample displaced a certain amount of liquid, which was measured as the volume of saline solution greater than the known empty vial volume of 0.618 mL. The volume of liquid displaced by the tissue sample is equal to the tissue sample volume (V_{LP}). Volume measurement was repeated on each tissue sample twice and averaged.

Tissue dehydration

Following volume measurement, the tissue samples were transferred to aluminum weighing dishes (Thermo Fisher Scientific, Waltham, Massachusetts). The VF LP samples were weighed using an electronic balance (Ohaus Corporation, Pine Brook, NJ) accurate to the milligram and placed into a vacuum oven (Isotemp Model 280A, Thermo Fisher Scientific, Waltham, Massachusetts) heated to 40° C for dehydration. This dehydration method was believed to dehydrate the tissue beyond the epithelium, as would be more consistent with physiological systemic tissue dehydration (Figure 2). Tissue samples were dehydrated to 30% or 70% of their baseline masses. These dehydration levels encompass a wide range in order to establish the extent of dehydration affecting tissue recovery upon rehydration, measured by the tissue mass recovery upon rehydration. While the dehydration levels are nonphysiological, they were repeatedly obtainable unlike physiological dehydration levels. In the physiological range of 0% to 15% dehydration, the tissue mass declined too abruptly to be replicated among all samples, which was necessary to compare the tissue recovery after different levels of dehydration.

During the dehydration period, the samples were weighed every two minutes. The sample mass was recorded and used to determine the percent dehydration by mass, which was computed by dividing the new mass by the original mass of the sample. This process continued until the sample was dehydrated to the desired mass percentage.

Rehydration

After dehydration to 30% or 70%, samples were immediately placed into 0.9% saline solutions to facilitate rehydration. Because 0.9% saline is isotonic to blood and other bodily fluids, it is assumed to be comparable to the rehydration process that would occur in the tissue under normal physiological conditions. In three minute increments, the tissue was dried of residual liquid, weighed, and then placed back into the saline to minimize the amount of time during which the tissue was not immersed in solution. After 45 minutes, measurements were spaced to once every 5 minutes. Measurements proceeded until the

tissue was no longer absorbing liquid, or there was no appreciable change in mass over a period of 15 minutes. Afterwards, the tissue was placed in the vacuum oven for complete dehydration.

Calculations

The definitions and computations of the following parameters were presented in our previous study (18). The tissue sample was kept in the vacuum oven for complete dehydration until it maintained a constant mass (m_s) as the dried solid component of the VF LP. The difference between the original mass of the VF LP tissue (m_{LP}) and the dry solid component mass represents the loss of liquid from the tissue. The mass of the liquid component (m_l) is computed as $m_l = m_{LP} - m_s$. We defined the solid mass fraction (ϕ_m^s) as the fraction of the total mass represented by the solid component, and the liquid mass fraction (ϕ_m^l) as the fraction of the total mass represented by the liquid component. From the measured m_s and m_{LP} , we then determined ϕ_m^s and ϕ_m^l as

$$\phi_m^s = \frac{m_s}{m_{LP}} \text{ and } \phi_m^l = \frac{m_{LP} - m_s}{m_{LP}}. \quad (1)$$

These parameters comply with $\phi_m^s + \phi_m^l = 1$. We can also determine the liquid:solid mass ratio $\frac{m_l}{m_s}$ as

$$\frac{m_l}{m_s} = \frac{m_{LP} - m_s}{m_s}. \quad (2)$$

The porous VF LP tissue was immersed in 0.9% saline, which mimicked the density and osmolarity of liquid in the tissue. The saline is considered to be isotonic to the tissue, preventing a net osmotic diffusion of water into or out of the tissue. Since liquid in the tissue can be viewed as in equilibrium with the saline, we assumed that the liquid density ρ_l approached the density 1.004 g/mL of the saline solution. The liquid volume is then calculated as $V_l = (m_{LP} - m_s) / \rho_l$. The difference between the original volume of the VF LP tissue sample and the liquid volume gives the solid volume as $V_s = V_{LP} - V_l$. We define the solid volume fraction (ϕ_v^s) as the fraction of the total volume represented by the solid component, and the liquid volume fraction (ϕ_v^l) as the fraction of the total volume occupied by the liquid phase. Thus, from the measured m_s , m_{LP} , and original volume V_{LP} of the lamina propria, we can determine ϕ_v^s and ϕ_v^l as

$$\phi_v^s = \frac{V_{LP} - (m_{LP} - m_s) / \rho_l}{V_{LP}} \text{ and } \phi_v^l = \frac{m_{LP} - m_s}{\rho_l V_{LP}}. \quad (3)$$

These parameters are defined such that $\phi_m^l + \phi_m^s = 1$ and $\phi_v^l + \phi_v^s = 1$. We can also determine the

liquid:solid volume ratio $\frac{V_l}{V_s}$ as,

$$\frac{V_l}{V_s} = \frac{\frac{m - m_s}{\rho_{su}}}{V_{LP} - \frac{m - m_s}{\rho_{su}}} \quad (4)$$

This equation holds true assuming that the liquid component of the tissue sample is completely saline solution.

Data Analysis

Data were analyzed and graphed using SigmaPlot v11.0. For data that passed normality and equal variance tests, a t-test was used to determine if there was a statistically significant difference in means between the two dehydration treatments. For data that failed the normality or equal variance test, the Mann-Whitney rank sum test was used to determine if there was a statistically significant difference in median values between the two treatments. A significance level of $\alpha = 0.05$ was used for all statistical procedures.

RESULTS

The average variation of the volume measurements was 6.1% of the total tissue volume. Figure 3 displays box plots for the liquid mass fractions after rehydration from 30% and 70% dehydration. The Mann-Whitney rank sum test suggested that there was a significant difference in median liquid mass fractions between the two treatments ($p=0.003$). Box plots for liquid:solid mass ratios after rehydration from 30% and 70% dehydration are shown in Figure 4. The median liquid:solid mass ratios between the two treatments were significantly different ($p=0.003$). Box plots for the liquid volume fractions after rehydration from 30% and 70% dehydration are displayed in Figure 5. The t-test suggested that mean liquid volume fractions between the two treatments were significantly different ($p<0.001$). Figure 6 shows box plots for the liquid:solid volume ratios after rehydration from 30% and 70% dehydration. The Mann-Whitney rank sum test suggested that median liquid:solid volume ratios between the two treatments were significantly different ($p=0.002$). Box plots for the fraction of the original masses after rehydration from 30% and 70% dehydration are shown in Figure 7. The median mass fractions between the two treatments were significantly different ($p=0.002$). Figure 8 depicts box plots for the fraction of the original volumes after rehydration from 30% and 70% dehydration. The median recovered volume fractions between treatments were significantly different ($p=0.006$).

DISCUSSION

Every biphasic parameter differed upon rehydration recovery from 30% and 70% dehydration. Half of the VF LP samples subjected to 30% dehydration recovered to their original volumes. One of the ten samples undergoing 70% dehydration recovered to its original volume upon rehydration. Only one VF LP sample, which underwent 30% dehydration, recovered to its original mass upon rehydration. These results suggest that the density of the saline solution absorbed by the tissue samples upon rehydration was less than the density of the liquid initially present in the tissue. Dehydration of the VF LP caused liquid loss that was often not fully recoverable upon rehydration and was less recovered at 70% dehydration than 30% dehydration.

The results help model the *ex vivo* rehydration of the canine VF LP at different dehydration levels. While the VF LP cannot be expected to behave the same under *in vivo* rehydration, the results provide preliminary evidence that the VF LP tissue cannot fully recover its liquid component after extensive dehydration. This may be important in a clinical setting, in order to emphasize the importance of hydration on the physiological properties and biphasic composition of the VFs. The inability of the VF LP to fully recover its liquid component after a certain extent of dehydration may affect rheologically dependent parameters such as PTP, PTF, jitter, shimmer, mean flow rate, acoustic intensity, and vocal efficiency (19,23,24).

A limitation of our study was the *ex vivo* dehydration and rehydration processes to examine the recovery of the VF LP tissue. Certainly, our procedure of dehydrating the VF LP tissue samples in a 40°C vacuum oven is not meant to mimic *in vivo* tissue dehydration; the procedure is meant to cause liquid loss within the tissue under controlled conditions that induce repeatable dehydration levels within reasonable experimental time constraints. Measures were taken to replicate *in vivo* conditions, such as using 0.9% saline during volume measurement and rehydration. Chan and Titze found that neither storing vocal fold tissue in 0.9% saline at room temperature nor quick freezing larynges followed by thawing affected vocal fold properties (22). Another limiting factor was the excision of the VF LP tissue using a scalpel. The excision process likely caused some degree of damage to the liquid and solid structures of the VFs, but the entire lamina propria was seemingly kept intact through careful separation of the vocalis muscle from the LP and inclusion of the epithelium with the LP in the tissue samples. While the *ex vivo* rehydration of dead VF LP may not generalize to live tissue, the biphasic parameters calculated during rehydration can be used with the biphasic theory to model VF vibration during rehydration. Since we only imposed two treatments before rehydration, 30% and 70% dehydration, we were unable to establish any correlations that may exist between dehydration level and recovery of the biphasic parameters upon rehydration. While the dehydration levels of 30% and 70% of the total tissue masses were nonphysiological, these levels were reproducible among the VF LP tissue samples, unlike physiological dehydration levels between 0% and 15%. Within the physiological dehydration range, the VF LP tissue mass declined too rapidly to be replicated among samples. The 40°C vacuum oven temperature used to dehydrate the VF LP samples may also affect protein interactions and folding within the tissue, although the canine body temperature reaches 39.5°C (25). This methodological study was used to examine the relationship between rehydration recovery after varying dehydration levels and the biphasic theory parameters. This study determined that there was a significant difference in VF LP recovery upon rehydration from the two dehydration levels. Our extensive literature search failed to find previous studies that have quantified the specific level of dehydration invoked on laryngeal tissue before rehydration. By quantifying the dehydration levels, our study presents preliminary evidence that higher dehydration levels cause more severe damage to the solid tissue structure and limit the tissue's ability to reabsorb liquid upon rehydration. Future studies should examine VF LP recovery after additional physiological dehydration levels in order to determine if there is a certain dehydration level after which complete recovery of the VF LP liquid component is no longer feasible. In order to repeatedly obtain physiological dehydration levels, the temperature of dehydration will need to be decreased below 40°C. The recovery of other VF tissues, such as the vocalis muscle, should also be examined and compared to these LP findings.

CONCLUSION

We have calculated the liquid volume and mass fractions, liquid:solid volume and mass ratios, and fractions of original masses and volumes from two sets of ten canine vocal fold lamina propria (VF LP) tissue samples upon rehydration from 30% and 70% dehydration. The parameters were calculated by initial volume measurement and incremental mass measurement of the tissue samples throughout the rehydration process. The biphasic parameters and fractions of original mass and volume recovered were significantly lower following rehydration after 70% dehydration compared to 30% dehydration. Half of the tissue samples subjected to 30% dehydration recovered to their original volumes, while only one of the ten samples subjected to 70% dehydration fully recovered its volume upon rehydration. Only one of the twenty tissue samples recovered to its original mass upon rehydration; this sample was subjected to the 30% dehydration treatment. The *ex-vivo* rehydration capability of the VF LP is dependent upon the dehydration level. These findings support the biphasic theory of the VFs and the importance of early rehydration of the VF LP

tissue before permanent damage and lowering of the liquid component occurs. The parameters may be used to model the biomechanical and physiological behavior of VF LP tissue upon rehydration.

Acknowledgments

Grant support: This study was supported by NIH Grant No. 1-R01DC05522 from the National Institute on Deafness and other Communication Disorders.

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Figure 1. Excised canine hemilarynx vocal fold excision en block with a scalpel. Excision area is outlined by the dotted lines.

Figure 2.

Canine vocal fold lamina propria (VF LP) before dehydration (A), after complete dehydration (B), after rehydration from 30% dehydration (C), and after rehydration from 70% dehydration (D). Image B represents the solid component of the VF LP tissue.

Figure 3.

Box plots for the liquid mass fractions of 2 sets of 10 canine vocal fold lamina propria tissue samples that were rehydrated after 30% or 70% dehydration by mass. Filled circles represent the mean, horizontal lines inside the boxes mark the median, error bars show the 5th and 95th percentiles, and unfilled circles show outliers.

Figure 4.

Box plots for the liquid:solid mass ratios of 2 sets of 10 canine vocal fold lamina propria tissue samples that were rehydrated after 30% or 70% dehydration by mass. Filled circles represent the mean, horizontal lines inside the boxes mark the median, error bars show the 5th and 95th percentiles, and unfilled circles show outliers.

Figure 5.

Box plots for the liquid volume fractions of 2 sets of 10 canine vocal fold lamina propria tissue samples that were rehydrated after 30% or 70% dehydration by mass. Filled circles represent the mean, horizontal lines inside the boxes mark the median, error bars show the 5th and 95th percentiles, and unfilled circles show outliers.

Figure 6.

Box plots for the liquid:solid volume ratios of 2 sets of 10 canine vocal fold lamina propria tissue samples that were rehydrated after 30% or 70% dehydration by mass. Filled circles represent the mean, horizontal lines inside the boxes mark the median, error bars show the 5th and 95th percentiles, and unfilled circles show outliers.

Figure 7.

Box plots for the fractions of the original masses of 2 sets of 10 canine vocal fold lamina propria tissue samples that were rehydrated after 30% or 70% dehydration by mass. Filled circles represent the mean, horizontal lines inside the boxes mark the median, error bars show the 5th and 95th percentiles, and unfilled circles show outliers.

Figure 8.

Box plots for the fractions of the original volumes of 2 sets of 10 canine vocal fold lamina propria tissue samples that were rehydrated after 30% or 70% dehydration by mass. Filled circles represent the mean, horizontal lines inside the boxes mark the median, error bars show the 5th and 95th percentiles, and unfilled circles show outliers.