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## In-Vivo Measurement of Vocal Fold Surface Resistance

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### Abstract

**Objective**—A custom-designed probe was developed to measure vocal fold surface resistance *in vivo*. The purpose of this study was to demonstrate proof of concept of using vocal fold surface resistance as a proxy of functional tissue integrity after acute phonotrauma using an animal model.

**Study Design**—Prospective animal study.

**Methods**—New Zealand White breeder rabbits received 120 minutes of airflow without vocal fold approximation (control), or 120 minutes of raised intensity phonation (experimental). The probe was inserted via laryngoscope and placed on the left vocal fold under endoscopic visualization. Vocal fold surface resistance of the middle 1/3 of the vocal fold was measured after 0 (baseline), 60, and 120 minutes of phonation. After the phonation procedure, the larynx was harvested and prepared for transmission electron microscopy.

**Results**—In the control group, vocal fold surface resistance values remained stable across time points. In the experimental group, surface resistance ( $X \pm Y\%$  relative to baseline) was significantly

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decreased after 120 minutes of raised intensity phonation. This was associated with structural changes using transmission electron microscopy, which revealed damage to the vocal fold epithelium after phonotrauma, including disruption of the epithelium and basement membrane, dilated paracellular spaces, and alterations to epithelial microprojections. In contrast, control vocal fold specimens showed well-preserved stratified squamous epithelia.

**Conclusion**—These data demonstrate the feasibility of measuring vocal fold surface resistance *in vivo* as a means of evaluating functional vocal fold epithelial barrier integrity. Device prototypes are in development for additional testing, validation and for clinical applications in laryngology.

### Keywords

epithelium; vocal fold surface resistance; phonotrauma; vocal fold; voice

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### Introduction

The vocal folds are exposed to a variety of environmental and physical threats, including industrial pollutants, gastric reflux, and the biomechanical stresses associated with airflow-induced vocal fold oscillation. Repeated exposure to these external threats has been implicated in the breakdown of the vocal fold epithelium and disorganization of the lamina propria, and may be a contributing factor in the development of benign phonotraumatic vocal fold lesions.<sup>1,2</sup> The vocal fold epithelium is made up of stratified, non-keratinized squamous cells, and serves as the outermost physical barrier. The structural integrity of the epithelium is maintained by junctional complexes which consist of tight junctions, adherens junctions, and desmosomes. In particular, tight junctions are responsible for intercellular signaling, while adherens junctions and desmosomes mechanically link adjacent cells to one another.<sup>3,4</sup> The integrity of the junctional complex is critical in the protection of the underlying lamina propria from environmental irritants and phonation-induced mechanical stresses.<sup>5</sup> Interestingly, dilated vocal fold epithelial intracellular spaces are a clinically-relevant characteristic of damage to the epithelium from laryngopharyngeal reflux<sup>6</sup>, and are thought to contribute to the development of vocal fold pathology.<sup>7</sup>

Our research group and others have reported evidence of a compromised vocal fold epithelial barrier after biomechanical tissue stress.<sup>5</sup> Scanning electron and transmission electron microscopy reveals structural changes to the epithelium, including reduced epithelial depth and microprojection height after transient episodes of phonotrauma (e.g., 30 minutes of raised intensity phonation), with complete obliteration of the epithelial cell surface after 120 minutes of experimentally induced phonation. However, very few studies have demonstrated the functional consequences of such changes to vocal fold structural integrity. Our overarching hypothesis is that epithelial damage and the subsequent changes to functional epithelial barrier integrity may contribute to the development of vocal fold pathology, including benign vocal fold lesions, inflammation, and scar.<sup>5,8</sup> Therefore, it is critically important to consider these structural changes in the context of functional epithelial barrier integrity in order to more effectively guide clinical management of these patients.

Functional epithelial barrier integrity has been measured using *in vitro* and *ex vivo* systems.<sup>7</sup> Transepithelial electrical resistance (TEER) is a measure of electrical resistance through the epithelium, and reflects the ionic conductance of the paracellular and transcellular pathways.<sup>9</sup> TEER has been measured from excised tissues using Ussing systems and *in vitro* using EVOM2 (epithelial volt-ohmmeter) systems.<sup>10–13</sup> *In vivo* approaches to measuring tissue resistance have been developed in other biological tissue systems. For example, Farré et al. have measured mucosal impedance in the esophagus of both animals and humans *in vivo*, using a multichannel intraluminal impedance tool that measures changes in conductivity to an electrical current by a pair of metallic rings mounted to a catheter.<sup>10</sup> These investigations have revealed a significant correlation between *in vivo* multichannel intraluminal impedance and *ex vivo* TEER measures. Farré et al. propose that mucosal impedance measures are a reflection of esophageal mucosal integrity. Similarly, Vaezi and colleagues have used *in vivo* real-time esophageal mucosal impedance and have reported decreased mucosal impedance at the site of erosive versus non-erosive mucosal regions, a finding of clinical importance in the treatment of patients with gastroesophageal reflux disease.<sup>14</sup>

Clinical laryngology currently lacks such a diagnostic instrument capable of measuring vocal fold surface resistance *in vivo* in real-time. The benefits of such a clinical tool that can be used to measure functional tissue integrity in patients in a repeatable fashion over the course of treatment has significant clinical implications in the management of patients with benign and malignant laryngologic disorders, including head and neck squamous cell carcinoma. To address this need, our research group has developed a new system that can be used to measure vocal fold surface resistance *in vivo*. This tool applies a constant current between two probes on the surface of the vocal fold to measure the electrical resistance (k $\Omega$ ) of the epithelial cell layer and surface secretions. To control for cell surface conditions across experiments, the use of humidified airflow and the clearing of surface secretions prior to probe contact with the epithelial surface is necessary to ensure accurate measurement within and between cell surface conditions.

In the current proof of concept study, we demonstrate the feasibility of using this system to measure *in vivo* changes in vocal fold surface resistance in response to acute phonotrauma within-subject and evidence that decreased surface resistance is associated with a structurally compromised vocal fold epithelial barrier, which has never been previously reported in the larynx. These data have important implications in the identification and management of patients with vocal fold disease.

## Materials and Methods

In this study, a novel custom-designed probe was used to collect vocal fold surface resistance *in vivo* at three separate time points over the course of 120 minutes of experimentally induced phonation. Following each phonation procedure, transmission electron microscopy of the vocal fold epithelium was used to investigate structural damage to the epithelial cell surface. To ensure measurement consistency across animals, all experiments were performed using the same custom-designed probe, data collection

protocol, and surgical procedures. The procedures used in this study were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center.

### Probe Design

A custom-designed resistance probe was engineered to measure electrical resistance *in vivo* across the surface of the rabbit vocal fold epithelium (Figure 1). The probe contained 2 leads, each made of 0.61-mm-diameter, 304 stainless steel rods. The leads were fixed center-to-center with a separation distance of 1 mm. The stainless steel leads were soldered to copper wires, which were routed inside a 150-mm-long stainless steel tube with an outside diameter of 3.0 mm and coupled to a control circuit. The probe was equipped with an instrument handle for phonomicrosurgery (Instrumentarium, Quebec, Canada).

An adjustable current source (ST Microelectronics LM134) provided a known current to the probe. The voltage across the leads of the probe was measured using a microcontroller. This allowed for the resistance between the probe leads to be calculated using a simple formula:  $R$  (resistance) =  $V$  (voltage)/ $I$  (current). The current source output was set to 6.77  $\mu\text{A}$ , which resulted in a resistance measurement range of 0–480 k $\Omega$ . In the current series of experiments, data were sampled at a rate of 19.6 Hz and streamed to a computer over a serial connection. The probe was calibrated using several known resistance values and piloted using an *ex vivo* rabbit larynx prior to use *in vivo*.

### In-Vivo Phonation and Measurement of Vocal Fold Surface Resistance

Ten New Zealand white breeder rabbits, weighing 2.6–3.4 kg were used in this experiment. Animals were randomized to receive 120 minutes of experimentally induced raised intensity phonation (experimental group) ( $n = 5$ ) or continuous humidified airflow in the absence of electrical stimulation to the cricothyroid muscle (control group) ( $n = 5$ ).

**Phonation**—The phonation procedure was performed as described in detail elsewhere.<sup>15</sup> Briefly, animals were anesthetized by intramuscular administration of ketamine (35 mg/kg), xylazine (5 mg/kg), and acepromazine (0.75 mg/kg). with maintenance doses of ketamine (17.5 mg/kg IM) and acepromazine (0.375 mg/kg IM) provided as needed. A midline incision was used to expose the larynx and trachea, the trachea was transected, and an endotracheal tube was inserted to provide a stable airway via a tracheostomy. A cuffed endotracheal tube (Mallinckrodt, Hennep, Germany) (3.5 mm) was inserted into the upper portion of the trachea to deliver humidified airflow to the glottis. Phonation was produced via electrical stimulation of the CT muscle. A Holinger-Tucker pediatric side-slotted laryngoscope was used to suspend the larynx and endoscopic video images of the larynx were captured using a 30° 2.7-mm rigid endoscope coupled to a Telecam-C camera (Karl Storz Endoscopy-America, Inc.).

**Acoustic Measures**—The acoustic measures were collected as described in detail elsewhere.<sup>1</sup> Briefly, acoustic measures of phonation intensity (dB SPL) were recorded at baseline and at 30-minute intervals. A microphone was positioned 10 cm from the opening of the laryngoscope. Recordings were digitized and the most stable 0.5- to 1.0-second portion of the acoustic waveform was analyzed to measure mean phonation intensity.

Phonation was recorded for each animal at modal intensity (humidified airflow = 85 cm<sup>3</sup>/sec, minimum stimulation required to achieve audible phonation) and raised intensity (humidified airflow = 144 cm<sup>3</sup>/sec, electrical stimulation increased to induce phonation >5 dB over modal intensity). For the control condition, 144 cm<sup>3</sup>/sec of humidified airflow was delivered to the vocal folds in the absence of electrical stimulation.

**Resistance Measures**—Vocal fold surface resistance (kΩ) was measured during both the experimental and control conditions after 0 (baseline), 60, and 120 minutes. The custom-designed probe equipped with an endoscopic instrument handle was passed intraorally under endoscopic visualization using a 30° 2.7-mm rigid endoscope to make contact with the left vocal fold surface. The leads of the probe were placed on the medial surface of the mid-membranous vocal fold and surface resistance was measured (Figure 2 A/B). To ensure the reliability of measurements from trial-to-trial within and across animals, all measurements were recorded from the epithelial surface after direct probe contact was made for over 2 seconds. This was used to confirm that adequate contact was made between the probes and the vocal fold surface, and thus allowing for the capture of stable resistance measures. The average surface resistance measures for each animal were normalized against its baseline (0 minutes) resistance measure, and reported as relative change from baseline. Immediately after the phonation procedure, animals were euthanized and larynges were harvested.

**Transmission Electron Microscopy**—Following harvest, the left vocal fold was prepared for transmission electron microscopy (TEM). The vocal fold tissues were soaked in 2.5 % Glutaraldehyde solution at room temperature for one hour and then stored at 4° C for fixation. Tissue processing for TEM has been described in detail elsewhere.<sup>12,16</sup> In brief, TEM images were acquired using a Philips CM-12 transmission electron microscope (FEI Company, Hillsboro, OR) for analysis of vocal fold epithelial surface damage.

**Statistical Analysis**—All data were analyzed using IBM SPSS 22.0 (International Business Machines Corp., Armonk, NY). The effect of raised-intensity phonation on vocal fold surface resistance was examined using the F-value in a one-way repeated measures analysis of variance (ANOVA) and pairwise comparisons made using Bonferroni post-hoc testing. The threshold for significance was determined using a p value of < 0.05.

## Results

*In vivo* vocal fold surface resistance was measured to identify changes in vocal fold tissue integrity after acute phonotrauma (Figure 3A). Acoustic measurement of phonation intensity revealed that mean phonation intensity during experimentally induced raised intensity phonation was 62.9 dB (SD = 3.1) at baseline and maintained at 64.6 dB (SD = 2.6) throughout the 120 minute experiment. Consistent with previous experiments from our laboratory, the intensity of raised intensity phonation was significantly increased relative to modal intensity phonation.<sup>1,2,17,18</sup>

During raised intensity phonation, there was a significant main effect of time on vocal fold surface resistance, as demonstrated by ANOVA [F value (2, 8) = 6.967, *p* = 0.018]. Post-hoc testing revealed that vocal fold surface resistance decreased significantly from 0 minutes

(baseline) to 120 minutes of experimentally induced raised intensity phonation (0.498 (95% CI, 0.165 to 0.831),  $p = 0.012$ ; Figure 3B). In the control condition, there were no significant differences in vocal fold surface resistance across time (F value (2, 8) = 0.239,  $p = 0.793$ ; Figure 3C).

TEM was used to evaluate structural changes to the vocal fold epithelial surface in response to 120 minutes of raised intensity phonation. The vocal fold epithelium in the control condition showed well-preserved stratified squamous epithelia with minimal desquamation or dilation of paracellular spaces (Figure 4A, C). In contrast, raised intensity phonation revealed extensive desquamation and dilation of paracellular spaces, which extended into the deep layers of the stratified squamous epithelial cell layers. Additionally, there was evidence of a disrupted basement membrane and dilated paracellular spaces in some specimens in the remaining intact epithelium (Figure 4B, D). Further, epithelial cell surface microprojections were fragmented and density was significantly decreased in specimens exposed to experimentally induced raised intensity phonation.

## Discussion

In this study, we describe the development of a custom-designed probe to measure vocal fold surface resistance *in vivo*. The purpose of the current series of experiments was to demonstrate proof of concept of using vocal fold surface resistance as a proxy of functional tissue integrity after acute phonotrauma. The experimental design involved the collection of repeated measures of vocal fold surface resistance over time to demonstrate intra-subject changes to the vocal fold surface in response to acute phonotrauma. Further, we investigated microscopic structural changes to the vocal fold epithelium in response to acute phonotrauma in order to determine the sensitivity of the custom-designed probe for detecting changes at the cellular level. Results revealed a strong relationship between reduced vocal fold surface resistance and transmission electron microscopic findings of extensive desquamation of the surface epithelium, disrupted basement membrane, dilated paracellular spaces, and alterations to epithelial surface microprojections after experimentally induced raised intensity phonation. In contrast, control vocal fold specimens showed well-preserved stratified squamous epithelia and intact paracellular spaces. Results revealed that the measurement of vocal fold surface resistance *in vivo* appears to provide a good proxy of functional vocal fold epithelial barrier integrity.

During raised intensity phonation, resistance values decreased significantly after 120 minutes of experimentally induced phonation. TEM after 120 minutes of raised intensity phonation revealed evidence of structural damage to the vocal fold epithelial surface. This finding was expected as the electrical current passing between the two probes on the vocal fold travels along the path of least resistance; therefore, damage to the epithelial cell surface and dilation of the paracellular spaces would result in decreased resistance and increased flow of current between the two measurement probes. These results provide preliminary evidence to demonstrate that damage to the vocal fold epithelial barrier is associated with decreased vocal fold surface resistance. Further, we have previously shown that the depth of the viable vocal fold epithelium is significantly decreased following 60 and 120 minutes of raised intensity phonation.<sup>8</sup> Thus, the strong relationship between morphological tissue

changes and surface resistance values provides support for the sensitivity of using vocal fold surface resistance as a proxy for functional vocal fold epithelial barrier integrity.

Previous studies of epithelial barrier integrity describe the use of Ussing chambers as a “gold standard” for determining TEER in *ex vivo* tissue<sup>10–13</sup>. This technique is inherently challenging to perform using rabbit vocal folds; indeed the small size of rabbit vocal fold tissue makes it particularly challenging to achieve a complete seal when mounting these tissues in lucite chambers using O rings. Further, the use of pins to mount these tissues in lucite chambers creates edge damage around the measurement surface area. Given these challenges, it was not possible to make comparisons between *in vivo* and *ex vivo* TEER using rabbit vocal folds. As such, porcine or canine vocal folds may be very useful for further validation of vocal fold surface resistance. The present study provides proof of concept for measurement of vocal fold surface resistance *in vivo*.

In our experiments, albeit we control for surface hydration by using humidified airflow across conditions, epithelial surface secretions may fluctuate in response to vocal fold injury. Therefore, the measurement of electrical resistance across the surface of the vocal fold is likely influenced by both tissue electrical conductivity, and by the conductivity of the secretions on the vocal fold surface and within the paracellular spaces. Based on what is known about the regulation of vocal fold surface fluid,<sup>7,19–22</sup> and the conductivity of surface secretions,<sup>23–25</sup> the influence of functional changes in surface fluid on vocal fold surface resistance should be controlled. Vocal fold surface fluid is maintained, in part, through ion and water transport<sup>7</sup>, and these biologic mechanisms are mediated by specific pumps and channel proteins located on epithelial cell membranes. This ion transport system is influenced by various challenges, including ionic and osmotic perturbations,<sup>26</sup> simulated reflux,<sup>13</sup> and pollutants<sup>27</sup>, and perturbations to ion transport result in subsequent changes to surface fluid. Of specific interest to our research group is the effect of phonotrauma on these epithelial ion transport mechanisms. This bioactive response of the vocal fold epithelium may serve a salient physiologic role in protection of the vocal folds from injury, as some studies have revealed an increase in secretory volume and viscosity in patients with vocal fold pathology.<sup>28,29</sup> The resistance probe developed in the present series of experiments may be useful to investigators interested in investigating the bioactive function of the vocal fold epithelium, and to clinicians as a diagnostic instrument used to assess mucosal tissue integrity before and after medical and surgical interventions. Indeed, during the development of vocal fold pathology, we hypothesize that changes to the epithelial cell surface precedes changes in vocal fold surface resistance. Therefore, we suspect that the excision of vocal fold lesions and restoration of the vocal fold surface to its non-diseased state would return surface resistance to more typical physiological values. Thus, the use of surface resistance may provide an effective assessment of vocal fold tissue pre- and post-treatment.

In a subsequent pilot experiment, the sensitivity of the resistance probe to changes in surface fluid was investigated. In this pilot study, the probe was able to detect a 19% decrease in surface resistance between laryngeal tissue maintained in a hydrated state using a topical saline drip and laryngeal tissue in a desiccated state from drying of the surface with gauze (0.19 +/- 0.04 (mean +/- standard error),  $t(10) = 5.063$ ,  $p = 0.0005$ ). These preliminary data provided increased confidence that any functional changes in the status of secretions of the

vocal fold surface would be detected by the resistance probe used in the present study given its sensitivity in detecting hydrated and desiccated vocal fold surface conditions. Interestingly, it has been suggested anecdotally that benign vocal fold lesions are often associated with the presence of thick secretions on the surface of the vocal folds. These surface secretions may serve an important physiologic role. However, excessively thick and purulent secretions on the vocal fold cell surface may be detrimental to phonation and warrant mucolytic or pharmacologic management. These areas remain ripe for future investigation.

While no differences in surface resistance were observed between baseline, 60 minutes and 120 minutes ( $p = 0.793$ ), intersubject variability was observed at 60 minutes ( $p < 0.0001$ ,  $0.99 \pm 0.05$ , mean  $\pm$  standard error) and 120 minutes ( $p < 0.0001$ ,  $1.02 \pm 0.04$ ; Figure 3C). As mentioned already, epithelial surface conditions likely impact surface resistance, in addition to other endogenous and exogenous factors, including but not limited to sampling location, the presence of mucus on the vocal folds, operator technique, and equipment precision. Fortunately, epithelial surface resistance between the experimental and control conditions were discrete enough to be picked up by the measurement probe in the present investigation; however users should utilize appropriate clinical judgement and care in future clinical applications of this measure to minimize variability and preserve measurement fidelity.

Yet another important consideration in the design and future development of the vocal fold surface resistance probe is that it is capable of insertion through the working channel of a flexible distal chip laryngoscope to allow for in-office procedures under local anesthesia. Measurement fidelity is also important and thus contact with the vocal fold surface for 2 seconds is ideal. However, improvements in the design and sensitivity of the probe should be incorporated in future design prototypes to allow for more rapid measurement of the epithelial cell surface. A tool that can provide reliable diagnostics in the office would be of great benefit to surgeons; eliminating the need to take patients to the operating room for exploratory direct microlaryngoscopy and reducing risk from general anesthesia. In order to assess the safety of the probe following repeated measurement of the mucosal surface of the vocal fold, control specimens were evaluated using TEM to assess for any changes to the vocal fold epithelium from probe insertion and repeat measurement. TEM images revealed no evidence of epithelial structural changes in control specimens, an important consideration for eventual clinical application.

With proof of concept demonstrated, studies are now underway to investigate the effects of phonotrauma on epithelial ion and water transport mechanisms. Experiments are also planned to investigate the effects of increasing time-doses and magnitude-doses of phonation on vocal fold surface resistance, functional epithelial barrier integrity, and the effects of phonotrauma on the composition and volume of excretory surface secretions. A recent study undertaken by our group to investigate repair mechanisms of the vocal folds after 120 minutes of raised intensity phonation identified structural damage to the microprojections of the vocal fold epithelium at 0, 4, 8, and 24 hours after phonotrauma<sup>30</sup>. At day 3 after phonotrauma complete regeneration of the epithelial surface with microprojection height and density similar to controls was observed. Thus, relative to surface resistance, we would

expect the surface damage observed within the first 24 hours after phonotrauma to be associated with decreased vocal fold surface resistance, followed by return of surface resistance values to more typical physiological levels by day 3 after phonotrauma (the time point in which recovery of surface projections is complete).

## Conclusions

The vocal fold epithelium is a biologically active and tightly regulated tissue that responds to internal and external cellular perturbations. Epithelial barrier integrity is thus an important and clinically salient variable that can provide relevant information about vocal fold tissue homeostasis and objective information to demonstrate the efficacy of medical and surgical management of vocal fold disease. The resistance probe developed in the present study provides proof of concept to demonstrate the feasibility of measuring vocal fold surface resistance *in vivo* to evaluate functional vocal fold epithelial barrier integrity. The measurement of vocal fold surface resistance *in vivo* may provide an important advance in clinical laryngology.

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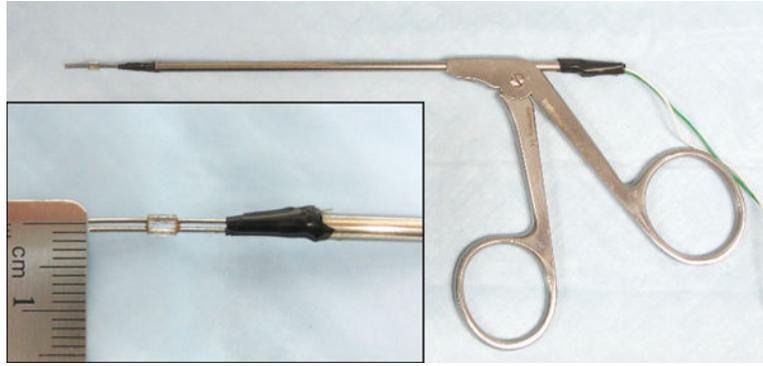
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## References

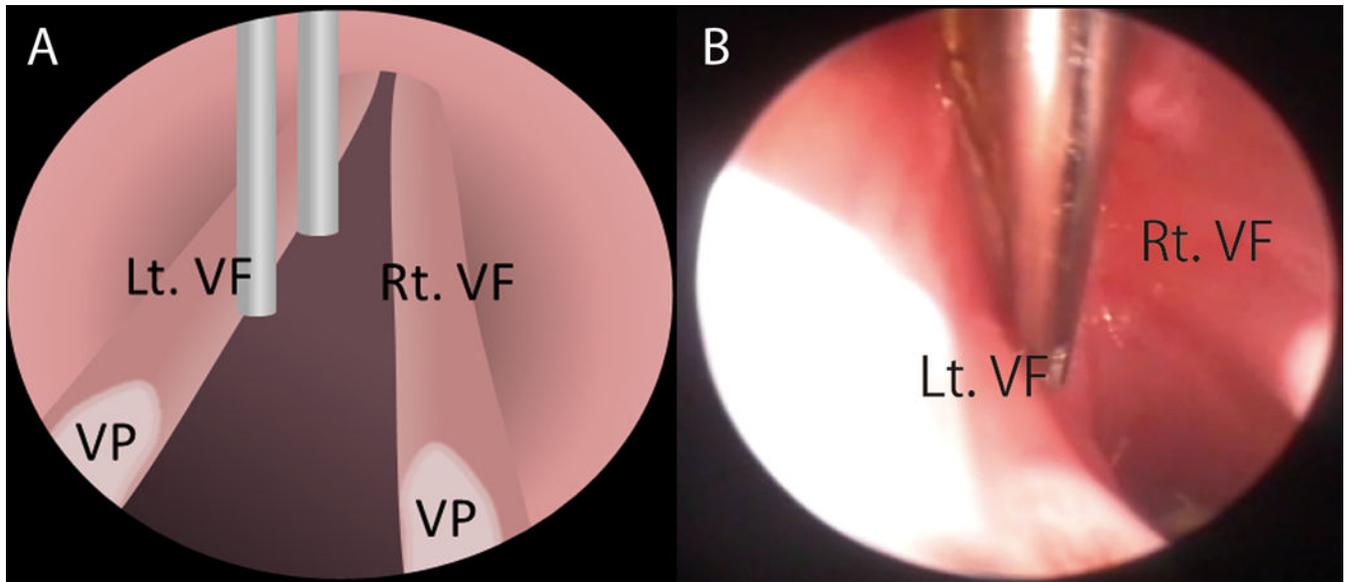
1. Swanson ER, Abdollahian D, Ohno T, Ge P, Zelear DL, Rousseau B. Characterization of raised phonation in an evoked rabbit phonation model. *The Laryngoscope*. 2009; 119(7):1439–1443. DOI: 10.1002/lary.20532 [PubMed: 19422027]
2. Kojima T, Valenzuela CV, Novaleski CK, et al. Effects of phonation time and magnitude dose on vocal fold epithelial genes, barrier integrity, and function. *The Laryngoscope*. 2014; 124(12):2770–2778. DOI: 10.1002/lary.24827 [PubMed: 25073715]
3. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol*. 2001; 2(4):285–293. DOI: 10.1038/35067088 [PubMed: 11283726]
4. Suzuki T. Regulation of intestinal epithelial permeability by tight junctions. *Cell Mol Life Sci*. 2013; 70(4):631–659. DOI: 10.1007/s00018-012-1070-x [PubMed: 22782113]
5. Rousseau B, Suehiro A, Echemendia N, Sivasankar M. Raised intensity phonation compromises vocal fold epithelial barrier integrity. *The Laryngoscope*. 2011; 121(2):346–351. DOI: 10.1002/lary.21364 [PubMed: 21271586]
6. Franchi A, Brogelli B, Massi D, Santucci M, Campora ED, Gallo O. Dilation of intercellular spaces is associated with laryngo-pharyngeal reflux: an ultrastructural morphometric analysis of laryngeal epithelium. *Eur Arch Otorhinolaryngol*. 2007; 264(8):907–911. DOI: 10.1007/s00405-007-0295-z [PubMed: 17436007]
7. Levendoski EE, Leydon C, Thibeault SL. Vocal Fold Epithelial Barrier in Health and Injury A Research Review. *J Speech Lang Hear Res JSLHR*. 2014; 57(5):1679–1691. DOI: 10.1044/2014\_JSLHR-S-13-0283 [PubMed: 24686981]

8. Kojima T, Van Deusen M, Jerome WG, et al. Quantification of Acute Vocal Fold Epithelial Surface Damage with Increasing Time and Magnitude Doses of Vibration Exposure. *PLoS ONE*. 2014; 9(3):e91615.doi: 10.1371/journal.pone.0091615 [PubMed: 24626217]
9. Srinivasan B, Kolli AR, Esch M, Abaci HE, Shuler ML, Hickman JJ. TEER Measurement Techniques for In Vitro Barrier Model Systems. *J Lab Autom*. 2015; 20(2):107–126. DOI: 10.1177/2211068214561025 [PubMed: 25586998]
10. Farré R, Blondeau K, Clement D, et al. Evaluation of oesophageal mucosa integrity by the intraluminal impedance technique. *Gut*. 2011; 60(7):885–892. DOI: 10.1136/gut.2010.233049 [PubMed: 21303918]
11. Alper R, Fu X, Erickson-Levendoski E, Zheng W, Sivasankar M. Acute stress to excised vocal fold epithelium from reactive oxygen species. *The Laryngoscope*. 2011; 121(10):2180–2184. DOI: 10.1002/lary.22157 [PubMed: 21898441]
12. 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(1):79–84. DOI: 10.1016/j.otohns.2009.09.011 [PubMed: 20096227]
13. Erickson E, Sivasankar M. Simulated Reflux Decreases Vocal Fold Epithelial Barrier Resistance. *The Laryngoscope*. 2010; 120(8):1569–1575. DOI: 10.1002/lary.20983 [PubMed: 20564752]
14. Saritas Yuksel E, Higginbotham T, Slaughter JC, et al. Use of Direct, Endoscopic-Guided Measurements of Mucosal Impedance in Diagnosis of Gastroesophageal Reflux Disease. *Clin Gastroenterol Hepatol*. 2012; 10(10):1110–1116. DOI: 10.1016/j.cgh.2012.05.018 [PubMed: 22642956]
15. Ge PJ, French LC, Ohno T, Zeale DL, Rousseau B. Model of Evoked Rabbit Phonation. *Ann Otol Rhinol Laryngol*. 2009; 118(1):51–55. DOI: 10.1177/000348940911800109 [PubMed: 19244964]
16. Jerome WG, Lewis JC, Taylor RG, White MS. Concurrent endothelial cell turnover and leukocyte margination in early atherosclerosis. *Scan Electron Microsc*. 1983; (Pt 3):1453–1459. [PubMed: 6648351]
17. Swanson ER, Ohno T, Abdollahian D, Rousseau B. Effects of raised-intensity phonation on inflammatory mediator gene expression in normal rabbit vocal fold. *Otolaryngol – Head Neck Surg*. 2010; 143(4):567–572. DOI: 10.1016/j.otohns.2010.04.264 [PubMed: 20879195]
18. Novalski CK, Kimball EE, Mizuta M, Rousseau B. Acute exposure to vibration is an apoptosis-inducing stimulus in the vocal fold epithelium. *Tissue Cell*. 2016; 48(5):407–416. DOI: 10.1016/j.tice.2016.08.007 [PubMed: 27577014]
19. Jeffery PK, Li D. Airway mucosa: secretory cells, mucus and mucin genes. *Eur Respir J*. 1997; 10(7):1655–1662. [PubMed: 9230262]
20. Fisher KV, Telser A, Phillips JE, Yeates DB. Regulation of vocal fold transepithelial water fluxes. *J Appl Physiol*. 2001; 91(3):1401–1411. [PubMed: 11509542]
21. Leydon C, Sivasankar M, Falciglia DL, Atkins C, Fisher KV. Vocal Fold Surface Hydration: A Review. *J Voice*. 2009; 23(6):658–665. DOI: 10.1016/j.jvoice.2008.03.010 [PubMed: 19114440]
22. Verdolini K, Branski RC, Rosen CA, Hebda PA. Shifts in Biochemical Markers Associated with Wound Healing in Laryngeal Secretions following Phonotrauma: A Preliminary Study. *Ann Otol Rhinol Laryngol*. 2003; 112(12):1021–1025. DOI: 10.1177/000348940311201205 [PubMed: 14703104]
23. Casiano RR, Zaveri V, Lundy DS. Efficacy of Videostroboscopy in the Diagnosis of Voice Disorders. *Otolaryngol Neck Surg*. 1992; 107(1):95–100. DOI: 10.1177/019459989210700115
24. Colton RH, Conture EG. Problems and pitfalls of electroglottography. *J Voice*. 1990; 4(1):10–24. DOI: 10.1016/S0892-1997(05)80077-3
25. Golla, ME., Moukalled, HJ., Orlikoff, RF., Deliyski, DD. Models and Analysis of Vocal Emissions for Biomedical Applications: 6th International Workshop: December 14–16, 2009. Firenze, Italy: Firenze University Press; 2009. Objective Comparison of the Electroglottogram to Synchronous High-Speed Images of Vocal Fold Contact During Vibration; p. 1000-1004.
26. Sivasankar M, Fisher KV. Vocal Folds Detect Ionic Perturbations on the Luminal Surface: An In Vitro Investigation. *J Voice*. 2008; 22(4):408–419. DOI: 10.1016/j.jvoice.2006.11.005 [PubMed: 17280815]

27. Liu X, Zheng W, Sivasankar MP. Acute Acrolein Exposure Induces Impairment of Vocal Fold Epithelial Barrier Function. PLOS ONE. 2016; 11(9):e0163237.doi: 10.1371/journal.pone.0163237 [PubMed: 27643990]
28. Hsiao T-Y, Liu C-M, Lin K-N. Videostrobolaryngoscopy of Mucus Layer during Vocal Fold Vibration in Patients with Laryngeal Tension-Fatigue Syndrome. Ann Otol Rhinol Laryngol. 2002; 111(6):537–541. DOI: 10.1177/000348940211100610 [PubMed: 12090710]
29. Deliyski DD, Petrushev PP, Bonilha HS, Gerlach TT, Martin-Harris B, Hillman RE. Clinical Implementation of Laryngeal High-Speed Videoendoscopy: Challenges and Evolution. Folia Phoniatr Logop. 2007; 60(1):33–44. DOI: 10.1159/000111802 [PubMed: 18057909]
30. Rousseau B, Kojima T, Novaleski CK, Kimball EE, Valenzuela CV, Mizuta M, Daniero JJ, Garrett CG, Sivasankar MP. Recovery of vocal fold epithelium after acute phonotrauma. Cells Tissues Organs. in press.



**Figure 1.** Custom-designed probe for measurement of mucosal resistance in *vivo*. The probe leads were 0.61 mm diameter stainless steel rods with a center-to-center separation distance of 1 mm. The leads were kept at a fixed separation distance by spacers and the probe was equipped with an instrument handle for laryngomicrosurgery.

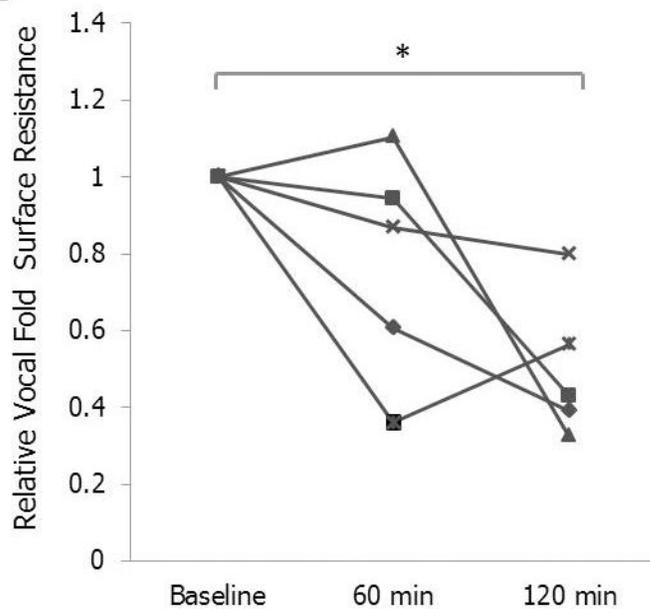


**Figure 2.** Demonstration of the measurement of mucosal resistance using the custom-designed probe in a rabbit larynx. A) Schematic of probe placement through the working channel of a side-slotted pediatric laryngoscope. B) Placement of the custom-designed probe leads on the middle 1/3 of the left vocal fold. The probe is shown in profile, with both leads placed upon the left vocal fold.

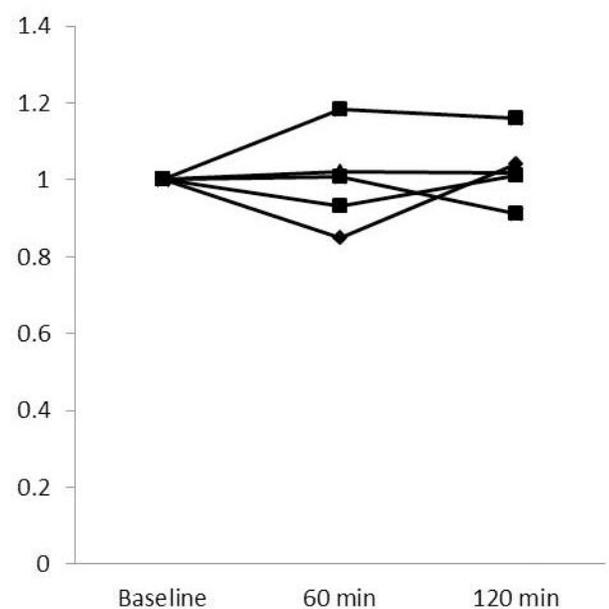
A

	Vocal Fold Surface Resistance (k $\Omega$ )			Relative Vocal Fold Surface Resistance		
	Baseline	60 mins	120 mins	Baseline	60 mins	120 mins
Raised Intensity Phonation	105.13	63.88	41.08	1.00	0.61	0.39
	54.85	51.74	23.70	1.00	0.94	0.43
	191.46	211.55	62.02	1.00	1.10	0.32
	189.12	164.38	151.27	1.00	0.87	0.80
	159.30	57.46	89.67	1.00	0.36	0.56
Control	54.08	45.85	56.25	1.00	0.85	1.04
	78.01	72.80	78.90	1.00	0.93	1.01
	140.86	143.98	143.55	1.00	1.02	1.02
	181.73	183.19	165.64	1.00	1.01	0.91
	130.09	154.01	151.06	1.00	1.18	1.16

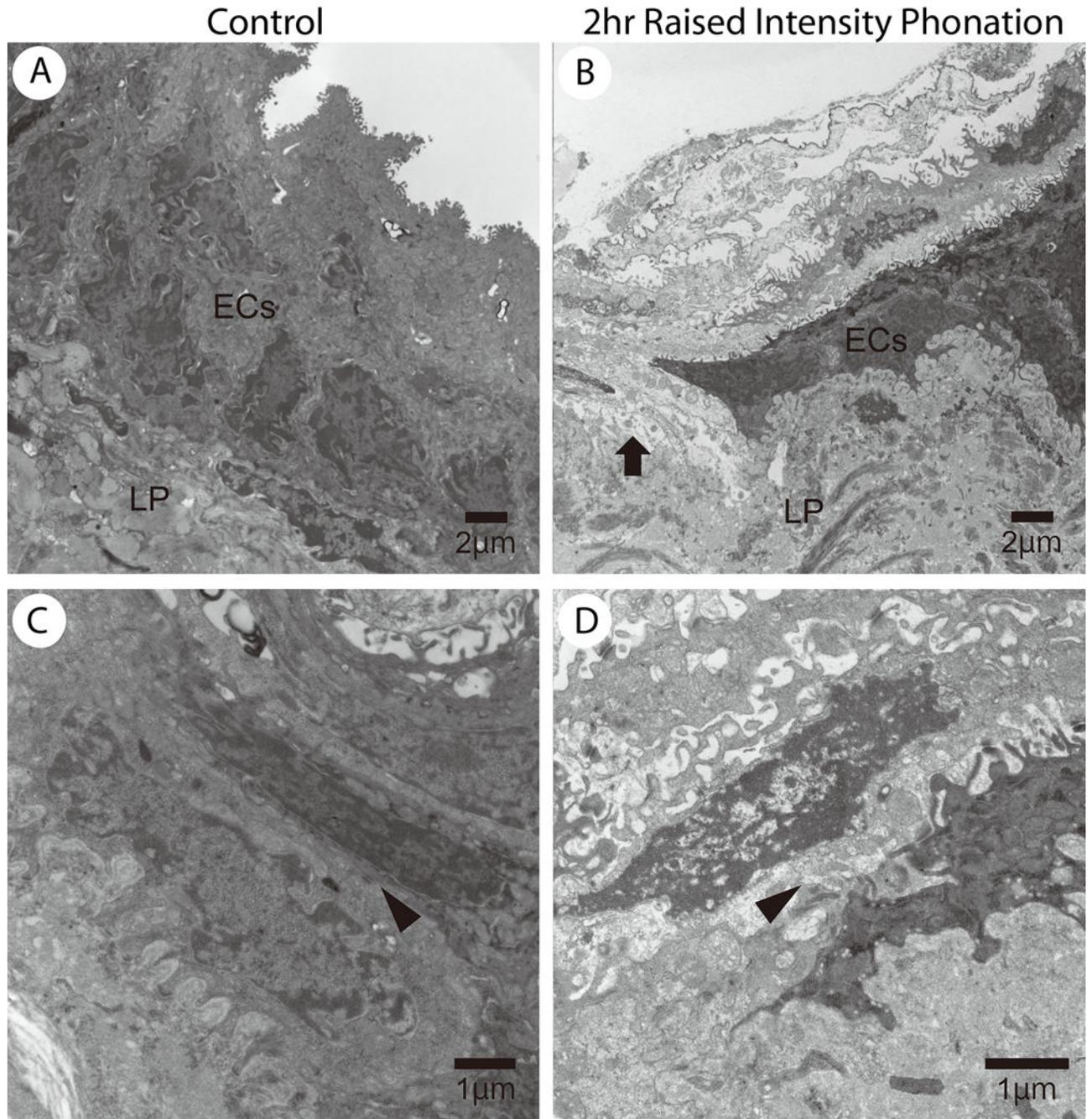
B



C

**Figure 3.**

Non-normalized (k $\Omega$ ) and relative vocal fold surface resistance (A). Relative vocal fold surface resistance after 0 (baseline), 60, and 120 minutes of experimentally induced raised intensity phonation (B) and control condition (C). Resistance was significantly decreased after 120 minutes of raised-intensity phonation ( $p = 0.012$ ). There were no significant differences in mucosal resistance across time in the control condition ( $p = 0.793$ ).



**Figure 4.** Transmission electron microscopy of the (A, C) control vocal fold and (B, D) Vocal fold following 2 hours of raised intensity phonation, revealing extensive desquamation and disruption of epithelial cells in the experimental vocal fold (B) compared to the control condition (A). The vocal fold epithelium in the (C) control condition showed well-preserved stratified squamous epithelia and paracellular spaces. Consistent with previous studies from our group, there was also evidence of basement membrane zone disruption and dilation of paracellular spaces in vocal folds exposed to (D) 2 hours of raised intensity phonation. The

lighter staining identified in TEM images of vocal folds after 2 hours of raised intensity phonation reveals apoptotic cells. ECs: Epithelial cells, LP: Lamina propria, Arrow: Disrupted basement membrane, Arrow head: paracellular space.

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