Recovery of Vocal Fold Epithelium after Acute Phonotrauma

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Electron microscopy · Epithelial barrier · Gene expression · Phonotrauma · Vocal fold

Abstract

We investigated the timeline of tissue repair of vocal fold epithelium after acute vibration exposure using an in vivo rabbit model. Sixty-five New Zealand white breeder rabbits were randomized to 120 min of modal- or raised-intensity phonation. After the larynges were harvested at 0, 4, 8, and 24 h, and at 3 and 7 days, the vocal fold tissue was evaluated using electron microscopy and quantitative real-time polymerase chain reaction. There was an immediate decrease in the microprojection depth and height following raised-intensity phonation, paired with upregulation of cyclooxygenase-2. This initial 24-h period was also characterized by the significant downregulation of junction proteins. Interleukin 1 β and transforming growth factor β 1 were upregulated for 3 and 7 days, respectively, followed by an increase in epithelial cell surface depth at 3 and 7 days. These data appear to demonstrate a shift from inflammatory response to the initiation of a restorative process in the vocal fold epithelium between 24 h and 3 days. Despite the initial damage from raised-intensity phonation, the vocal fold epithelium dem-

onstrates a remarkable capacity for the expeditious recovery of structural changes from transient episodes of acute phonotrauma. While structurally intact, the return of functional barrier integrity may be delayed by repeated episodes of phonotrauma and may also play an important role in the pathophysiology of vocal fold lesions. © 2017 S. Karger AG, Basel

Introduction

The vocal folds are a multilayered connective tissue structure comprised of stratified squamous epithelium, lamina propria, and muscle [Hirano, 1974]. The vocal fold epithelium forms the outermost layer of the vocal

Abbreviations used in this paper

COX-2	cyclooxygenase-2
IL-1 β	interleukin 1 β
qRT-PCR	quantitative real-time polymerase chain reaction
SDHA	succinate dehydrogenase complex subunit A
TEM	transmission electron microscopy
TGF- β 1	transforming growth factor β 1
ZO-1	zonula occludens-1

fold and acts as a physical barrier to various threats from the external environment. The impact, stretching, and shearing forces that occur during vocal fold vibration comprise a major category of physical threats to the epithelial barrier [Titze, 1994]. These repeated cycles of oscillatory collision produce biomechanical tissue stresses of differing magnitudes, directions, and distributions. In a previous study, we found evidence of epithelial desquamation, microhole formation, and the obliteration of surface microprojections after transient episodes of raised-intensity phonation in an in vivo rabbit model [Rousseau et al., 2011]. These findings supported the notion of compromised structural integrity of the vocal fold epithelial barrier after acute phonotrauma. In subsequent experiments, we incorporated the use of objective imaging measures to quantify phonation-related structural damage to the vocal fold epithelial surface. These experiments revealed significant decreases in microprojection density, microprojection height, and the depth of the remaining cell surface layer, along with the increasing duration and magnitude of the doses of vibration exposure [Kojima et al., 2014a]. In some instances, damage to the epithelium extended down to the basal cell layer, exposing the microfilaments of the cytoskeleton and basement membrane anchoring fibers [Kojima et al., 2014a]. Given the susceptibility of the vocal folds to repeated cycles of oscillatory collision and strong shearing forces during vibration, we suspect that the tissue-reparative response of the epithelium is critically important in the maintenance of a functional epithelial barrier. However, the precise nature and timing of the epithelial-reparative response after phonotrauma remains unknown. Our overarching hypothesis is that the epithelium is responsive to the physical threats imposed by vocal fold vibration, and exhibits the capacity to repair itself after phonotrauma. We further hypothesize that inefficient or prolonged repair of the epithelium may expose the tissue to increased risk of further injury.

The purpose of this study was to investigate temporal changes in vocal fold gene expression and epithelial barrier repair after acute phonotrauma. Specifically, given the previous findings of compromised vocal fold barrier integrity after phonotrauma, we were interested in the timing of epithelial recovery following acute vibration exposure. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure gene transcript levels of the junctional complex proteins, occludin, zonula occludens-1 (ZO-1), and E-cadherin; the inflammatory and profibrotic mediators, transforming growth factor β 1 (TGF- β 1), interleukin 1 β (IL-1 β), and cyclooxygenase-2 (COX-2); and the extracellular matrix glycoprotein fibro-

Table 1. Experimental design

	0 h	4 h	8 h	24 h	3 days	7 days
Modal intensity, <i>n</i>	5	5	5	5	5	5
Raised intensity, <i>n</i>	5	5	5	5	5	5
Normal (control), <i>n</i>	5					
Total						65

nectin. Transmission electron microscopy (TEM) was used to examine the epithelial cell layer and quantify the microprojection density, microprojection height, and the depth of the remaining epithelial cell surface. All dependent measures were collected at 0, 4, 8, and 24 h, and at 3 and 7 days after modal- or raised-intensity phonation.

Materials and Methods

Animals

The procedures used in this study were in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, National Institutes of Health Guide for the Care and Use of Laboratory Animals, and Animal Welfare Act (7 U.S.C. et seq.). The animal use protocol was approved by the Vanderbilt University Institutional Animal Care and Use Committee. The study involved 65 adult male New Zealand white breeder rabbits weighing 2.8–3.8 kg. The animals were anesthetized by intramuscular administration of ketamine (35 mg/kg), xylazine (5 mg/kg), and acepromazine (0.75 mg/kg). To maintain a steady state of anesthesia, ketamine (17.5 mg/kg) and acepromazine (0.375 mg/kg) were administered as needed. Oxygen saturation level, temperature, and heart rate were continually monitored throughout the procedure to assess the safety and well-being of the animals.

A between-groups research design was used to measure vocal fold gene expression, microprojection density, microprojection height, and the depth of the epithelial cell surface at specific time points following exposure to doses of phonation of different magnitudes. Sixty animals were randomized to receive either 120 min of modal- or raised-intensity phonation, and 5 were assigned to a normal-control group (Table 1). The phonation protocol used was based on previous work conducted in our laboratory, with the raised-intensity phonation elicited using airflow rates of approximately 144 cm³/s and stimulation currents of 0.2–0.6 mA above modal-intensity phonation. After the phonation procedures, the larynges were harvested at 0, 4, 8, and 24 h, and at 3 and 7 days after each experiment, and then preserved for either electron microscopy or qRT-PCR. Normal vocal fold tissue was harvested from animals not undergoing phonation to serve as controls.

Phonation Procedures

Animals were placed in the supine position on an operating platform and shaved at the neck from the submentum down to the chest. As described previously, the neck was dissected at midline from the hyoid bone to the sternal notch to expose the larynx and

Fig. 1. TEM measurements. **a** Vocal fold microprojection density (Dn) and height (H). Bar, 500 nm. **b** Vocal fold epithelial depth (Dp). Bar, 2 μ m.

trachea. The trachea was transected proximal to the sternum [Ge et al., 2009; Swanson et al., 2009, 2010; Rousseau et al., 2011; Kojima et al., 2014a, b]. Sutures were placed to suspend the lower portion of the trachea to the sternal fascia and provide a stable airway through a tracheostomy. A 3.5-mm cuffed endotracheal tube (Mallinckrodt, Hennep, Germany) was inserted into the upper portion of the bisected trachea and placed 2 cm below the opening of the glottis. The cuff was then inflated to seal the trachea and deliver continuous airflow through the glottis.

Two pairs of custom stainless-steel hooked electrodes were prepared to deliver electrical stimulation to the laryngeal musculature. One electrode was inserted into the belly of each cricothyroid muscle, perpendicular to the muscle fibers (cathodes). A second pair of electrodes was inserted into the cricothyroid membrane on each side of the intersection of a longitudinal line 1 mm lateral to the midline and a transverse line 1 mm inferior to the thyroid cartilage (anodes). Compressed, humidified airflow at 37°C was delivered to the glottis using a flowmeter (GF-8522-1700; Gilmont Instruments, Barrington, IL, USA) and a Concha Therm Neptune humidifier (Hudson, RCI, Temecula, CA, USA). To induce electrical stimulation to the laryngeal apparatus, a Grass S-88 stimulator (SA Instrumentation, Encinitas, CA, USA) and a constant-current isolation unit (Grass Telefactor, model PSIU6; West Warwick, RI, USA) were used. The total duration was 10 s: 3 s of electrical stimulation and 7 s of rest. Phonation was elicited for 120 min. During the procedure, laryngeal structures were visualized endoscopically using a 30°, 2.7-mm, rigid endoscope and a Telecam-C camera (both from Karl Storz Endoscopy-America, Inc., El Segundo, CA, USA).

Acoustic measures were collected to determine mean vocal intensity and to confirm differences between the modal- and raised-intensity phonation groups. Acoustic signals were recorded at baseline and at 15-min intervals throughout the phonation procedure using a Perception 170 Condenser Microphone (AKG, Vienna, Austria) that was positioned 10 cm from the opening of the laryngoscope. Mouth-to-microphone distance and amplitude gain were held constant throughout the audio recordings. Recordings were digitized in Computerized Speech Lab Model 4500 (Kay-PENTAX, Montvale, NJ, USA) using the system's factory-calibrated settings. The most stable 0.5- to 1.0-s portion of the acoustic

waveform was selected and analyzed to confirm the dose magnitude (modal versus raised intensity).

Larynges were harvested at 0, 4, 8, and 24 h, and at 3 and 7 days after phonation. One vocal fold was soaked in 2.5% glutaraldehyde solution, stored at room temperature for 1 h, and then at 4° for TEM. The contralateral vocal fold was soaked and stored in RNALater (Life Technologies).

Objective Examination by TEM

Routine methods were used to process tissue for TEM evaluation as described previously [Jerome et al., 1983; Sivasankar et al., 2010]. Briefly, TEM images were acquired using a Philips CM-12 transmission electron microscope (FEI Co., Hillsboro, OR, USA). For each tissue specimen, 5 randomized regions were selected for the measurement of mean microprojection density, microprojection height, and the depth of the epithelial cell surface using ImageJ (Fig. 1a, b) [Schneider et al., 2012; Kojima et al., 2014a]. Microprojection density was quantified as the number of microprojections within a distance of 10 μ m. This was calculated by counting 5 consecutive microprojections and measuring the distance of these using an original magnification of $\times 15,000$ – $53,000$. A calculation was then performed in which 5 was divided by the distance of 5 consecutive microprojections and multiplied by 10. Microprojection height was measured as the entire length of the microprojection using an original magnification of $\times 4,400$ – $8,800$. The depth of the remaining cell layer was measured from the surface of the cell to the basal layer using an original magnification of $\times 4,400$ – $8,800$ (Fig. 1b).

Quantitative Real-Time Polymerase Chain Reaction

qRT-PCR was used to measure the gene expression of inflammatory mediators (TGF- β 1, IL-1 β , and COX-2), junctional complex proteins (occludin, ZO-1, and E-cadherin), and the extracellular matrix glycoprotein, fibronectin. The epithelium and lamina propria of the middle third of the RNALater-soaked vocal fold was used for this analysis. The vocalis muscle was excluded. Standard protocols for RNA extraction, reverse transcription, and qRT-PCR were used. Rabbit-specific primers for IL-1 β , COX-2, TGF- β 1, occludin, ZO-1, E-cadherin, fibronectin, and succinate dehydrogenase complex subunit A (SDHA) were synthesized by Sigma-

Table 2. Primer sequences

Occludin	forward	5'-GCT TCT GGA TCT ATG TAT GGC TCA C-3'
	reverse	5'-TCA TAG CGG TCC ATC TTT CTT CGA G-3'
ZO-1	forward	5'-CGT AAC ACC AAA TGC AGT AGA TCG TC-3'
	reverse	5'-CTG TTG CTG GAT TGC TTC CTT CAA C-3'
E-cadherin	forward	5'-AGG ACA CAG ACT ATG TCA AGA ACA G-3'
	reverse	5'-GGA TCG ATG ATG TTT ATG AGC TGA G-3'
IL-1 β	forward	5'-GAA TCT GAA CCA ACA AGT GG-3'
	reverse	5'-ATG TAC CAG TTG GGG AAC T-3'
COX-2	forward	5'-CAA ACT GCT CCT GAA ACC CAC TC-3'
	reverse	5'-GCT ATT GAC GAT GTT CCA GAC TCC-3'
TGF- β 1	forward	5'-CGG CAG CTG TAC ATT GAC TT-3'
	reverse	5'-AGC GCA CGA TCA TGT TGG AC-3'
Fibronectin	forward	5'-CTC ACC CGA GGC GCC ACC TA-3'
	reverse	5'-TCG CTC CCA CTC CTC TCC AAC G-3'
SDHA	forward	5'-CAT TTA GCA GAA CTG AAG AC-3'
	reverse	5'-GGT ATC ATA TCG CAG AGA C-3'

ZO-1, zonula occludens-1; IL-1 β , interleukin 1 β ; COX-2, cyclooxygenase-2; TGF- β 1, transforming growth factor β 1; SDHA, succinate dehydrogenase complex subunit A.

Aldrich Corp. (St. Louis, MO, USA). Specific primer sequences are displayed in Table 2. SDHA served as the control gene. Standard curves were used to determine the relative ratio of gene expression. Target gene ratios from all groups were normalized using the expression ratios of the internal control gene.

Statistical Analysis

Data were analyzed using IBM SPSS 23.0 (IBM Corp., Armonk, NY, USA). For each dependent variable, a series of Kruskal-Wallis tests were performed to investigate the overall main effect of time and phonation magnitude dose. If the overall main effect was significant, a series of post hoc pairwise comparisons were performed using the Mann-Whitney U test for 2 independent samples. All α levels were set at 0.05 using two-tailed p values.

Results

Acoustic Analysis

Mean vocal intensity was 59.50 dB (SD 1.98) during modal-intensity phonation and 66.97 dB (SD 2.95) during raised-intensity phonation. As expected, an independent-samples t test confirmed significantly increased mean vocal intensity during raised-intensity phonation compared to during modal-intensity phonation ($t(81) = -13.57, p = 0.000$).

Transmission Electron Microscopy

Objective Examination of Epithelial Surface Microprojection Height

The results revealed a significant main effect of time ($\chi^2(6) = 16.43, p = 0.012$) and phonation magnitude dose ($\chi^2(2) = 6.85, p = 0.033$) on the vocal fold epithelial surface microprojection height (Figure 2a). Post hoc pairwise comparisons revealed a significantly decreased microprojection height after raised-intensity phonation compared to the control at 0 ($U = 1.00, p = 0.015$), 8 ($U = 2.00, p = 0.028$), and 24 h ($U = 3.00, p = 0.047$).

Objective Examination of Epithelial Surface Microprojection Density

The results revealed a significant main effect of phonation magnitude dose on vocal fold epithelial surface microprojection density ($\chi^2(2) = 10.57, p = 0.005$; Fig. 2b). Post hoc pairwise comparisons revealed that microprojection density was significantly decreased after raised-intensity phonation compared to modal-intensity phonation and the control at 0 (modal intensity: $U = 3.00, p = 0.034$; control: $U = 0.00, p = 0.007$) and 8 h (modal intensity: $U = 3.00, p = 0.047$; control: $U = 2.00, p = 0.028$). At 24 h, the microprojection density was significantly decreased after raised-intensity phonation compared to the control ($U = 2.00, p = 0.028$).

Objective Examination of the Depth of the Remaining Cell Surface

Results revealed a significant main effect of time on the depth of the remaining vocal fold epithelial cell surface ($\chi^2(6) = 26.39, p = 0.000$; Fig. 2c). Post hoc pairwise comparisons revealed that, compared to the control, epithelial depth was significantly increased after modal- and raised-intensity phonation at 3 (modal intensity: $U = 3.00, p = 0.047$; raised intensity: $U = 0.00, p = 0.009$) and 7 days (modal intensity: $3.00, p = 0.047$; raised intensity: $U = 3.00, p = 0.047$).

Quantitative Real-Time Polymerase Chain Reaction Gene Expression of Inflammatory Mediators

IL-1 β . The results revealed a significant main effect of time ($\chi^2(6) = 48.76, p = 0.000$) and phonation magnitude dose ($\chi^2(6) = 10.17, p = 0.006$) on IL-1 β gene transcript levels (Fig. 3a). Post hoc pairwise comparisons revealed that IL-1 β gene expression was significantly upregulated after modal- ($U = 0.00, p = 0.009$) and raised-intensity ($U = 12.00, p = 0.003$) phonation compared to the control at 0, 4, 8, and 24 h, and 3 days.

Fig. 2. Microprojection height (**a**) and density (**b**), and epithelial depth (**c**) in control specimens (circles) 0, 4, 8, and 24 h, and 3 and 7 days (d) after modal- (diamonds) and raised-intensity (triangles) phonation. Horizontal bars display the mean for each condition. * $p < 0.05$ compared to control condition. † $p < 0.05$ compared to corresponding modal-intensity phonation time point.

COX-2. The results revealed a significant main effect of time on COX-2 gene transcript levels ($\chi^2(6) = 45.08$, $p = 0.000$; Fig. 3b). Post hoc pairwise comparisons revealed that COX-2 gene expression was significantly up-

regulated after modal- and raised-intensity phonation compared to the control at 0 (modal intensity: $U = 0.00$, $p = 0.009$; raised intensity: $U = 3.00$, $p = 0.047$), 4 ($U = 0.00$, $p = 0.009$), 8 ($U = 0.00$, $p = 0.009$), and 24 h (modal

Fig. 3. log-transformed mRNA expression ratios for IL-1 β (a), COX-2 (b), and TGF- β 1 (c) in control specimens (circles) 0, 4, 8, and 24 h, and 3 and 7 days (d) after modal- (diamonds) and raised-intensity (triangles) phonation. Horizontal bars display the mean for each condition. * $p < 0.05$ compared to control condition.

intensity: $U = 1.00$, $p = 0.016$; raised intensity: $U = 0.00$, $p = 0.009$).

TGF- β 1. The results revealed a significant main effect of time ($\chi^2(6) = 32.72$, $p = 0.000$) and phonation magnitude dose ($\chi^2(6) = 6.22$, $p = 0.045$) on TGF- β 1 gene tran-

script levels (Fig. 3c). Post hoc pairwise comparisons revealed that TGF- β 1 gene expression was significantly up-regulated after modal- and raised-intensity phonation compared to the control at 4 and 8 h ($U = 0.00$, $p = 0.009$). TGF- β 1 gene expression was significantly upregulated af-

ter raised-intensity phonation compared to the control at 24 h ($U = 0.00$, $p = 0.009$), and at 3 ($U = 0.00$, $p = 0.009$) and 7 days ($U = 2.00$, $p = 0.028$).

Gene Expression of Tight and Adherens Junction Proteins

Occludin. The results revealed a significant main effect of time ($\chi^2(6) = 24.35$, $p = 0.000$) and phonation magnitude dose ($\chi^2(6) = 7.30$, $p = 0.026$) on occludin gene transcript levels (Fig. 4a). Post hoc pairwise comparisons revealed that occludin gene expression was significantly downregulated after raised-intensity phonation compared to modal-intensity phonation and the control at 0 h ($U = 0.00$, $p = 0.009$). Occludin gene expression was significantly downregulated after modal- and raised-intensity phonation compared to the control at 4 h ($U = 2.00$, $p = 0.028$), 8 h ($U = 0.00$, $p = 0.009$), and 24 h (modal intensity: $U = 1.00$, $p = 0.016$; raised intensity: $U = 0.00$, $p = 0.008$). Occludin gene expression was significantly downregulated after modal-intensity phonation compared to the control at 3 days ($U = 1.00$, $p = 0.016$).

ZO-1. The results revealed a significant main effect of time on ZO-1 gene transcript levels ($\chi^2(6) = 28.39$, $p = 0.000$; Fig. 4b). Post hoc pairwise comparisons revealed that ZO-1 gene expression was significantly downregulated after modal- ($U = 3.00$, $p = 0.047$) and raised-intensity ($U = 0.00$, $p = 0.009$) phonation compared to the control at 24 h and 3 days.

E-Cadherin. The results revealed a significant main effect of time ($\chi^2(6) = 18.23$, $p = 0.006$) and phonation magnitude dose ($\chi^2(6) = 6.87$), $p = 0.032$) on E-cadherin gene transcript levels (Fig. 4c). Post hoc pairwise comparisons revealed that E-cadherin gene expression was significantly downregulated after modal- and raised-intensity phonation compared to the control at 8 ($U = 0.00$, $p = 0.009$) and 24 h (raised: $U = 0.00$, $p = 0.009$; modal: $U = 1.00$, $p = 0.016$). E-cadherin gene expression was significantly downregulated after raised-intensity phonation compared to the control at 0 h ($U = 0.00$, $p = 0.009$) and 7 days ($U = 3.00$, $p = 0.047$).

Gene Expression of Fibronectin

The results revealed a significant main effect of time ($\chi^2(6) = 35.50$, $p = 0.000$) and phonation magnitude dose ($\chi^2(6) = 6.56$, $p = 0.038$) on fibronectin gene transcript levels (Fig. 5). Post hoc pairwise comparisons revealed that fibronectin gene expression was significantly upregulated after modal- and raised-intensity phonation ($U = 0.00$, $p = 0.009$) compared to the control at 4 and 8 h. Fibronectin gene expression was significantly upregulated

after modal-intensity phonation compared to the control at 3 days ($U = 2.00$, $p = 0.028$). Fibronectin gene expression was significantly upregulated after raised-intensity phonation compared to the control at 24 h ($U = 0.00$, $p = 0.009$) and 7 days ($U = 3.00$, $p = 0.047$).

Discussion

Epithelial responses to injury have been studied in various models including the intestine, skin, and cornea. However, similarities between these tissues and the vocal folds in the response to injury must be interpreted with caution, as the vocal folds represent a unique structure in the body that undergoes repeated biomechanical trauma during phonation. Unlike other stratified squamous epithelia, the vocal fold epithelium may be especially well-equipped to withstand these repeated tissue stresses, and its tolerance to these may be critical to the maintenance and protection of the vocal folds from injury. While the epidermal response to injury has been well-studied, less is known about the reparative response of the vocal fold epithelium to repeated cycles of oscillatory collision and shearing forces; we suspect that this response is critically important in the maintenance of a functional epithelial barrier. Epidermal wound-healing has been characterized into the following 4 phases, i.e., the inflammatory, migratory, proliferative, and maturation phases [Tortora et al., 2008]. During the inflammatory phase, a clot is formed, and inflammatory cells including neutrophils, monocytes, and fibroblasts infiltrate the tissue with the assistance of dilated vessels. During the migratory phase, the surrounding epithelial cells migrate to cover the injured surface, and fibroblasts begin to deposit a disorganized but robust collagen matrix. During the proliferative phase, there is division of epithelial cells to replace the damaged stratified and differentiated epithelial cell layers, and the continued deposition of collagen by fibroblasts, creating a scar. This is accompanied by an increase in vascularity to provide nutrients to the rapidly dividing cells. During the maturation phase, there is decreased vascularization and continued remodeling of the disorganized collagen matrix. While there is great variation in the time sequelae, depending on the tissue type and other factors, the general progression through these phases is relatively comparable across tissues [Mutsaers et al., 1997].

While the various phases of the vocal fold wound-healing response after iatrogenic injury have been well documented, the specific timing and recovery of structural changes after phonotrauma remains unknown [Branski

Fig. 4. log-transformed mRNA expression ratios for occludin (**a**), ZO-1 (**b**), and E-cadherin (**c**) in control specimens (circles) 0, 4, 8, and 24 h, and 3 and 7 days (d) after modal- (diamonds) and raised-intensity (triangles) phonation. Horizontal bars display the mean for each condition. * $p < 0.05$ compared to control condition. † $p < 0.05$ compared to corresponding modal-intensity phonation time point.

et al., 2005; Ling et al., 2010]. In this study, we investigated the temporal repair and regeneration of the vocal fold epithelial surface after acute episodes of modal- and raised-intensity phonation. A primary goal of the study was to determine the timing of recovery of the physical

structures (epithelial microprojection height and density and epithelial cell layer depth) over time after exposure to modal- or raised-intensity phonation [Kojima et al., 2014a]. We also evaluated gene transcript levels during the recovery period. Data were collected at 0, 4, 8, and

Fig. 5. log-transformed mRNA expression ratios for fibronectin in control specimens (circles) 0, 4, 8, and 24 h, and 3 and 7 (d) days after modal- (diamonds) and raised-intensity (triangles) phonation. Horizontal bars display the mean for each condition. * $p < 0.05$ compared to control condition.

24 h, and at 3 and 7 days after exposure to a 120-min-long dose of modal- or raised-intensity phonation using an in vivo rabbit phonation model.

Our results revealed a significant decrease in the height and density of the epithelial microprojections immediately after injury and for up to 24 h (at 0, 8, and 24 h) after exposure to a 120-min-long dose of raised-intensity phonation compared to the control condition. No significant structural changes were observed during the first 24 h for modal-intensity phonation, compared to the control. These results are consistent with previous findings by our group, and Kojima et al. [2014a] reported significantly decreased height and density of vocal fold epithelial microprojections 30 min after exposure to a 120-min-long dose of raised-intensity phonation. Our study findings on the raised-intensity phonation condition revealed incomplete recovery of the vocal fold epithelial surface microprojections during the first 24 h, with complete regeneration of an epithelial surface with a microprojection height and density similar to the control by 3 days [Kojima et al., 2014a].

Despite the complete regeneration of epithelial surface structures at 3 days, TEM revealed marked hypertrophy of the epithelial cell layer, as measured by a significant increase in the depth of this layer at 3 and 7 days, following 120 min of modal- and raised-intensity phonation, compared to the control. Our findings are consistent with reports of extensive cellular proliferation and epithelial hypertrophy during the wound-healing of the vocal fold epithelium following iatrogenic injury [Branski et al., 2005; Leydon et al., 2014]. Specifically, Branski et al., [2005]

noted considerable cellular proliferation at the wound site 3 days after injury in the healing rabbit vocal fold, followed by marked epithelial hypertrophy at 5 days. Similarly, Leydon et al. [2014] reported a peak in epithelial cell proliferation at 3 days, with continued elevation and epithelial proliferation at 5 days after injury in the healing rat vocal fold; in addition, a thin epithelium consistent with uninjured rat vocal fold was not evident until 14 days after injury.

Our results suggest that while rapid regeneration of the epithelial surface microprojection structure can be achieved in as few as 3 days following phonotraumatic injury, cell proliferation, stratification, and differentiation are evident for at least 7 days after phonotrauma. So although the epithelial surface appeared to be repaired by day 3, the thin epithelial phenotype consistent with normal epithelia was not observed, suggesting that continued remodeling of the epithelium beyond 7 days was likely.

The 24-h period immediately following phonotrauma was characterized by the downregulation of occludin and E-cadherin gene expression. E-cadherin was significantly downregulated after exposure to a 120-min-long dose of modal- and raised-intensity phonation compared to the control at 8 and 24 h. Occludin gene expression was significantly downregulated after exposure to a 120-min-long dose of modal- and raised-intensity phonation compared to the control for up to 24 h (at 4, 8, and 24 h). Despite between-subject variability, our results revealed an overall pattern of downregulation of both occludin and E-cadherin throughout the first 24 h to 3 days after phonotrauma. Downregulation of E-cadherin has been cor-

related with epithelial-mesenchymal transition in airway epithelia and, in this case, epithelial-mesenchymal transition may have been contributing to the restitution of the epithelial surface [Câmara and Jarai, 2010]. During epithelial-mesenchymal transition, epithelial cells take on the characteristics of mesenchymal cells, a process induced by the loss of E-cadherin and the activation of inflammatory cytokines such as TNF- α [Yan et al., 2010].

ZO-1 gene expression was significantly downregulated after exposure to a 120-min-long dose of modal- and raised-intensity phonation compared to the control at 24 h. ZO-1 is often associated with cell-to-cell junctions and barrier integrity, but unlike occludin and E-cadherin, it is also involved in intercellular signaling [González-Mariscal et al., 2000]. Our findings appear to suggest that cell-to-cell communication pathways may initially be spared, and ZO-1 is then subsequently downregulated as the migratory phase and proliferative phases ensue and inflammation subsides. The downregulation of junctional complex protein expression has been reported in epithelial injury models [Costantini et al., 2009; Shen et al., 2013]. In this study, these expression levels returned to control levels between 3–7 days following phonotrauma. The time window of 24 h to 3 days seems to be an important period in the transition and recovery of the vocal fold epithelial surface. These findings are consistent with previous experiments in our laboratory, showing a return of vibratory function and the attenuation of the inflammatory process by day 3, with evidence of active tissue remodeling at 3–7 days after microflap incision [Kojima et al., 2014c; Mitchell et al., 2014].

Modal- and raised-intensity phonation were characterized by significantly upregulated gene transcript levels of COX-2 and IL-1 β immediately after injury, with peak expression at 8 h. COX-2 gene expression was significantly upregulated for 24 h (at 0, 4, 8, and 24 h), while IL-1 β gene expression was significantly upregulated for up to 3 days, compared to the control. The expression of IL-1 β immediately after phonotrauma supports the notion of early cellular infiltration, and is consistent with the potential presence of inflammatory cells in the tissue because IL-1 β is produced primarily by macrophages. The continued upregulation of IL-1 β for 3 days coincides with the inflammatory phase of repair and suggests active recruitment of immune cells for up to 3 days during recovery from acute phonotrauma. Wilson et al. [2001] describe the corneal wound-healing pathway, in which IL-1 β plays a major role. IL-1 β has negative chemotactic effects on mesenchymal cells and mediates the deposition of fibrous tissue components early in the wound-healing

process [Wilson et al., 2001]. Sturm and Dignass [2008] suggest that IL-1 enhances epithelial restitution by increasing the concentration of bioactive TGF- β . Beck et al. [2003] found that, compared to a control group with intact TGF- β signaling pathways in which a near-complete recovery of the intestinal epithelium was achieved, the injured area in the pathway-inhibited group failed to completely reepithelialize during the time period studied (10 days after injury), thus supporting the role of TGF- β in the regulation of epithelial cell proliferation and repair.

During acute injury, cells receive chemotactic cues from cytokines, such as IL-1 β and TGF- β , to stimulate neomatrix formation. A number of cells are involved in the secretion of TGF- β 1, including epithelial cells, fibroblasts, macrophages, and platelets. During wound-healing, TGF- β 1 plays an important role in the recruitment of inflammatory cells and fibroblasts to the site of injury to orchestrate tissue repair [Liu et al., 2006]. TGF- β 1 and fibronectin play an important role in the preservation of cellular integrity and tissue function, including cell adhesion, growth, migration, and differentiation. TGF- β 1 enhances the synthesis and deposition of fibronectin, and these 2 proteins are well-linked in the epithelial wound-healing response [Jester et al., 1999]. Increased fibronectin expression has been reported in corneal epithelial wound-healing models at the site of injury [Suda et al., 1981]. Our study revealed the upregulation and downregulation of fibronectin and TGF- β 1 to be tightly correlated during the 7 days following injury. Fibronectin gene expression peaked at 8 h after exposure to the 120-min-long doses of modal- and raised-intensity phonation, and remained elevated at 3 (modal) and 7 (raised) days, compared to the control. TGF- β 1 gene expression peaked at 8 h and had returned to control levels by 24 h after modal-intensity phonation. In contrast, TGF- β 1 gene expression peaked at 24 h and remained elevated for up to 7 days (at 24 h, 3 days, and 7 days) after exposure to a 120-min-long dose of raised-intensity phonation, compared to the control.

Our results support the role of COX-2 in the early orchestration of the inflammatory response after phonotrauma. COX-2 is an important precursor to many prostaglandins, specifically prostacyclin, which is a vasodilator and inhibits platelet sticking. The period between 24 h, when COX-2 (a specific marker for stimuli-induced cell activation and inflammation) is upregulated, and 3 days, when COX-2 signaling returns to baseline, supports the idea of a critical period during the inflammatory and proliferative response of the vocal fold after injury – a transition from epithelial restitution orchestrated by innate cells to a proliferative phase in which new cells are

recruited to regain a normal macrostructure. In the context of phonotraumatic injury, we postulate that the disruption of the epithelial barrier and junctional complex proteins provides an important stimulus that signals cells in the extracellular matrix to initiate tissue repair. Critical to the maintenance of cellular integrity and tissue function are the tight junction and adherens junction proteins that make up the adhesive structures, characteristic of all epithelia [McCormack et al., 2013].

Interestingly, despite the differences in the degree of epithelial cell surface damage between modal- and raised-intensity phonation, downstream changes in gene expression and cell proliferation were roughly analogous between groups. This lends support to the idea that homeostatic regulation is a key biological feature of vocal fold tissue. That is to say that, even in the absence of overt structural damage to the epithelial cell surface, the tissue is continually responding to external threats in an attempt to maintain a constant internal environment. The vocal folds undergo continual repair as the tissues respond to the repeated tensile, shearing, and impact stresses during vocal fold vibration exposure. It has been shown that the integrity of the basement membrane determines the regenerative nature of corneal epithelial repair [Stramer et al., 2003]. For transient episodes of phonotrauma, the vocal fold epithelium appears to be remarkably tolerant and capable of expeditious recovery of the structural integrity. However, the return of functional barrier integrity may delay the restitution of structural features, particularly relevant during repeated episodes of phonotrauma and in the development of vocal fold pathology.

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Ethics Statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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