Effects of vocal fold epithelium removal on vibration in an excised human larynx model

Justin R. Tse, Zhaoyan Zhang, and Jennifer L. Long[a)]
Department of Head and Neck Surgery, David Geffen School of Medicine, University of California, Los Angeles, CHS Room 62-132, 650 Charles Young Drive, Los Angeles, California 90095, USA
jrtse@mednet.ucla.edu, zyzhang@ucla.edu, jlong@mednet.ucla.edu

Abstract: This study investigated the impact of selective epithelial injury on phonation in an excised human larynx apparatus. With intact epithelium, the vocal folds exhibited a symmetrical vibration pattern with complete glottal closure during vibration. The epithelium was then enzymatically removed from one, then both vocal folds, which led to left-right asymmetric vibration and a decreased closed quotient. Although the mechanisms underlying these vibratory changes are unclear, these results demonstrate that some component of an intact surface layer may play an important role in achieving normal symmetric vibration and glottal closure.

[AL]
Date Received: June 29, 2014 **Date Accepted:** June 6, 2015

1. Introduction

The epithelium is the most superficial layer of the vocal fold and serves as a physical, protective barrier for deeper tissues. In humans, the epithelium forms a 50–80 μm thick cell layer, with an elastic modulus of about 100 kPa (Hirano and Kakita, 1985). The epithelium is much thinner and stiffer than the deeper lamina propria, which is 2 mm thick with an estimated elastic modulus of about 4 kPa (Kutty and Webb, 2009). As the vocal folds undergo continuous cycles of phonation, the tissue is particularly susceptible to deformation and trauma. Thus, one hypothesized function of the epithelium is to protect the soft, deeper layers of the vocal folds (Murray and Thomson, 2012) and to augment wound healing after trauma (Tateya *et al.,* 2006).

Recent modeling work has suggested that the epithelium may also play an intimate role in voice production in addition to tissue protection. Using a physical vocal fold model, Xuan and Zhang (2014) demonstrated that the presence of a stiff outer layer (simulating the epithelium) led to complete glottal closure along the anterior-posterior (A-P) direction. Isotropic one-layer models did not achieve complete A-P closure and had lower closed quotient (Xuan and Zhang, 2014; Mendelsohn and Zhang, 2011). Models with the simulated epithelial layer also demonstrated an in-phase medial-lateral motion along the anterior-posterior length, unlike the out-of-phase vibrations of single layer models. Resulting acoustic spectra demonstrated stronger excitation of high-order harmonics in the epithelial models, with a difference in perceived phonatory quality. However, it is unclear whether the epithelium exerts such a large effect on phonation in more complex anisotropic vocal folds such as in humans.

The goal of this study was to investigate the effects of the epithelium on phonation in human larynges. The excised larynx was selected as the most relevant model system because it allows controlled manipulation of the human anatomy, which would be impossible to perform in live persons. The study design was to selectively remove the epithelium, comparing phonatory vibration before and after. The enzyme trypsin was chosen for epithelium removal, because its action is specific to cell-cell adhesion proteins, thus sparing the extracellular matrix of the underlying lamina propria. This method produces finer disruption than achievable with dissection or thermal injury techniques.

2. Methods

2.1 Larynx preparation

Two adult cadaveric human larynges from a 72-year-old male (72M) and 63-year-old female (63F) were harvested from autopsy within 24 h of death and were kept frozen

[a)]Author to whom correspondence should be addressed. Also at: Research Service, VA Greater Los Angeles Healthcare System, Los Angeles, CA 90074, USA.

at −80 °C until use. Causes of death were unrelated to any laryngeal pathology. The larynges were thawed at 4 °C overnight prior to phonation. The remainder of the experiment was conducted at room temperature. Larynges were prepared by resecting the epiglottis and false vocal folds, to provide an unobstructed view of the superior aspect of the glottis. A single stitch was placed through the arytenoid cartilages bilaterally, adducting the vocal folds.

2.2 Excised larynx phonation

The excised larynges were mounted onto an air supply pipe to simulate tracheal air movement, as described in previous experiments (Zhang *et al.*, 2006). Briefly, the setup consisted of a pressurized and regulated airflow supply, an expansion chamber that simulated the lungs (rectangular cross-section 23.5 cm × 25.4 cm × 50.8 cm), and a straight circular PVC tube to simulate the trachea (11 cm-long cylinder with an inner diameter of 2.54 cm). The larynx was held motionless with a hose clamp over the trachea and non-penetrating circumferential pins. No cricothyroid tension was applied.

For each larynx, the subglottal pressure was gradually increased in discrete increments from 0% to about 120% of the phonation threshold pressure. At each step, with a delay of about 1–2 s after the flow rate change, the following parameters were measured: mean subglottal pressure (measured at 2 cm from the entrance of the glottis using a Baratron 220D pressure transducer, MKS Instruments, Andover, MA), mean flow rate (MKS 558A mass-flow meter, MKS Instruments, Andover, MA), subglottal acoustic pressure inside the tracheal tube (2 cm from the entrance of the glottis using a B&K 4182 probe microphone, Bruel & Kjaer North America Inc., Norcross, GA), and outside acoustic pressure (20 cm downstream and 30° off axis using a B&K 2669 microphone, Bruel & Kjaer North America Inc., Norcross, GA). Quantitative aerodynamic parameters of phonation threshold pressure, onset flow rate, resistance, and onset frequency were then extracted as the mean subglottal pressure, the mean flow rate, mean resistance, and the fundamental frequency at onset, as described in Zhang *et al.* (2006). Measurements were recorded at phonation onset to be consistent with the methodology of previously published techniques (Xuan and Zhang, 2014; Zhang *et al.*, 2006). The glottal resistance R was calculated as the ratio between the mean subglottal pressure and the mean flow rate.

A high-speed camera (Fastcam-Ultima APX, Photron Unlimited, Inc., San Diego, CA) recorded vocal fold vibration from a superior view at 2000 frames per second. Qualitative parameters of vibrational symmetry and glottal closure were assessed from the video images via visual inspection. A MATLAB-based code extracted the glottal area waveform for a 1-s segment of video, and the closed quotient was calculated.

2.3 Enzymatic de-epithelialization

After baseline phonation (i.e., with intact epithelia), the epithelium was enzymatically removed from one randomly chosen vocal fold by applying 5–10 mL of 0.25% Trypsin-EDTA (Sigma-Aldrich, St. Louis, MO) for 15 min at room temperature ("unilateral de-epithelialization"). Paraffin paper was placed between the two vocal folds to form an application pocket and to prevent trypsin contact on the contralateral vocal fold. Phonation as described above was repeated immediately after unilateral enzymatic de-epithelialization. The contralateral vocal fold was then de-epithelialized ("bilateral de-epithelialization") and phonation was repeated once more. The right vocal fold from the 73M larynx was de-epithelialized first, while the left vocal fold from the 63F was de-epithelialized first.

To verify complete de-epithelialization, the vocal folds were fixed with 3.7% formaldehyde in phosphate buffered saline, paraffin-embedded, and then sectioned with a thickness of 5 μm via microtome. The vocal folds were cut at the coronal plane to expose all three vocal fold layers: the epithelium, lamina propria, and thyroarytenoid muscle. Standard histological staining was performed with solutions hematoxylin and eosin (H&E).

3. Results

3.1 Enzymatic de-epithelialization

Histological staining with H&E revealed near-complete removal of the epithelium and the underlying basement membrane. Lamina propria and thyroarytenoid muscle layers were unperturbed by trypsin treatment (Fig. 1).

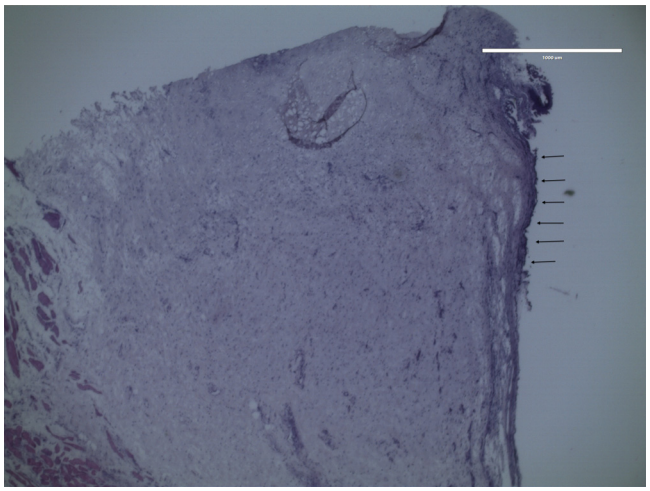

Fig. 1. (Color online) Histologic section of vocal fold after trypsin de-epithelialization, 4×. Coronal view demonstrates near-complete removal of epithelial cells from superior and medial surfaces. Residual epithelium is marked with arrows.

3.2 High-speed video analysis of vibration

Baseline phonation for both the 63F and 72M larynges demonstrated normal and symmetric mucosal waves with complete glottal closure along the anterior-posterior direction [Figs. 2(a) and 2(b), first row of each]. Specifically, the vocal folds exhibited in-phase medial-lateral vibration with both vocal folds moving toward and away from the glottal midline simultaneously. Also, the entire vocal fold edge moved in synchrony and the entire edge participated in closure. After enzymatic removal of unilateral epithelium with trypsin, both larynges demonstrated reduced glottal closure and asymmetric vibration with loss of the organized mucosal wave [second row in Figs. 2(a) and 2(b)]. The opposing vocal folds no longer vibrated in phase with each other, and the medial-lateral movement was not coordinated along the vocal fold edge. This resulted in a disorganized mucosal wave, with different regions of each vocal fold vibrating out-of-phase with each other. This impairment occurred in both vocal folds, even though only one side was treated with trypsin. After the epithelium was removed on the contralateral side, vibration continued to be asymmetric and exhibit additional

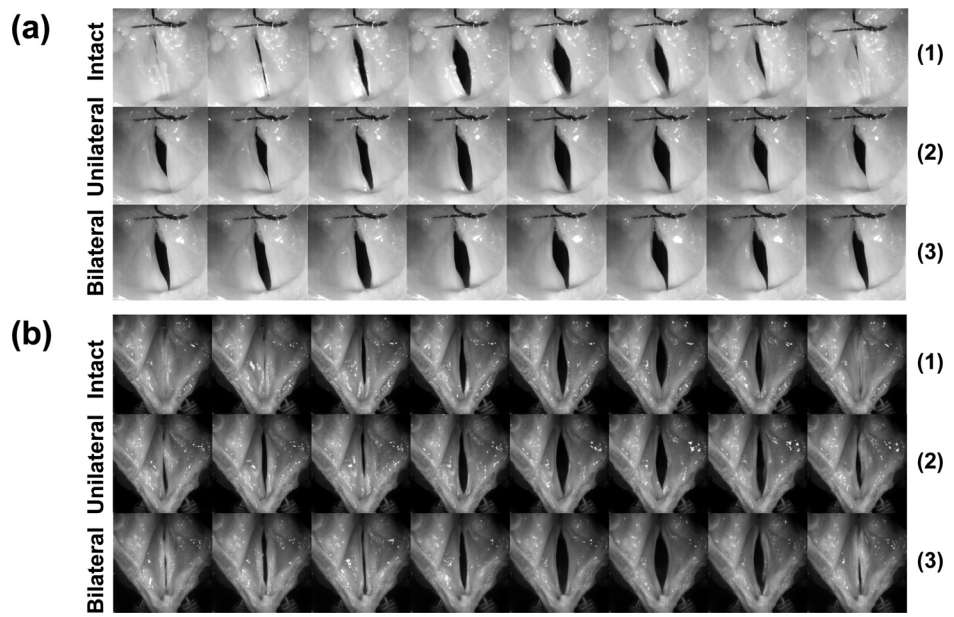

Fig. 2. Superior-view images of larynges from 63-year-old female (a) and 72-year-old male (b). Anterior commissure is at the image bottom. For each larynx, images are shown at equal intervals during one oscillation cycle at baseline (row 1), after unilateral de-epithelialization (row 2), and after bilateral de-epithelialization (row 3). The larynx in (a) shows a black adduction stitch between arytenoid cartilages; in (b) the adduction stitch is neutral-colored and limited to the vocal processes.

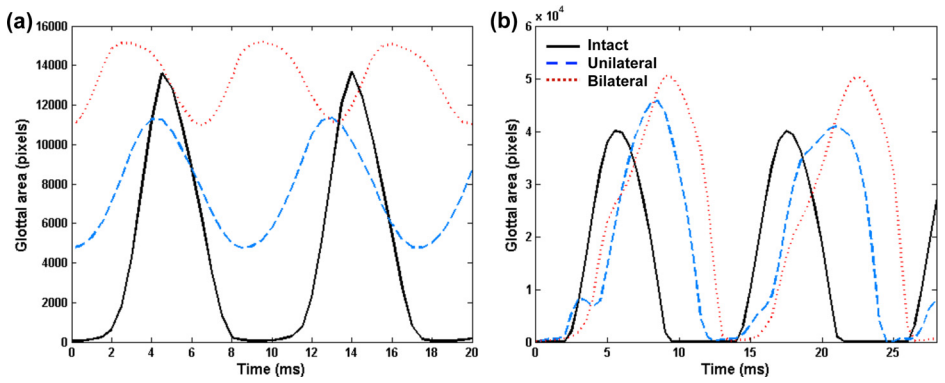

Fig. 3. (Color online) Glottal area waveform, shown for two glottal cycles of the (a) 63F larynx and (b) 72M larynx. Both show decreasing glottal closure with de-epithelialization.

anterior-posterior modes with impaired glottal closure [third row in Figs. 2(a) and 2(b)]. When closure did occur (in the 72M larynx only), it was incomplete along the anterior-posterior direction.

3.3 Quantitative assessment of aerodynamic and vibratory parameters

MATLAB extraction of glottal area waveform demonstrated quantitative decrease in glottal closure for both larynges after trypsin treatment (Fig. 3). Table 1 shows the closed quotient, onset flow rate, glottal resistance, fundamental frequency (F0) at onset, and phonation onset pressure for the baseline conditions, unilateral treatment, and bilateral treatment. The main effects of epithelium removal were to decrease the closed quotient and consequently decrease glottal resistance and increase flow rate. Fundamental frequency at onset tended to increase with de-epithelialization and phonation threshold pressure was unchanged. Of note, the female larynx did have a low F0 at phonation onset, but this rose to a more physiologic level (167 Hz) as airflow increased.

4. Discussion and conclusions

The role of vocal fold epithelium in laryngeal health and disease is an area of active investigation. The epithelial layer functions in water and ion transport, and in exclusion of irritants. Epithelial integrity also has been shown to be impacted by phonotrauma (Rousseau *et al.,* 2011), leading some to postulate a role in formation of benign vocal fold pathology such as polyps (Levendoski *et al.,* 2014). Because of its intimate association with the lamina propria, we have proposed that therapies for severe vocal fold scarring should consider this layer as well (Long, 2010). Despite the growing interest, a role for the epithelial layer in normal phonation has not previously been demonstrated experimentally. In this study, selectively removing the epithelium from human vocal folds impaired phonatory vibration. Glottal closure was consistently impaired, both quantitatively in the closed quotient, and qualitatively in the character of the vocal fold edge apposition. Loss of left-right vibrational symmetry, and of anterior-posterior vibrational unity, also occurred.

The underlying mechanisms for these vibratory changes are unclear. One possible explanation is that the epithelial layer may indeed serve a biomechanical function in normal phonation. Similar function of the epithelium layer has been observed in

Table 1. Assessment of aerodynamic and vibratory parameters.

	Closed Quotient, %	Onset flow rate, mL/s	Glottal resistance, Pa-s/mL	Fundamental frequency at onset, Hz	Phonation threshold pressure, kPa
63F					
Intact	33	64	7.2	88	0.46
Unilateral	0	118	2.9	134	0.34
Bilateral	0	169	2.7	131	0.46
72M					
Intact	38	223	1.7	153	0.39
Unilateral	15	281	1.3	156	0.36
Bilateral	23	474	0.9	183	0.44

physical model experiments by Xuan and Zhang (2014), in which adding a stiff superficial epithelium layer to isotropic single-layer models led to complete glottal closure with in-phase vibration along the anterior-posterior length. The absence of this layer, i.e., isotropic single-layer models, led to incomplete glottal closure with out-of-phase vibration along the anterior-posterior length. However, it is possible that other mechanisms may be involved as well. For example, the enzymatic disruption of the epithelium might produce other surface changes in the vocal folds. Surface mucus viscosity and fluid characteristics have been suggested to impact phonation in limited situations (Roy *et al.,* 2003; Ayache *et al.,* 2004). Removing the protective epithelial cell layer is very likely to also have a dehydrating effect on the vocal folds, so separating those changes from the loss of the cellular layer is difficult. Future studies, including a more detailed time course and re-hydration efforts, are required to further clarify the specific mechanisms underlying the observed vibratory changes.

Acknowledgments

This study was supported by Research Grant No. R01DC011299 from the National Institute on Deafness and Other Communication Disorders at the National Institutes of Health (Z.Z.); Research Grant No. IK2BX001944 from the Department of Veteran's Affairs Career Development Award (J.L.L.); and the AΩA Carolyn L. Kuckien Student Research Fellowship (J.R.T.). We would like to thank Dr. Yue Xuan for technical support.

References and links

Ayache, S., Ouaknine, M., Dejonkere, P., Prindere, P., and Giovanni, A. (**2004**). "Experimental study of the effects of surface mucus viscosity on the glottic cycle," J. Voice **18**, 107–115.

Hirano, M., and Kakita, Y. (**1985**). "Cover-body theory of vocal fold vibration," in *Speech Science: Recent Advances*, edited by R. G. Daniloff (College-Hill Press, San Diego, CA), pp. 1–46.

Kutty, J. K., and Webb, K. (**2009**). "Tissue engineering therapies for the vocal fold lamina propria," Tissue Eng. Part B Rev. **15**(3), 249–262.

Levendoski, E., Leydon, C., and Thibeault, S. L. (**2014**). "Vocal fold epithelial barrier in health and injury: A research review," J. Speech Language Hearing Res. **57**, 1679–1691.

Long, J. (**2010**). "Tissue engineering for treatment of vocal fold scar," Curr. Opin. Oto. **18**, 521–525.

Mendelsohn, A., and Zhang, Z. (**2011**). "Phonation threshold pressure and onset frequency in a two-layer physical model of the vocal folds," J. Acoust. Soc. Am. **130**, 2961–2968.

Murray, P. R., and Thomson, S. L. (**2012**). "Vibratory responses of synthetic, self-oscillating vocal fold models," J. Acoust. Soc. Am. **132**, 3428–3438.

Rousseau, B., Suehiro, A., Echmendia, N., and Sivasankar, M. (**2011**). "Raised intensity phonation compromises epithelial barrier integrity," Laryngoscope **121**, 346–351.

Roy, N., Tanner, K., Gray, S. D., Blomgren, M., and Fisher, K. V. (**2003**). "An evaluation of the effects of three laryngeal lubricants on phonation threshold pressure (PTP)," J. Voice **17**, 331–342.

Tateya, I., Tateya, T., Lim, X., Sohn, J. H., and Bless, D. M. (**2006**). "Cell production in injured vocal folds: A rat study," Ann. Oto. Rhinol. Larngol. **115**(2), 135–143.

Xuan, Y., and Zhang, Z. (**2014**). "Influence of embedded fibers and an epithelium layer on glottal closure pattern in a physical vocal fold model," J. Speech Language Hearing Res. **57**, 416–425.

Zhang, Z., Neubauer, J., and Berry, D. (**2006**). "The influence of subglottal acoustics on laboratory models of phonation," J. Acoust. Soc. Am. **120**(3), 1558–1569.