

RESEARCH ARTICLE

Magnetic resonance imaging quantification of dehydration and rehydration in vocal fold tissue layers

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Abstract

Clinicians commonly recommend increased hydration to patients with voice disorders. However, effects on clinical voice outcome measures have been inconsistent. Hydration-induced change within different layers of vocal fold tissue is currently unknown. Magnetic Resonance Imaging (MRI) is a promising method of noninvasively measuring water content in vocal folds. We sought to image and quantify changes in water content within vocal fold mucosa and thyroarytenoid muscle after dehydration and rehydration. Excised porcine larynges were imaged using proton density (PD) weighted MRI (1) at baseline and (2) after immersion in one of five hypertonic, isotonic, or hypotonic solutions or in dry air. Larynges dehydrated in hypertonic solutions or dry air were rehydrated and imaged a third time. Scans revealed fluid-rich vocal fold mucosa that was distinct from muscle at baseline. Baseline normalized signal intensity in mucosa and muscle varied by left vs. right vocal fold ($p < 0.01$) and by anterior, middle, or posterior location ($p < 0.0001$). Intensity changes in the middle third of vocal fold mucosa differed by solution after immersion ($p < 0.01$). Hypertonic solutions dehydrated the middle third of mucosa by over 30% ($p < 0.001$). No difference from baseline was found in anterior or posterior mucosa or in muscle after immersion. No association was found between intensity change in mucosa and muscle after immersion. After rehydration, intensity did not differ by solution in any tissue, and was not different from baseline, but post-rehydration intensity was correlated with post-immersion intensity in both mucosa and muscle ($p < 0.05$), suggesting that degree of change in vocal fold water content induced by hypertonic solutions *ex vivo* persists after rehydration. These results indicate that PD-MRI can be used to visualize large mammalian vocal fold tissue layers and to quantify changes in water content within vocal fold mucosa and thyroarytenoid muscle independently.

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Introduction

Voice disorders affect roughly 30% of the population during the lifespan [1]. Voice therapy for the treatment of voice disorders frequently includes patient education regarding vocal hygiene [2]. A common component of vocal hygiene, echoed by speech-language pathologists, physicians, and singing teachers, is the recommendation to increase systemic hydration by increasing dietary water and avoiding caffeine and alcohol. Two literature reviews and a meta-analysis concluded that hydration and vocal function are likely associated, but that evidence is currently limited [3–5]. One systematic review concluded that acoustic measures of voice production improve with fluid ingestion and worsen with water deprivation [6]; however, direction and significance of change in each parameter was inconsistent among the studies cited. Overall, changes in acoustic, aerodynamic, stroboscopic, perceptual, and self-reported voice outcome measures have been inconsistent and transient following changes in systemic hydration [3,5]. Limitations of clinical studies include failure to control baseline medications and hydration status and insufficient and inappropriately timed measures validating hydration levels [3,6]. Long-term effects of commonly recommended quantities of daily water intake such as “eight 8-ounce glasses” and “half the body weight in ounces” have not been tested for effects on voice production or prevention or treatment of voice disorders.

Vocal folds, housed within the larynx, have high water content. Vocal fold mucosa, which comprises epithelium, basement membrane, and lamina propria, is 83% water [7]. Vocal folds oscillate hundreds of times per second during voice production, and their biomechanics are dependent on the composition and organization of the lamina propria extracellular matrix (ECM) [8,9]. Theoretically, increased water in the superficial layer of the lamina propria could reduce viscosity and therefore reduce phonation threshold pressure (PTP) [10], a measure of ease of voice production defined as the lowest subglottic pressure required to initiate and sustain vocal fold oscillation [11]. However, a meta-analysis found that effects of systemic and/or surface hydration interventions on PTP measures were not significant at the 95% confidence level in people with voice disorders or normal voices [5]. The intermediate layer of the lamina propria is rich in hyaluronic acid, a strongly hydrophilic glycosaminoglycan that regulates tissue viscosity and absorbs shock [12–14], as well as elastic fibers, which impart recoil without deformation to tissues through strong hydrophobicity of elastin [15]. Disordered ECM is characteristic of many voice diagnoses [16]. While biomechanical properties of some of its components are water-dependent on a molecular level, there is no evidence that drinking more water restores vocal fold ECM to a healthy state or prevents its degradation. Deep to the vocal fold mucosa is the thyroarytenoid muscle. Water content in the thyroarytenoid has not been directly measured, but is likely 75–80%, consistent with other striated muscle [17,18]. Vocal fold muscle and mucosa are distinct anatomical entities with different effects on voice function [19]. Systemic or superficial hydration-induced change within different layers of vocal fold tissue is unknown.

Magnetic Resonance Imaging (MRI) is a noninvasive modality that is highly sensitive to water and has been used to image vocal fold layers *ex vivo* in human cadavers, dogs, ferrets, and rats [20–24]. Proton-density weighted MRI (PD-MRI) is a protocol that provides high signal intensity in tissues with high water or fat content [25]. In tissues without abundant fat, such as the ocular lens, it is well-accepted that PD-weighted signal is proportional to water content [26]. In voice science, this is an emerging area of study. Recently, PD-MRI was used to quantify the effect of systemic dehydration on water content of rat vocal folds *in vivo* [27]. Water deprivation that resulted in loss of mean 10.89% body weight decreased signal intensity in vocal folds by a mean of 11.38% and in salivary glands by a mean of 10.74%. Since mean PD-weighted signal change in vocal folds approximated weight loss due to water deprivation

and since fat is not abundant in vocal folds, PD-MRI signal is likely proportional to water content in vocal folds, as in other tissues in the body. However, tissue layers within the vocal folds were unable to be resolved in this rodent model [27].

Ex vivo studies of larger mammalian larynges hold promise for validating imaging parameters that can reveal vocal fold tissue layers for eventual use in clinical research. Porcine vocal folds, in particular, resemble human vocal folds in overall dimensions and tissue composition, including depth of mucosa [28–30]. Water balance in healthy people is well-regulated by the kidneys such that total body water varies by only 1% over a typical 3-day period [31]. Therefore, the method of dehydration in an imaging study must produce small changes in vocal fold water content, and imaging parameters must be sufficiently sensitive to detect these. There are many excised larynx studies of dehydration and rehydration, but few quantify water loss, and those that do report excessively high dehydration levels. For example, removing less than 30% of water from vocal fold mucosa is not possible using a vacuum oven, a common procedure reported in the literature [7,32,33]. However, immersion in 30% sodium chloride (NaCl) produced a smaller mass decrease in vocal fold mucosa than other methods, calculated at roughly 23%, which is still unacceptably high [34]. In contrast, healthy participants in imaging studies of the effect of acute dehydration on the brain do not lose more than 2–3% body weight over 1–2 days [35–38]. Case reports of death in adolescent wrestlers include 7–9% water weight loss over 3–4 days [39], and 10% is the threshold for severe dehydration in children [40]. In *ex vivo* studies, concentration of hypertonic solutions can be controlled to produce smaller changes, and 5% NaCl was sufficient to induce morphological change in rabbit subglottic lamina propria [41]. Since hypertonic solutions decrease transepithelial resistance [42] and water flux continues in vocal fold epithelium *ex vivo* [43], hypertonic solutions are likely to penetrate epithelium and dehydrate vocal fold lamina propria.

The objective of this study was to validate PD-MRI as a tool to image and quantify changes in water content within separate vocal fold tissue layers after dehydration and rehydration in an excised porcine larynx model. We hypothesized that PD-MRI would allow visualization and measurement of water content in vocal fold mucosa and thyroarytenoid muscle of a large mammalian larynx. We further hypothesized that immersing larynges in solutions of varying concentrations of NaCl would induce physiologic levels of water loss in vocal fold tissues, thus facilitating translation of imaging parameters to *in vivo* large animal and eventual clinical studies.

Materials and methods

This study was exempt from the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison and Brigham Young University, because tissues were obtained from the slaughterhouse after pigs were slaughtered for human consumption.

Tissue preparation

A total of 30 porcine larynges were transported immediately postmortem on ice from the slaughterhouse (Circle V Meat Co., Spanish Fork, UT). Larynges were dissected to remove esophagus, excess fat, and strap muscles (omohyoid, thyrohyoid, cricothyroid and sternohyoid) from the thyroid cartilage and the trachea was cut below the second cartilaginous ring. Larynges were inspected to ensure no damage affecting the intrinsic muscles, vocal folds, cricothyroid membrane, or thyroid cartilage in order to prevent basolateral penetration of solution to the vocal folds. To prevent vocal folds from touching and confounding results with capillary action of surface fluid, the posterior cricoid cartilage was hemisected in the superior to inferior direction and held open with a 15-mL plastic centrifuge tube (Fig 1A).

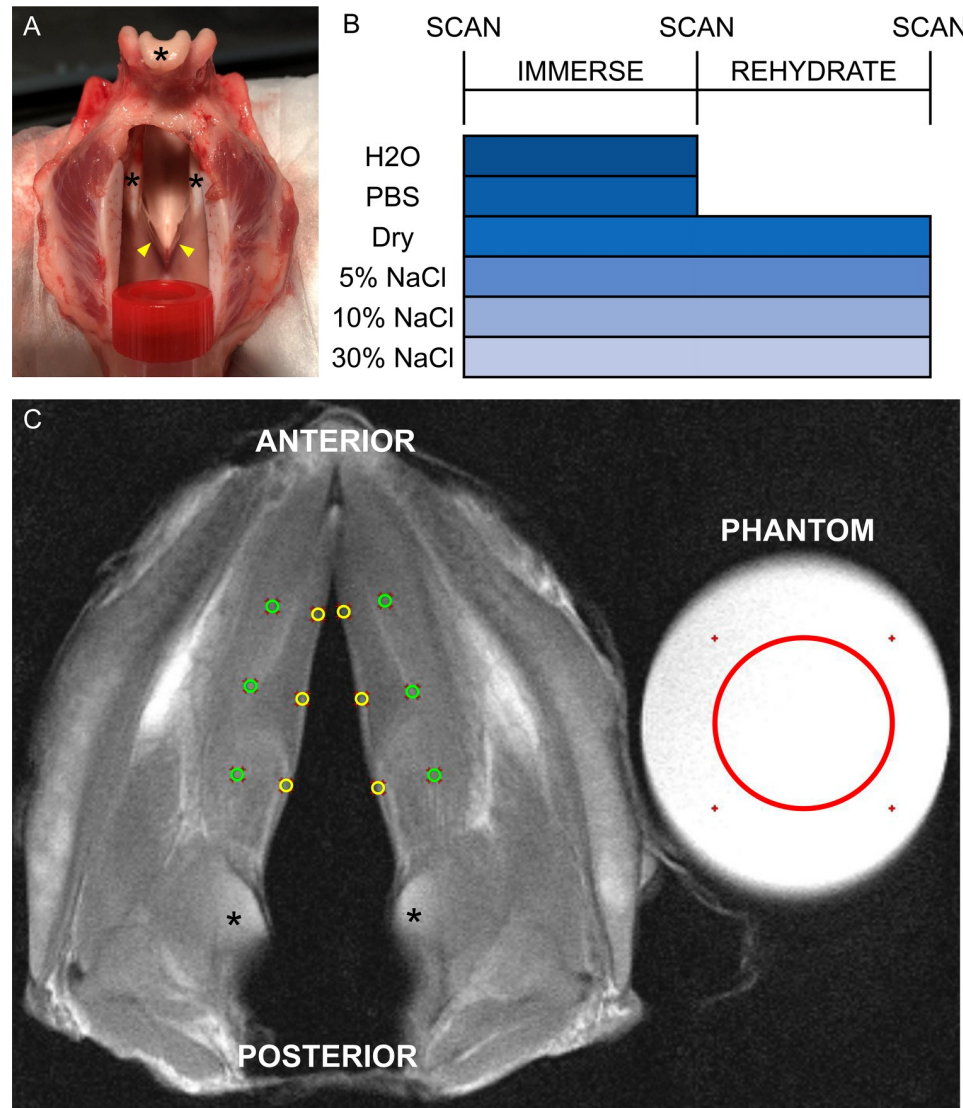


Fig 1. Study procedures. (A): Posterior view of porcine larynx hemisected through the posterior cricoid cartilage and held open with a 15-mL plastic centrifuge tube. Asterisk: arytenoid complex. Arrowheads: membranous vocal folds. (B): Timeline of study procedures. 30 porcine larynges, 5 per group, underwent PD-MRI scans at baseline and after immersion in water (H₂O), phosphate-buffered saline (PBS), 5%, 10%, or 30% NaCl, or dry air. Larynges dehydrated in NaCl solutions or dry air were rehydrated in H₂O and scanned a third time. (C): Exemplar selections for intensity measurement in a vocal fold slice. Asterisks: arytenoid complex. Yellow circles (D = 0.8–0.9 mm): intensity in anterior, middle, and posterior thirds of membranous vocal fold mucosa. Green circles (D = 0.8–0.9 mm): intensity in anterior, middle, and posterior thirds of thyroarytenoid muscle. Red circle (D = 1.5 cm): intensity of phantom.

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Immersion and rehydration

For the initial immersion condition, 5 larynges each were immersed in 500 mL of one of five solutions (Fig 1B): hypotonic deionized water (H₂O), isotonic phosphate-buffered saline (PBS, pH 7.4, Fisher Scientific, Fair Lawn, NJ, USA), and hypertonic 5%, 10%, and 30% NaCl (Fisher Scientific, Fair Lawn, NJ, USA). Five additional larynges were left dry in a sealed 500-mL container for 30 minutes as controls. We expected that water in vocal fold mucosa would increase after immersion in H₂O, remain stable after immersion in PBS, decrease in direct proportion to NaCl content of hypertonic solutions, and slightly decrease in dry air. Larynges dehydrated

in hypertonic solutions or dry air were then immersed in 500 mL H₂O for 30 minutes to represent rehydration conditions (Fig 1B).

MRI

A 3 Tesla (3T) TIM-Trio MRI scanner (Siemens Healthineers, Erlangen, Germany) was used for image acquisition. All larynges were imaged at baseline and after immersion. Dehydrated larynges were imaged a third time after rehydration (Fig 1B). Larynges were positioned within a 32-channel head coil with the thyroid prominence superior and the posterior cricoid cartilage inferior. They were wrapped in blue disposable underpads to protect the machinery from the tissues and solutions, then were set on a series of wedges to lift them to isocenter in the coil and stabilized with wedges on top. A vial of H₂O was included within the scanner to serve as a phantom for signal normalization. Images were aligned at localization, and 2D axial images of the larynx parallel to the true vocal fold with 4-mm slice thickness were acquired for PD-weighted sequences (echo time (TE) 43 ms, repetition time (TR) 1740 ms) with 6 averages and 2 concatenations giving a scan duration of 14–16 minutes, including localization and scan setup. The sequence field of view was 88mm, base resolution was 384 and slice thickness was 4mm giving a voxel dimension of 0.2mm x 0.2mm x 4mm. Additional parameters include 25% phase oversample, generalized auto-calibrating partial parallel acquisition (GRAPPA) turbo factor of 12 and flip angle of 150.

Image analysis

Digital Imaging and Communications in Medicine (DICOM) files were opened in the RadiANT DICOM Viewer (Medixant, Poznan, Poland). One slice per file was selected just inferior to the vocal process of the arytenoid cartilage to capture the medial surface of the vocal fold. Codes were created with a random number generator (Microsoft Excel, Redmond, WA, USA) and a key was saved in a separate file. Slices were renamed with codes and saved in a separate folder. A total of 80 slices were acquired, 20% of which were saved twice using different codes for determining intrarater reliability, resulting in a total of 96 slices used for measurement.

Coded slices were reopened in RadiANT with annotations off, thus masking group and timepoint during measurement. Measures were taken by a clinician with five years' experience studying laryngeal anatomy and biology following initial consultation with the senior authors who each have over a decade of experience in medical imaging and porcine vocal fold surgery respectively. Since vocal folds are subject to different forces during vibration along the anterior-posterior axis [44,45], particularly in the "striking zone" of the middle third where phonotraumatic lesions are likely to occur [46,47], vocal folds were measured at multiple locations within mucosa and muscle. Raw signal intensity (arbitrary units) was taken at 13 points per slice: one each in the middle of the anterior, middle, and posterior thirds of left and right vocal fold mucosa and thyroarytenoid muscle, and one in the phantom (Fig 1C). Because porcine vocal fold mucosa is approximately 0.9 mm thick [28,48], mucosa was measured using circular selections with $D = 0.8\text{--}0.9$ mm beginning at the vocal fold edge and moving laterally toward the thyroid cartilage. Muscle was measured using circular selections of the same dimensions placed laterally to the corresponding points in the mucosa. A circle with $D = 1.5$ cm was used to measure intensity of the phantom. For each slice, normalized intensity for each point within tissue was calculated as a percentage of intensity of the phantom. After normalized intensity was calculated, the key was used to decode data.

Statistical analysis

Anatomical and group differences in baseline tissue intensity were analyzed using mixed models for mucosa and thyroarytenoid separately with normalized tissue intensity (% of phantom)

as the response variable. Fixed effects were side (left or right), location within the vocal fold (anterior, middle, or posterior), and group (H₂O, PBS, Dry, 5% NaCl, 10% NaCl, or 30% NaCl). Intercepts for each larynx were included as repeated measures. Tukey's honestly significant difference (HSD) test was used for post hoc analysis. Post-immersion intensity and post-rehydration intensity were calculated as percentage of baseline, with baseline intensity set at 100%. For analysis of change from baseline, values were averaged between left and right vocal folds for each location within mucosa and muscle. Intensity post-immersion and post-rehydration was analyzed using one-way analysis of variance (ANOVA). Welch's ANOVA was used for data that failed Levene's test for equality of variances at $\alpha = 0.05$. Games-Howell test was used for post hoc pairwise comparisons. Welch's T-test with Bonferroni correction was used to test the hypothesis that intensity in various subgroups of tissue was not equal to 100%. The Pearson correlation coefficient was used to analyze correlations between mucosa and muscle intensity in immersion and rehydration conditions and correlations between changes in dehydration and rehydration within mucosa and muscle. For correlation analyses of changes in intensity, data were paired by side and location (e.g. dehydrated left anterior mucosa was paired with rehydrated left anterior mucosa and with dehydrated left anterior thyroarytenoid). Intrarater reliability was analyzed using intraclass correlation coefficient (ICC) estimates and 95% confidence intervals (CI) calculated based on a single-rater, absolute-agreement, two-way mixed effects model [49]. Statistical analyses were performed with SAS Studio 3.5 (SAS Institute, Inc., Cary, NC, USA) and RStudio 1.0.143 (RStudio, Inc., Boston, MA USA). The α level for significance was 0.05.

Results

At baseline, vocal fold appearance in PD-MRI scans was characterized by a bright line at the vocal fold edge, indicative of high fluid content in mucosa (Fig 2A). Muscle and cartilage appeared in shades of gray as expected with PD-MRI. After dehydration in hypertonic solutions, fluid in mucosa was no longer visible (Fig 2B) but was restored after rehydration (Fig 2C). In all scans, the phantom was bright white.

Intrarater reliability

ICC between first and second raw measurements of tissue intensity (arbitrary units) was 0.9566 with 95% CI = 0.9428–0.9672. ICC between first and second calculated values of normalized tissue intensity (% of phantom) was 0.9533 with 95% CI = 0.9384–0.9646.

Baseline signal intensity in vocal fold tissue

Baseline intensity, representative of water content [25], was analyzed within mucosa and muscle for differences by side, anterior-posterior location, and group. Within the mucosa, type 3 tests of fixed effects in the mixed model revealed a significant interaction effect of location by group (Table 1). Post hoc testing revealed a significant effect of location within larynges in Dry, 5% NaCl, and 30% NaCl groups, but not those in H₂O, PBS, or 10% NaCl groups (Table A in S1 File). There was a significant effect of group within the middle third of the mucosa (Table B in S1 File); however, no pairwise comparisons were significantly different (Table C in S1 File). In both mucosa and muscle, there were significant main effects of side and location on normalized intensity (Table 1). Left vocal fold was brighter than right in both tissue types (Fig 3A and 3D). In both mucosa and muscle, intensity increased from anterior to middle third and from middle to posterior third (Fig 3B and 3E). There was no main effect of group (i.e., planned immersion solution) on normalized intensity of mucosa or muscle (Table 1; Fig 3C and 3F). Taken together, these data indicate that there were no between-group differences in vocal fold intensity

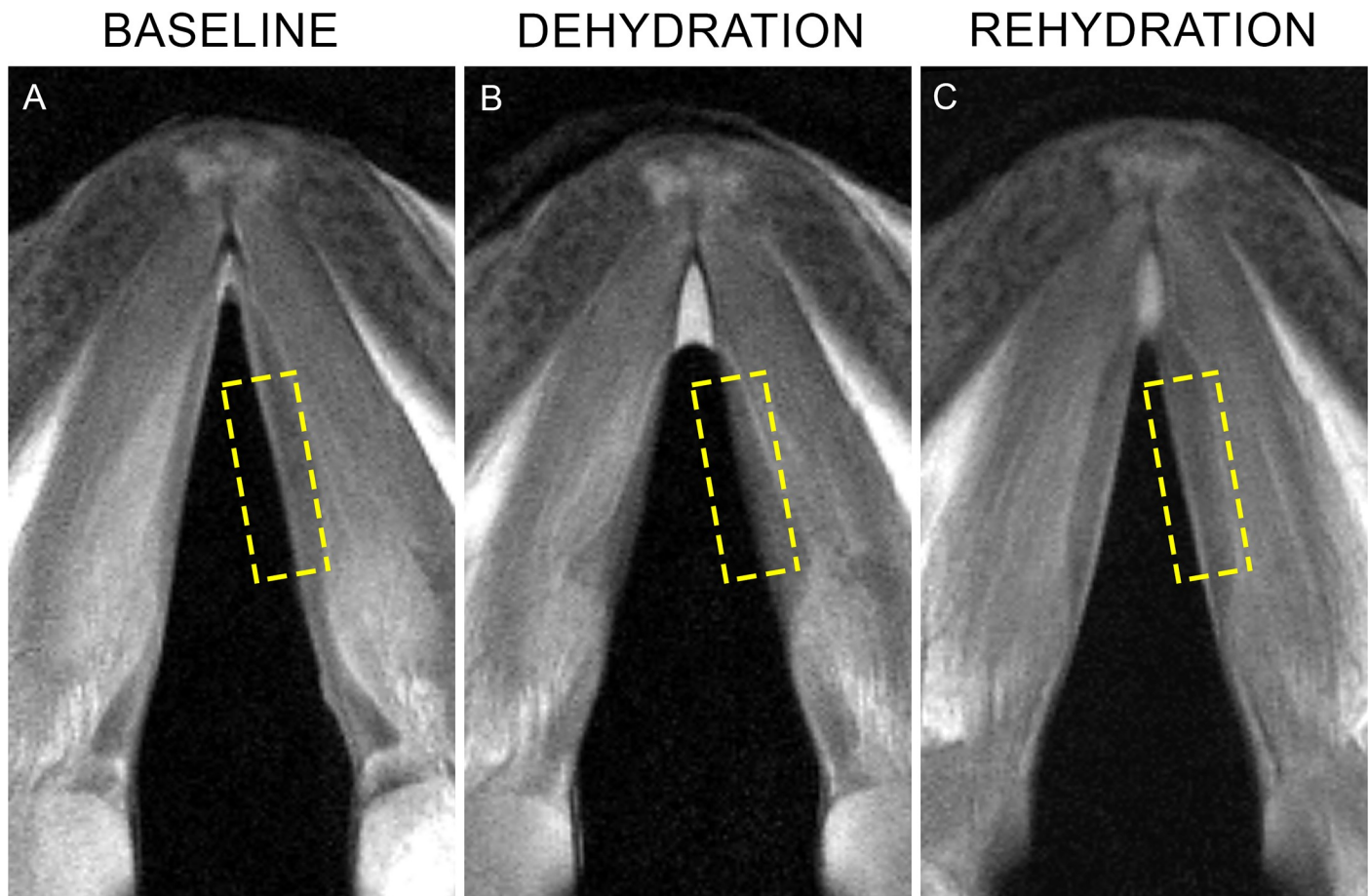


Fig 2. PD-MRI image of porcine vocal fold. Representative images of slices through vocal fold of the same larynx. (A): Baseline. (B): After dehydration in 30% NaCl. (C): After rehydration in H₂O. Dashed boxes highlight mucosa at the vocal fold edge.

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at baseline once side and location were accounted for. Therefore, group differences in intensity after immersion and rehydration resulted from experimental conditions.

Immersion

Post-immersion intensity in the middle third of vocal fold mucosa failed Levene's test for equality of variances ($F(5, 24) = 4.40, p = 0.0055$). Welch's ANOVA revealed significant differences by group after immersion (Fig 4; $F(5, 10.8726) = 8.55, p = 0.0017, \omega^2 = 0.55$). Midfold mucosa was brighter in larynges immersed in H₂O than in those immersed in 5% and 10% NaCl (Fig 4B; adjusted $p = 0.011$ and 0.012 , respectively). Immersion in 5% and 10% NaCl decreased intensity in midfold mucosa (Table 2). There was no evidence that other immersion solutions produced differences from baseline intensity.

Post-immersion intensity did not vary by solution in anterior or posterior mucosa (Fig 4A and 4C; Table A in S2 File) and did not vary from baseline intensity (Table B in S2 File). There was no evidence for differences in intensity change by solution in any location in thyroarytenoid muscle (Fig 4D and 4F; Table A in S2 File), or for changes from baseline intensity (Table B in S2 File). Taken together, our data indicate that brief immersion in hypertonic solutions can induce water loss in vocal fold mucosa at the midfold, but not in thyroarytenoid muscle.

Table 1. Anatomical and group differences in baseline vocal fold tissue intensity.

Effect	Result	Significance (p)
Mucosa		
Side	F(1,24) = 17.95	0.0003***
Location	F(2,96) = 20.25	< .0001***
Group	F(5,24) = 1.97	0.1190
Side × Location	F(2,96) = 0.14	0.8665
Side × Group	F(5,24) = 0.37	0.8666
Location × Group	F(10,96) = 0.33	0.0193*
Side × Location × Group	F(10,96) = 2.28	0.9719
Muscle		
Side	F(1,24) = 9.51	0.0051**
Location	F(2,96) = 78.81	< .0001***
Group	F(5,24) = 1.06	0.4051
Side × Location	F(2,96) = 0.10	0.9082
Side × Group	F(5,24) = 1.53	0.2178
Location × Group	F(10,96) = 1.38	0.1989
Side × Location × Group	F(10,96) = 0.30	0.9786

Type 3 tests of fixed effects on vocal fold tissue intensity in mixed models with intercepts for each larynx included as repeated measures.

*p < .05

**p < .01

***p < .001.

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Rehydration

After rehydration, there were no group differences in intensity of any location in vocal fold mucosa or posterior thyroarytenoid by one-way ANOVA (Table A and Fig in [S3 File](#)). Anterior and middle thyroarytenoid failed Levene’s test for equality of variances (F(3,16) = 5.19 and 3.37, p = 0.0108 and 0.0445). Welch’s ANOVA revealed no differences in post-rehydration intensity by group in anterior or middle thyroarytenoid (Table A in [S3 File](#)). There was no evidence that intensity after rehydration was different from baseline in any group (Table B in [S3 File](#)). Therefore, rehydration restored intensity close to baseline levels.

Correlations

Correlation coefficient between mucosa and muscle intensity after immersion was not significant (r = 0.0353, p = 0.7412, [Fig 5A](#)), indicating that various solutions produced different effects in separate tissue layers. In dehydrated larynges, the correlation coefficient between rehydrated mucosa and muscle was 0.3300 (p = 0.0100, [Fig 5B](#)). Correlation coefficient between dehydrated and rehydrated intensity was 0.3014 (p = 0.0193) in mucosa and 0.3908 (p = 0.0020) in muscle ([Fig 5C and 5D](#)), implying that despite intensity approaching baseline after rehydration, some effects of dehydration persisted.

Discussion

This is the first study to our knowledge to expose vocal fold tissue layers on MRI scans without contrast and with clinically feasible acquisition times. Herrera et al. [20] were able to distinguish vocal fold epithelium, lamina propria, muscle, and cartilage, as well as scar and implanted biomaterials, using 11.7T scans of paraformaldehyde-fixed dog and ferret larynges. However, stated limitations included scanning duration that was too long for clinical

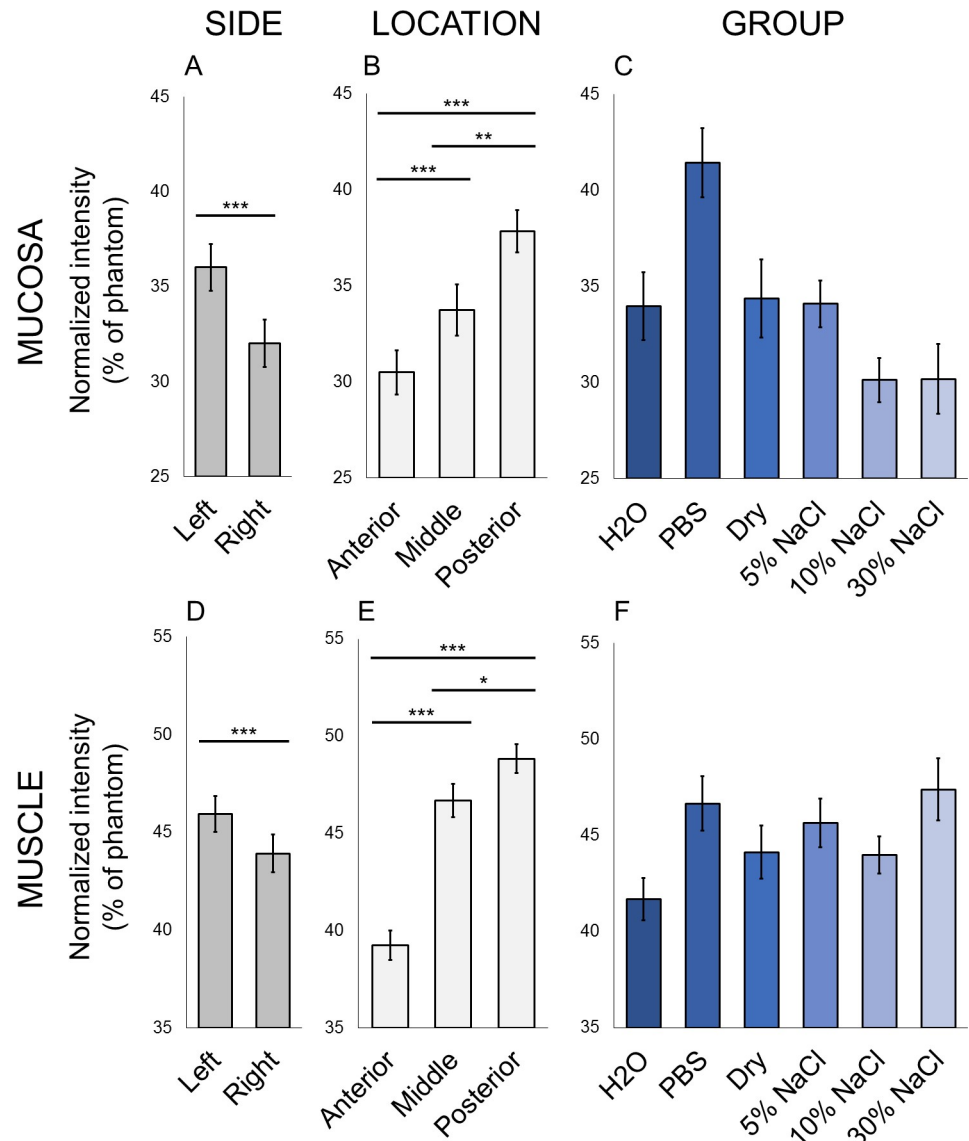


Fig 3. Baseline tissue intensity varied by side and location within vocal folds. Normalized intensity (% of phantom) at baseline in mucosa (A-C) and muscle (D-F). (A) and (D): Left and right vocal fold. (B) and (E): Location in vocal fold. (C) and (F): Experimental group. Data plotted as mean \pm SEM. $n = 5$ larynges per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significance of pairwise comparisons of group means using Tukey's HSD test after significant main effects were found in mixed model ANOVAs.

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application. Kishimoto et al. [21] resolved mucosa, muscle, cartilage, and scar tissue in excised rat larynges in a 10-minute scan at 9.4T, but tissues were immersed for 10 days in gadolinium contrast. *In vivo* scans in the same study did not reveal vocal fold tissue layers at 9.4T, even with IV contrast. Chen et al. [22], Klepavec et al. [23], and Wu and Zhang [24] each distinguished mucosa from muscle in human cadaveric larynges without contrast; however, field strength ranged from 4.7-7T and scan times ranged from 2-18 hours. Oleson et al. [27] used a 7T system to demonstrate pre-post changes in rat vocal fold and salivary gland signal intensity after water deprivation, but again could not distinguish vocal fold tissue layers. The scans in the present study were acquired with a 3T system that is widely clinically available and acquisition times of only 14-16 minutes. We revealed a bright, fluid-rich line at the vocal fold edge

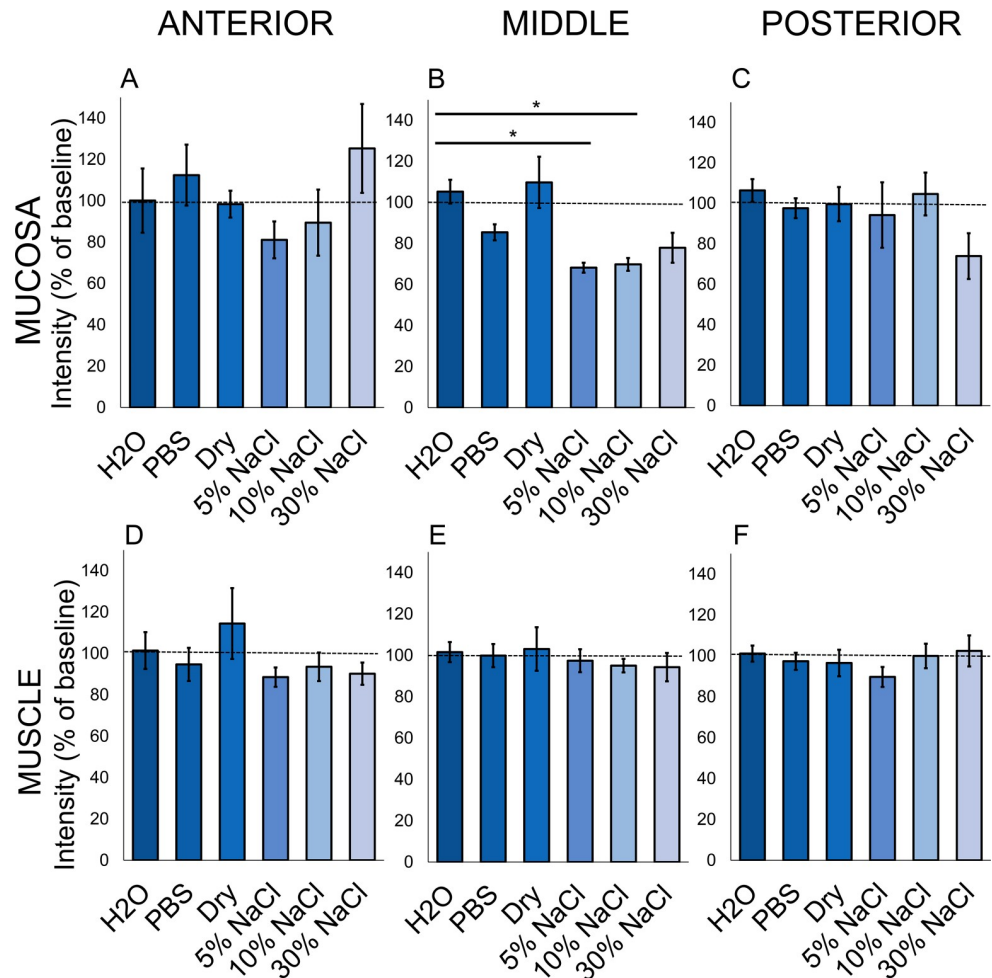


Fig 4. Intensity in vocal fold mucosa and thyroarytenoid after immersion in hypo-, iso-, and hypertonic solutions. Intensity (% of baseline) as a function of immersion solution in mucosa (A-C) and in thyroarytenoid muscle (D-F). Data plotted as mean \pm SEM. $n = 5$ larynges per group. Pairwise comparison using Games-Howell test: * $p < 0.05$.

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Table 2. Intensity in middle third of vocal fold mucosa after immersion in hypo-, iso-, and hypertonic solutions.

Group	Intensity (% of baseline)		Significance (p)
	Mean	95% CI	
H2O	105.26	89.22–121.29	0.4142
PBS	85.44	74.67–96.21	0.0199
Dry	109.75	75.05–144.46	0.4789
5% NaCl	68.20	61.51–74.89	0.0002*
10% NaCl	69.81	61.12–78.49	0.0006*
30% NaCl	77.90	57.63–98.16	0.0389

Results of Welch’s T-test of the hypothesis that intensity after immersion was not equal to 100% of baseline. $n = 5$ larynges per group.

* $p < \text{Bonferroni-adjusted } \alpha = 0.05/6 = 0.0083$.

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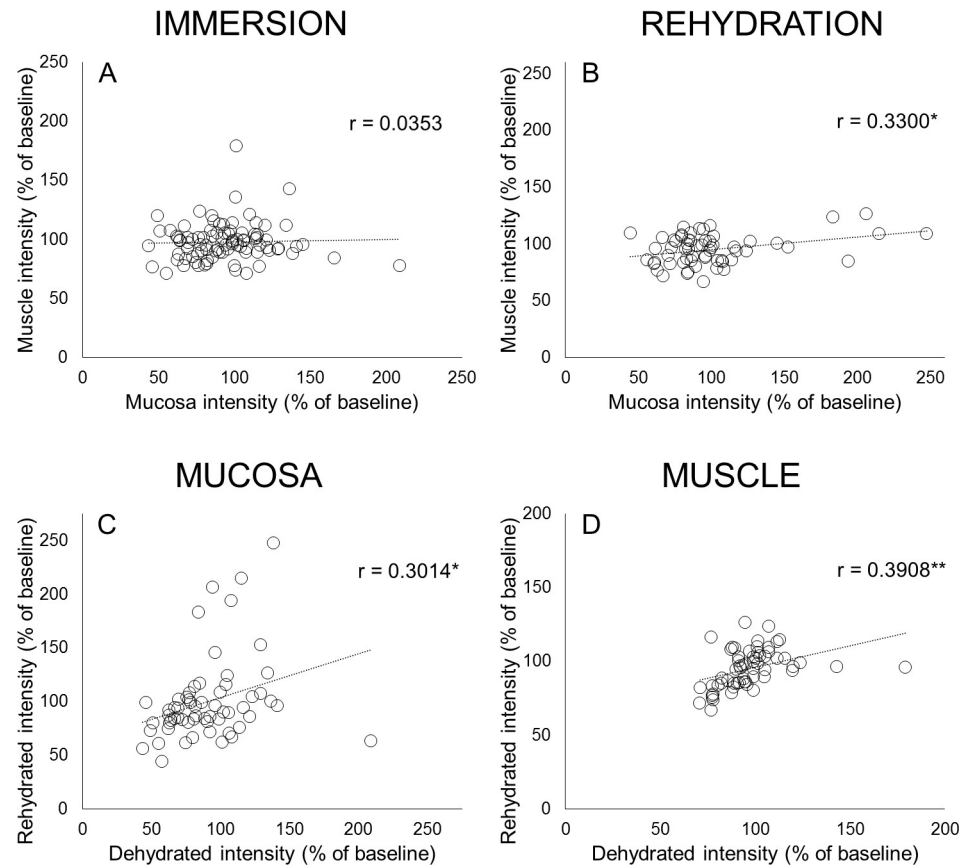


Fig 5. Correlations between intensity changes between tissues and across conditions. (A-B): Correlation between intensity (% of baseline) in mucosa and thyroarytenoid muscle after immersion (A) and after rehydration (B). (C-D): Correlation between intensity (% of baseline) after dehydration and after rehydration in mucosa (C) and muscle (D). Pearson correlation coefficient: * $p < .05$, ** $p < 0.01$.

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(Fig 2) consistent with known thickness of porcine vocal fold mucosa that was clearly distinct from muscle.

High variability in intensity within vocal fold tissue at baseline (Fig 3) is consistent with other *ex vivo* studies and hydration literature in general, but could be due to procedural limitations. Specifically, larynges were positioned supine within the scanner with the phantom on the right, so the increase in intensity from anterior to posterior and from right to left may be explained by gravitational flow of water within tissue. Body position affects clinical measures of total body water as well [50]. There was an interaction effect of location by group, with anterior-posterior differences in some groups but not others. There was evidence for group difference in the middle third of the mucosa at baseline, but no pairwise differences were significant and there were no group differences otherwise. By far, the strongest effects on baseline intensity were side, which we accounted for by calculating mean values, and location, which we accounted for by analyzing anterior, middle, and posterior vocal fold locations separately.

We immersed larynges in hypo-, iso- and hypertonic solutions with varying solute concentrations in order to produce changes in vocal fold lamina propria water content through osmosis. Different solutions produced different levels of water content only in the middle third of vocal fold mucosa (Fig 4B). We found that 5% and 10% NaCl dehydrated this tissue 32% and 31% respectively (Table 2), which are non-physiologic levels of water loss. Changes induced by

other solutions were not significant. Heterogeneity and variability of hydration-induced change in vocal fold tissues and functional voice measures is a common challenge in *ex vivo*, *in vivo*, and clinical studies [3–6]. For example, in the study by Oleson et al. [27], some rat vocal folds and salivary glands actually increased in intensity post dehydration. Similarly, in a clinical study, 3 out of 16 patients had increased vocal fold thickness on endoscopy after hemodialysis despite weight loss [51]. Our findings thus indicate that a higher number of larynges should be included in future work. There were no significant post-immersion intensity changes in anterior or posterior mucosa (Fig 4A and 4C). Due to the relatively steep angle of the porcine vocal fold within the thyroid cartilage [28,52–54] and 4-mm thickness of our slices, it is possible that selected points within anterior and posterior vocal folds included other tissue types (e.g. cartilage or fat) that were not the focus of this study. By contrast, the middle third of the mucosa was relatively isolated and measurements were more likely to include only mucosa or muscle. Taken together, these results demonstrate that response of vocal fold mucosa to solutions of varying tonicity is highly variable, and that 30-minute immersion in low-solute concentrations produces excessively high dehydration levels in the middle third of the mucosa.

There were no significant post-immersion intensity changes in muscle (Fig 4D and 4F), possibly due to limited depth of solution penetration for the duration of immersion in this study. Using hypertonic solutions for longer durations to dehydrate thyroarytenoid muscle is ill-advised, as this is the basis of brining meat, which results in increased moisture. Increased NaCl concentration below 5 molarity (M), roughly 29%, causes myofibril swelling, which is maximized at 1 M (5.8%) [55]. Therefore hypertonic immersion has little relevance to the effects of dehydration on muscle *in vivo*. Intensity change in mucosa after immersion in hypo-, iso-, and hypertonic solutions was not associated with change in muscle at the same anterior-posterior location (Fig 5A), which provides further evidence that this method can be used to analyze hydration-induced changes in these tissues separately.

After rehydration, vocal folds dehydrated in different hypertonic solutions or dry air did not differ in intensity in mucosa or muscle (S3 File). However, we found a positive association between intensity after dehydration and after rehydration in both mucosa and muscle (Fig 5C and 5D), indicating that the degree of change in water content due to immersion in hypertonic solutions persisted even after rehydration. Karamzadeh et al. [41] found that after dehydrating rabbit subglottis in 5% NaCl for 25 minutes, immersion for the same duration in H₂O increased subglottic lamina propria from its dehydrated thickness. They did not quantify changes, so it is unclear whether tissue returned to baseline. Hanson et al. [33] dehydrated excised canine vocal fold mucosa by 30% and 70% of mass using a vacuum oven. Using 0.9% saline, they were able to restore mass of only half of tissues dehydrated by 30% and 1 of 10 dehydrated by 70%. Chan and Tayama [56] immersed canine vocal fold mucosa in a 25% sucrose solution for 25 min and rehydrated in H₂O for 30 minutes. Rheology revealed that dehydration increased stiffness and viscosity, which was only partially recovered after rehydration. Consistent with these studies, our results suggest that *ex vivo* dehydration methods induce irreversible changes in vocal fold mucosa, which limits generalization to clinical settings.

This study has several limitations. Laryngeal position within the scanner may have influenced fluid content within vocal fold locations and limited our ability to draw conclusions based on anatomical variation. It is possible that moisture could have evaporated from the surfaces of the vocal folds during scanning, but applying a barrier to prevent this would have concentrated surface liquid in place by capillary action and confounded our measures. A single rater completed all measurements; however, intrarater reliability was excellent (ICC > 0.95). Mucosa was difficult to distinguish in dehydrated larynges, potentially resulting in imprecise

measurements. Our dehydration method could not produce clinically applicable change in muscle [57] and produced excessively high dehydration of mucosa.

Water deprivation has different effects on cellular and extracellular fluid compartments of the body [58]. For example, ventricular volume in the brain paradoxically increases after acute dehydration, possibly due to diffusion of water out of cells and into extracellular space [35,36,38]. Thyroarytenoid muscle is largely cellular, and vocal fold mucosa comprises thick, largely acellular lamina propria and relatively thin cellular epithelium [12]. Due to the different effects of these tissues on phonation, it is important to understand hydration-induced changes in vocal fold mucosa and muscle as separate entities. We have demonstrated that PD-MRI can distinguish separate vocal fold tissue layers in a large mammalian larynx. Future studies should (1) focus on the middle third of mucosa, which has clinical relevance in biomechanical load and lesion occurrence [44–47], (2) include a higher number of larynges per condition, and (3) use shorter duration of immersion to produce less extreme changes in water content. *In vivo* studies are needed to quantify the relationship between change in vocal fold tissue and overall systemic hydration level using multiple measurements such as weight change, urine specific gravity, and serum osmolality [58].

Conclusions

PD-MRI can be used to visualize porcine vocal fold tissue layers and to quantify changes in water content within vocal fold mucosa and thyroarytenoid muscle. Hypertonic solutions dehydrate the middle third of vocal fold mucosa but produce excessively high water loss in 30 minutes. Degree of change in vocal fold water content induced by hypertonic solutions *ex vivo* persists after rehydration.

Supporting information

S1 File. Post hoc testing within location × group interaction in mucosa intensity at baseline.

(PDF)

S2 File. Intensity differences from baseline after immersion.

(PDF)

S3 File. Intensity differences from baseline after rehydration.

(PDF)

S4 File. Raw data. Signal intensity of vocal fold tissues and phantom across baseline, immersion, and rehydration. Slices 1–80: experimental data. Slices 81–96: repeated measurements used to calculate intrarater reliability.

(XLSX)

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References

1. Roy N, Merrill RM, Gray SD, Smith EM. Voice disorders in the general population: Prevalence, risk factors, and occupational impact. *The Laryngoscope*. 2005; 115: 1988–1995. <https://doi.org/10.1097/01.mlg.0000179174.32345.41> PMID: 16319611
2. Gartner-Schmidt JL, Roth DF, Zullo TG, Rosen CA. Quantifying component parts of indirect and direct voice therapy related to different voice disorders. *J Voice*. 2013; 27: 210–216. <https://doi.org/10.1016/j.jvoice.2012.11.007> PMID: 23352061
3. Hartley NA, Thibeault SL. Systemic hydration: Relating science to clinical practice in vocal health. *J Voice*. 2014; 28: 652.e1–652.e20. <https://doi.org/10.1016/j.jvoice.2014.01.007> PMID: 24880674
4. Leydon C, Sivasankar M, Falciglia DL, Atkins C, Fisher KV. Vocal fold surface hydration: A review. *J Voice*. 2009; 23: 658–665. <https://doi.org/10.1016/j.jvoice.2008.03.010> PMID: 19111440
5. Leydon C, Wroblewski M, Eichorn N, Sivasankar M. A meta-analysis of outcomes of hydration intervention on phonation threshold pressure. *J Voice*. 2010; 24: 637–643. <https://doi.org/10.1016/j.jvoice.2009.06.001> PMID: 20359862
6. Alves M, Krüger E, Pillay B, van Lierde K, van der Linde J. The effect of hydration on voice quality in adults: A systematic review. *J Voice*. 2017; 0. <https://doi.org/10.1016/j.jvoice.2017.10.001> PMID: 29122414
7. Phillips R, Zhang Y, Keuler M, Tao C, Jiang JJ. Measurement of liquid and solid component parameters in canine vocal fold lamina propria. *J Acoust Soc Am*. 2009; 125: 2282–2287. <https://doi.org/10.1121/1.3086276> PMID: 19354403
8. Gray SD, Titze IR, Chan R, Hammond TH. Vocal fold proteoglycans and their influence on biomechanics. *The Laryngoscope*. 1999; 109: 845–854. <https://doi.org/10.1097/00005537-199906000-00001> PMID: 10369269
9. Gray SD, Alipour F, Titze IR, Hammond TH. Biomechanical and histologic observations of vocal fold fibrous proteins. *Ann Otol Rhinol Laryngol*. 2000; 109: 77–85. <https://doi.org/10.1177/000348940010900115> PMID: 10651418
10. Chan RW, Titze IR. Dependence of phonation threshold pressure on vocal tract acoustics and vocal fold tissue mechanics. *J Acoust Soc Am*. 2006; 119: 2351–2362. <https://doi.org/10.1121/1.2173516> PMID: 16642848
11. Titze IR. The physics of small-amplitude oscillation of the vocal folds. *J Acoust Soc Am*. 1988; 83: 1536–1552. <https://doi.org/10.1121/1.395910> PMID: 3372869
12. Gray SD. Cellular physiology of the vocal folds. *Otolaryngol Clin North Am*. 2000; 33: 679–697. [https://doi.org/10.1016/S0030-6665\(05\)70237-1](https://doi.org/10.1016/S0030-6665(05)70237-1) PMID: 10918654
13. Laurent TC, Laurent UB, Fraser JR. Functions of hyaluronan. *Ann Rheum Dis*. 1995; 54: 429–432. PMID: 7794055
14. Ward PD, Thibeault SL, Gray SD. Hyaluronic acid: Its role in voice. *J Voice*. 2002; 16: 303–309. [https://doi.org/10.1016/S0892-1997\(02\)00101-7](https://doi.org/10.1016/S0892-1997(02)00101-7) PMID: 12395982

15. Moore J, Thibeault S. Insights into the role of elastin in vocal fold health and disease. *J Voice*. 2012; 26: 269–275. <https://doi.org/10.1016/j.jvoice.2011.05.003> PMID: 21708449
16. Thibeault SL. Advances in our understanding of the Reinke space. *Curr Opin Otolaryngol Head Neck Surg*. 2005; 13: 148–151. PMID: 15908811
17. Iseri LT, Alexander LC, McCaughey RS, Boyle AJ, Myers GB. Water and electrolyte content of cardiac and skeletal muscle in heart failure and myocardial infarction. *Am Heart J*. 1952; 43: 215–227. [https://doi.org/10.1016/0002-8703\(52\)90212-3](https://doi.org/10.1016/0002-8703(52)90212-3) PMID: 14894423
18. Ward SR, Lieber RL. Density and hydration of fresh and fixed human skeletal muscle. *J Biomech*. 2005; 38: 2317–2320. <https://doi.org/10.1016/j.jbiomech.2004.10.001> PMID: 16154420
19. Hirano M. Morphological structure of the vocal cord as a vibrator and its variations. *Folia Phoniatr (Basel)*. 1974; 26: 89–94.
20. Herrera VLM, Viereck JC, Lopez-Guerra G, Kumai Y, Kobler J, Karajanagi S, et al. 11.7 Tesla magnetic resonance microimaging of laryngeal tissue architecture. *The Laryngoscope*. 2009; 119: 2187–2194. <https://doi.org/10.1002/lary.20643> PMID: 19824052
21. Kishimoto AO, Kishimoto Y, Young DL, Zhang J, Rowland IJ, Welham NV. High and ultrahigh-field magnetic resonance imaging of naïve, injured, and scarred vocal fold mucosae in rats. *Dis Model Mech*. 2016; dmm.026526. <https://doi.org/10.1242/dmm.026526> PMID: 27638667
22. Chen T, Chodara AM, Sprecher AJ, Fang F, Song W, Tao C, et al. A new method of reconstructing the human laryngeal architecture using micro-MRI. *J Voice*. 2012; 26: 555–562. <https://doi.org/10.1016/j.jvoice.2011.03.012> PMID: 21816571
23. Klepacek I, Jirak D, Duskova Smrckova M, Janouskova O, Vampola T. The human vocal fold layers. Their delineation inside vocal fold as a background to create 3D digital and synthetic glottal model. *J Voice*. 2016; 30: 529–537. <https://doi.org/10.1016/j.jvoice.2015.08.004> PMID: 26432357
24. Wu L, Zhang Z. A parametric vocal fold model based on magnetic resonance imaging. *J Acoust Soc Am*. 2016; 140: EL159–EL165. <https://doi.org/10.1121/1.4959599> PMID: 27586774
25. Jackson EF, Ginsberg LE, Schomer DF, Leeds NE. A review of MRI pulse sequences and techniques in neuroimaging. *Surg Neurol*. 1997; 47: 185–199. [https://doi.org/10.1016/S0090-3019\(96\)00375-8](https://doi.org/10.1016/S0090-3019(96)00375-8) PMID: 9040824
26. Vaghefi E, Pontre BP, Jacobs MD, Donaldson PJ. Visualizing ocular lens fluid dynamics using MRI: Manipulation of steady state water content and water fluxes. *Am J Physiol-Regul Integr Comp Physiol*. 2011; 301: R335–R342. <https://doi.org/10.1152/ajpregu.00173.2011> PMID: 21593426
27. Oleson S, Lu K-H, Liu Z, Durkes AC, Sivasankar MP. Proton density-weighted laryngeal magnetic resonance imaging in systemically dehydrated rats. *The Laryngoscope*. 2018; 128: E222–E227. <https://doi.org/10.1002/lary.26978> PMID: 29114904
28. Kurita S, Nagata K, Hirano M. A comparative study of the layer structure of the vocal fold. In: Bless DM, Abbs JH, editors. *Vocal fold physiology: Contemporary research & clinical issues*. San Diego, CA: College-Hill Press; 1983. pp. 3–21.
29. Hirano M, Kurita S, Nakashima T. The structure of the vocal folds. In: Stevens KN, Hirano M, editors. *Vocal fold physiology*. Tokyo: University of Tokyo Press; 1981. pp. 33–43.
30. Hirano M, Kurita S, Nakashima T. Growth, development, and aging of human vocal folds. In: Bless DM, Abbs JH, editors. *Vocal fold physiology: Contemporary research & clinical issues*. San Diego, CA: College-Hill Press; 1983. pp. 23–43.
31. Cheuvront SN, Ely BR, Kenefick RW, Sawka MN. Biological variation and diagnostic accuracy of dehydration assessment markers. *Am J Clin Nutr*. 2010; 92: 565–573. <https://doi.org/10.3945/ajcn.2010.29490> PMID: 20631205
32. Hanson KP, Zhang Y, Jiang JJ. Parameters quantifying dehydration in canine vocal fold lamina propria. *The Laryngoscope*. 2010; 120: 1363–1369. <https://doi.org/10.1002/lary.20927> PMID: 20564654
33. Hanson KP, Zhang Y, Jiang JJ. Ex vivo canine vocal fold lamina propria rehydration after varying dehydration levels. *J Voice*. 2011; 25: 657–662. <https://doi.org/10.1016/j.jvoice.2010.06.005> PMID: 20951551
34. Miri AK. Mechanical characterization of vocal fold tissue: A review study. *J Voice*. 2014; 28: 657–667. <https://doi.org/10.1016/j.jvoice.2014.03.001> PMID: 25008382
35. Dickson JM, Weavers HM, Mitchell N, Winter EM, Wilkinson ID, Van Beek EJR, et al. The effects of dehydration on brain volume—Preliminary results. *Int J Sports Med*. 2005; 26: 481–485. <https://doi.org/10.1055/s-2004-821318> PMID: 16037892
36. Kempton MJ, Ettinger U, Schmechtig A, Winter EM, Smith L, McMorris T, et al. Effects of acute dehydration on brain morphology in healthy humans. *Hum Brain Mapp*. 2009; 30: 291–298. <https://doi.org/10.1002/hbm.20500> PMID: 18064587

37. Watson P, Head K, Pitiot A, Morris P, Maughan RJ. Effect of exercise and heat-induced hypohydration on brain volume. *Med Sci Sports Exerc.* 2010; 42: 2197–2204. <https://doi.org/10.1249/MSS.0b013e3181e39788> PMID: 20421835
38. Streitbürger D-P, Möller HE, Tittgemeyer M, Hund-Georgiadis M, Schroeter ML, Mueller K. Investigating structural brain changes of dehydration using voxel-based morphometry. *PLOS ONE.* 2012; 7: e44195. <https://doi.org/10.1371/journal.pone.0044195> PMID: 22952926
39. Office of Regulatory Affairs; Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Div of Environmental Hazards and Health Effects, National Center for Environmental Health; Div of Nutrition and Physical Activity, National Center for Chronic Disease Prevention and Health Promotion, CDC, editors. Hyperthermia and dehydration-related deaths associated with intentional rapid weight loss in three collegiate wrestlers—North Carolina, Wisconsin, and Michigan, November–December 1997. *Morb Mortal Wkly Rep.* 1998;47. Available: <https://www.cdc.gov/mmwr/preview/mmwrhtml/00051388.htm>
40. Friedman JN, Goldman RD, Srivastava R, Parkin PC. Development of a clinical dehydration scale for use in children between 1 and 36 months of age. *J Pediatr.* 2004; 145: 201–207. <https://doi.org/10.1016/j.jpeds.2004.05.035> PMID: 15289767
41. Karamzadeh AM, Jackson R, Guo S, Ridgway JM, Wong HS, Ahuja GS, et al. Characterization of submucosal lesions using optical coherence tomography in the rabbit subglottis. *Arch Otolaryngol Head Neck Surg.* 2005; 131: 499–504. <https://doi.org/10.1001/archotol.131.6.499> PMID: 15967882
42. Sivasankar M, Erickson E, Rosenblatt M, Branski RC. Hypertonic challenge to porcine vocal folds: Effects on epithelial barrier function. *Otolaryngol—Head Neck Surg.* 2010; 142: 79–84. <https://doi.org/10.1016/j.otohns.2009.09.011> PMID: 20096227
43. Fisher KV, Telsner A, Phillips JE, Yeates DB. Regulation of vocal fold transepithelial water fluxes. *J Appl Physiol.* 2001; 91: 1401–1411. <https://doi.org/10.1152/jappl.2001.91.3.1401> PMID: 11509542
44. Jiang JJ, Diaz CE, Hanson DG. Finite element modeling of vocal fold vibration in normal phonation and hyperfunctional dysphonia: Implications for the pathogenesis of vocal nodules. *Ann Otol Rhinol Laryngol.* 1998; 107: 603–610. <https://doi.org/10.1177/000348949810700711> PMID: 9682857
45. Tao C, Jiang JJ. Mechanical stress during phonation in a self-oscillating finite-element vocal fold model. *J Biomech.* 2007; 40: 2191–2198. <https://doi.org/10.1016/j.jbiomech.2006.10.030> PMID: 17187805
46. Hochman I, Hillman RE, Sataloff RT, Zeitels SM. Ectasias and varices of the vocal fold: Clearing the striking zone. *Ann Otol Rhinol Laryngol.* 1999; 108: 10–16. <https://doi.org/10.1177/000348949910800102> PMID: 9930535
47. Dikkers FG, Nikkels PGJ. Benign lesions of the vocal folds: Histopathology and phonotrauma. *Ann Otol Rhinol Laryngol.* 1995; 104: 698–703. <https://doi.org/10.1177/000348949510400905> PMID: 7661518
48. Woodson G. Developing a porcine model for study of vocal fold scar. *J Voice.* 2012; 26: 706–710. <https://doi.org/10.1016/j.jvoice.2012.03.003> PMID: 22727125
49. Koo TK, Li MY. A guideline of selecting and reporting intraclass correlation coefficients for reliability research. *J Chiropr Med.* 2016; 15: 155–163. <https://doi.org/10.1016/j.jcm.2016.02.012> PMID: 27330520
50. Thurlow S, Taylor-Covill G, Sahota P, Oldroyd B, Hind K. Effects of procedure, upright equilibrium time, sex and BMI on the precision of body fluid measurements using bioelectrical impedance analysis. *Eur J Clin Nutr.* 2017; <https://doi.org/10.1038/ejcn.2017.110> PMID: 28722029
51. Ori Y, Sabo R, Binder Y, Weinstein T, Korzets A, Ori G, et al. Effect of hemodialysis on the thickness of vocal folds: A possible explanation for postdialysis hoarseness. *Nephron Clin Pract.* 2006; 103: c144–c148. <https://doi.org/10.1159/000092911> PMID: 16636582
52. Knight MJ, McDonald SE, Birchall MA. Intrinsic muscles and distribution of the recurrent laryngeal nerve in the pig larynx. *Eur Arch Otorhinolaryngol.* 2005; 262: 281–285. <https://doi.org/10.1007/s00405-004-0803-3> PMID: 15322831
53. Jiang JJ, Raviv JR, Hanson DG. Comparison of the phonation-related structures among pig, dog, white-tailed deer, and human larynges. *Ann Otol Rhinol Laryngol.* 2001; 110: 1120–1125. <https://doi.org/10.1177/000348940111001207> PMID: 11768701
54. Alipour F, Jaiswal S. Phonatory characteristics of excised pig, sheep, and cow larynges. *J Acoust Soc Am.* 2008; 123: 4572–4581. <https://doi.org/10.1121/1.2908289> PMID: 18537405
55. Knight P, Parsons N. Action of NaCl and polyphosphates in meat processing: Responses of myofibrils to concentrated salt solutions. *Meat Sci.* 1988; 24: 275–300. [https://doi.org/10.1016/0309-1740\(88\)90040-X](https://doi.org/10.1016/0309-1740(88)90040-X) PMID: 22056057
56. Chan RW, Tayama N. Biomechanical effects of hydration in vocal fold tissues. *Otolaryngol—Head Neck Surg.* 2002; 126: 528–537. <https://doi.org/10.1067/mhn.2002.124936> PMID: 12075228

57. Hackney KJ, Cook SB, Fairchild TJ, Plutz-Snyder LL. Skeletal muscle volume following dehydration induced by exercise in heat. *Extreme Physiol Med.* 2012; 1: 3. <https://doi.org/10.1186/2046-7648-1-3> PMID: [23849266](https://pubmed.ncbi.nlm.nih.gov/23849266/)
58. Armstrong LE. Assessing hydration status: The elusive gold standard. *J Am Coll Nutr.* 2007; 26: 575S–584S. <https://doi.org/10.1080/07315724.2007.10719661> PMID: [17921468](https://pubmed.ncbi.nlm.nih.gov/17921468/)