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Effects of Phonation Time and Magnitude Dose on Vocal Fold Epithelial Genes, Barrier Integrity, and Function

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Abstract

Objective—To investigate the effects of increasing time and magnitude doses of vibration exposure on transcription of the vocal fold's junctional proteins, structural alterations, and functional tissue outcomes.

Study Design—Animal study.

Methods—100 New Zealand White breeder rabbits were studied. Dependent variables were measured in response to increasing time doses (30, 60, or 120 minutes) and magnitude doses (control, modal intensity, and raised intensity) of vibration exposure. Messenger RNA expression of occludin, zonula occluden-1 (ZO-1), E-cadherin, β -catenin, interleukin 1 β (IL-1 β), cyclooxygenase-2 (COX-2), transforming growth factor β -1 (TGF β 1), and fibronectin were measured. Tissue structural alterations were assessed using transmission electron microscopy (TEM). Transepithelial resistance was used to measure functional tissue outcomes.

Results—Occludin gene expression was downregulated in vocal folds exposed to 120 minute time doses of raised intensity phonation, relative to control, and modal intensity phonation. ZO-1 gene expression was upregulated following a 120 minute time dose of modal intensity phonation, compared to control, and downregulated after a 120 minute time dose of raised intensity phonation, compared to modal intensity phonation. E-cadherin gene expression was

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downregulated after a 120 minute time dose of raised intensity phonation, compared to control and modal intensity phonation. TEM revealed extensive desquamation of the stratified squamous epithelial cells with increasing time and magnitude doses of vibration exposure. A general observation of lower transepithelial resistance measures was made in tissues exposed to raised intensity phonation, compared to all other groups.

Conclusions—This study provides evidence of vocal fold tissue responses to varying time and magnitude doses of vibration exposure.

Level of Evidence—N/A

Keywords

epithelial barrier; junctional complex; phonotrauma; vocal fold

Introduction

The vocal fold epithelium undergoes significant biomechanical stresses during vibration exposure. Although phonation is a necessary biological constituent of oral communication, phonotrauma impairs the vocal folds unique native microarchitecture and impedes vibration. Clinical evidence of phonotrauma may include hemorrhage, sub-epithelial edema, and nodular formations of the vocal folds. In a systematic series of investigations, our laboratory has been discerning the often subtle ultrastructural changes underlying acute phonotrauma¹⁻³. Our studies thus far have focused on quantifying structural tissue alterations accounting for varying time and magnitude doses of vibration exposure. We have detected phonation associated reductions in the density and height of epithelial microprojections, protrusions of the epithelial surface, believed to be responsible for normal homeostatic tissue functions, such as water absorption, traction, and tissue lubrication³. These studies have led to a theoretical account of acute phonotrauma, which our laboratory has been testing empirically, using a unique combination of measurement considerations, including gene transcription, electron microscopy, and tissue electrophysiology.

The regulation of homeostasis in the vocal fold epithelium requires epithelial cells to adhere to their neighboring counterparts and polarize. Underneath the surface of the epithelium, epithelial cells adhere tightly to one another via specialized adhesive structures. Inappropriate regulation of signaling pathways subsequently disrupts epithelial adhesion properties, and may underlie the maturation of pathology. The presence of specialized adhesive structures is characteristic of all epithelia, providing an important means of cell adhesion, that is critical for maintaining cellular integrity and tissue function⁴. Tight junction and adherens junction proteins make up these adhesive structures. The former are maintained by the interaction of transmembrane proteins (claudins and occludins) and scaffolding proteins (Zonula family of proteins), that together assist in regulating ion/solute transport across the epithelium⁵. Tight junctions serve an important barrier function, providing fortification between cells and protecting the underlying tissue from invading pathogens^{6,7}. The latter are formed by cadherin receptors (E-cadherin, P-cadherin) and indirectly associate with the cortical actin cytoskeleton^{4,8}. Cytoplasmic proteins (α -catenin, β -catenin), in conjunction with additional junction-associated proteins, are believed to

contribute essential anchoring support for E-cadherin adhesion to the cortical actin cytoskeleton⁹.

Studies conducted in other tissues highlight the importance of these epithelial adhesive structures in preventing transepithelial migration of invading organisms, which can lead to pulmonary inflammation and edema¹⁰, intestinal disease¹¹, and cerebral ischemia¹². For example, the hallmarks of Crohn's disease and ulcerative colitis, both inflammatory diseases, include the passage of neutrophils through an altered epithelial barrier. Further, tumor necrosis factor- α (TNF- α), a potent regulator of immune cells capable of inducing epithelial cell shedding in the intestine, has been implicated in the redistribution of tight junction proteins as a means of fashioning an intercellular barrier between the lateral membrane walls of the intestine, and in so doing, preserving mucosal function¹¹. In the context of these findings from epithelia in other tissues of the human body, it is clear that conservation of epithelial adhesive structures and regulation of the inflammatory factors mediating phonation-induced tissue reparative mechanisms warrants closer attention. The response of the vocal fold epithelium to a variety of challenges is now a commonly used approach in laryngology research to assess tissue permeability^{13, 14, 15}.

Several questions remain unanswered. Although, our laboratory has demonstrated alterations in paracellular width and barrier integrity following transient time doses of vibration exposure, the effects of varying time and magnitude doses of vibration exposure on vocal fold epithelial and inflammatory gene transcription remains unknown². Further, it is unclear whether these empirical alterations in paracellular width, results in increased epithelial permeability, which as described above, may carry a host of significant consequences. Therefore, the purpose of the present study was to investigate the effects of increasing phonation time- and magnitude-doses of vibration exposure on the vocal fold junctional complex at the transcript, structural, and functional tissue levels.

Methods

Animals

The procedures used in this study were performed 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 Institutional Animal Care and Use Committee of Vanderbilt University Medical Center. A total of 100 New Zealand white breeder rabbits were utilized, with weights ranging from 2.8-3.8 kg. Rabbits were induced with an anesthetic cocktail of ketamine (35 mg/kg), xylazine (5 mg/kg), and acepromazine (0.75 mg/kg), administered intramuscularly, and given maintenance anesthesia of ketamine (17.5 mg/kg) and acepromazine (.375 mg/kg) as needed. Animals were randomized to nine groups ($n = 10$, per group), including a control group (vocal fold approximation in the absence of airflow), modal intensity phonation, or raised intensity phonation for 30 minutes, 60 minutes, or 120 minutes, as described in our previous studies³. A separate group of 10 rabbits were used to establish normative values for transepithelial resistance of the normal rabbit vocal fold epithelium.

Phonation Procedure and Tissue Harvest

Experimentally induced phonation was elicited from animals as described in our previous studies¹⁶⁻¹⁹. Briefly, modal intensity phonation was elicited from animals, yielding 59.60 dB (SD = 0.83) mean intensity at baseline and a mean frequency of 782 Hz (SD = 175). Intensity was maintained at 59.74 dB (SD = 1.70) throughout the modal intensity phonation procedure. Raised intensity phonation was elicited from animals, yielding 69.98 dB (SD = 3.40) mean intensity at baseline and a mean frequency of 735 Hz (SD = 190) during raised intensity phonation. Intensity was maintained at 68.97 dB (SD = 4.59) throughout the raised-intensity phonation procedure. Thirty-minutes after the completion of each procedure, larynges were harvested and animals were humanely sacrificed. Five larynges from each group were used for gene expression analysis and to assess functional tissue outcomes (i.e., transepithelial resistance). One vocal fold from each larynx was used in quantitative real-time polymerase chain reaction (qRT-PCR) experiments, in which the central region of the middle one-third portion of the vocal fold was dissected and immediately stabilized in RNAlater (Ambion, Austin, TX) for analysis of gene transcription. This dissection was limited to the mucosal layers of the vocal fold above the thyroarytenoid muscle and did not include muscle. The contralateral vocal fold from each larynx was used to measure TER immediately after the harvest. Ten normal healthy rabbit vocal fold epithelia were used to determine normative transepithelial resistance values for the rabbit vocal fold. The dissection was limited to the epithelium and did not include lamina propria or muscle. A separate analysis involving 45 animals undergoing the phonation procedure described above were used to quantify structural changes of the vocal fold in the context of gene transcript alterations and functional tissue outcomes³.

Gene Expression Analysis

qRT-PCR was used to measure gene expression of junctional complex proteins (occludin, ZO-1, E-cadherin, β -catenin), inflammatory mediators (TGF β 1, IL-1 β , COX-2), and the multifunctional extracellular matrix glycoprotein, fibronectin, a protein of the extracellular matrix involved in embryogenesis and important tissue repair functions, such as cell adhesion, growth, cell migration, and cell differentiation. Protocols for RNA extraction, reverse transcription, and qRT-PCR were followed as described previously^{2,16}. Rabbit-specific primers for occludin, ZO-1, E-cadherin, β -catenin, IL-1 β , COX-2, TGF β 1, fibronectin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Sigma-Aldrich Corp. (St. Louis, MO) using previously published sequences^{2,16,19,20}. Specific primer sequences are displayed in Table 1. Standard curves were used to determine the relative ratio of gene expression. Target gene ratios from all groups were normalized using expression ratios of the internal control gene (GAPDH).

Transepithelial Resistance (TER)

Freshly dissected vocal folds were mounted on a customized Lucite chamber (Jim's Instruments, Iowa City, IA) and measurements were taken using an Ussing System (World Precision Instruments, Sarasota, FL). Two voltage and two current electrodes (Ag+/AgCl with 3 mol/L KCl/agar salt bridges) were placed on either side of the tissue for measurement of open-circuit potential difference and short-circuit current by means of a current-voltage

clamp. TER was measured, from Ohms law, as the ratio of potential difference/short-circuit current by measuring the current deflection to an imposed 3-mV pulse. TER was measured at baseline (45-60 minutes) after mounting the tissue¹⁵. The system was calibrated prior to the start of each experiment. Data were collected using two separate Ussing chambers (2 and 4 mm) and normalized using the following equation: TER Value / Average Normal TER value (N=10) $\times$ 100 (%).

Transmission Electron Microscopy

Procedures describing tissue processing, cell imaging, and the quantification of TEM images are described in detail elsewhere^{3,15,21}. Vocal fold epithelial samples were visualized using a Philips CM-12 transmission electron microscope (FEI Company, Hillsboro, OR).

Statistical Analysis

A series of non-parametric Kruskal-Wallis tests were computed to assess for overall main effects of time-dose and magnitude-dose, with an appropriate alpha level correction of 0.025 to control for type I error (0.05/2). In the presence of significant overall main effects, post-hoc pairwise comparisons were performed between groups using a series of Mann-Whitney *U* tests for two independent samples to assess for between group differences in time-dose and magnitude-dose. Alpha levels were again adjusted during post-hoc testing to 0.0167 to control for multiple pairwise comparisons (0.05/3). All analyses were performed using two-tailed *p* values. Data were analyzed using IBM SPSS 22.0 (International Business Machines Corp., Armonk, NY).

Results

Gene Expression of Tight Junction Proteins

Gene transcript levels of the tight junction proteins occludin and ZO-1 were quantified using qRT-PCR to examine the vocal fold epithelial gene response to increasing time and magnitude doses of vibration exposure (Figure 1). No gene transcript level changes were detected at 30 or 60 minute time doses of phonation, for either gene, irrespective of magnitude dose. Conversely, 120 minute time doses of raised intensity phonation resulted in significantly downregulated occludin gene expression, relative to control ($P=0.008$), and modal intensity phonation ($P=0.008$). Likewise, gene transcript level changes were observed for ZO-1 following a 120 minute time dose of phonation. Results revealed significantly upregulated ZO-1 gene expression following a 120 minute time dose of modal intensity phonation, relative to control ($P=0.016$), and significantly downregulated ZO-1 gene expression following a 120 minute time dose of raised intensity phonation, relative to modal intensity phonation of the same duration ($P=0.008$).

Gene Expression of Adherens Junction Proteins

Gene transcript levels of the adherens junction proteins E-cadherin and β -catenin were quantified using qRT-PCR to examine the vocal fold epithelial gene response to increasing time and magnitude doses of vibration exposure (Figure 2). No gene transcript level changes were detected at 30 or 60 minute time doses of phonation, for either gene, irrespective of magnitude dose. In contrast, 120 minute time doses of raised intensity phonation resulted in

significantly downregulated E-cadherin gene expression, relative to control ($P=0.008$) and modal intensity phonation ($P=0.008$) of the same duration. Additionally, an effect of time-dose was observed for gene transcript levels of E-cadherin, such that E-cadherin gene expression was significantly downregulated following 120 minutes of raised intensity phonation, compared to a 60 minute time dose of the same magnitude ($P=0.008$). No gene transcript level changes were detected for β -catenin for either of the time-doses or magnitude-doses investigated.

Gene Expression of Interleukin-1 β , Cyclooxygenase-2, Transforming Growth Factor β 1, and Fibronectin

Gene transcript levels of the inflammatory mediators Interleukin-1 β (IL-1 β) and Cyclooxygenase-2 (COX-2), the multifunctional peptide Transforming Growth Factor β 1 (TGF β 1), and the multifunctional extracellular matrix glycoprotein Fibronectin were quantified using qRT-PCR to examine transcription of key genes involved in the vocal fold inflammatory, cell proliferative, and cellular differentiation processes, in response to increasing time and magnitude doses of vibration exposure (Figure 3). No gene transcript level changes were detected for IL-1 β , COX-2, TGF β 1, and fibronectin as a function of time or magnitude-dose.

Transepithelial Resistance

Normal healthy rabbit vocal fold epithelium ($n=10$ rabbits) were used to establish normative values for transepithelial resistance of the rabbit vocal fold. Vocal folds harvested from animals undergoing the *in vivo* phonation procedure were used to investigate the functional vocal fold epithelial response to increasing time and magnitude doses of vibration exposure (Figure 4). No functional changes were detected for either of the time-doses or magnitude-doses investigated. However, a general observation of lower adjusted transepithelial resistance measures was made in tissues exposed to raised intensity phonation, compared to modal intensity phonation, control, and normal.

Transmission Electron Microscopy

Transmission electron microscopy was used to quantify structural changes of the vocal fold epithelium in response to increasing time and magnitude doses of vibration exposure as reported previously³ (Figure 5). Measurement involved examination of the stratified squamous epithelial layer, basement membrane, and paracellular spaces. Results revealed well-preserved stratified squamous epithelia, paracellular spaces, and an intact basement membrane in the control animals, (irrespective of time dose) and in animals receiving modal intensity phonation of a short time dose (i.e., 30 minute duration). Further examination of the epithelial cell surface revealed normal desquamation processes, as evidenced by undisturbed surface microprojections on desquamating cells. In contrast, modal intensity phonation of significantly longer time doses (e.g., 60 and 120 minutes) revealed desquamation of extensive maturation. Interestingly, phonation of higher magnitudes (e.g., raised intensity phonation) required significantly shorter time-doses (e.g., 30 minute time dose) to reveal desquamation comparable to modal intensity phonation of longer durations (e.g., 60 and 120 minutes). That is, advanced desquamation processes were observed much

earlier in epithelia exposed to raised intensity phonation, compared to modal intensity phonation. Despite the desquamation evident in epithelia exposed to modal intensity phonation at 60 and 120 minute time doses, and in epithelia exposed to raised intensity phonation at 30 minute time doses, it is important to note that these tissues revealed a relatively intact basement membrane and paracellular spaces. This is in sharp contrast to longer time doses of raised intensity phonation (e.g., 60 and 120 minute time dose), where extensive desquamation of the stratified squamous epithelial cells was often associated with disruption of the basement membrane and exposure of microfilaments of the actin cytoskeleton.

Discussion

In the present study, we evaluated the effects of varying time- and magnitude-doses of vibration exposure on vocal fold gene transcript levels, tissue ultrastructure, and functional tissue outcomes using an in vivo rabbit phonation model. To our knowledge, these data represent the first attempt to quantify messenger RNA expression, vocal fold ultrastructure, and associated impairment in functional tissue outcomes in response to increasing time and magnitude doses of vocal fold vibration exposure in vivo. We focused on gene transcript levels of the tight junction proteins (occludin and ZO-1) and adherens junction proteins (E-cadherin and β -catenin) because of their key roles in cell to cell adhesion and importance in maintaining cellular integrity and tissue function. The gene transcript levels of inflammatory mediators (IL-1 β , COX-2) were also of interest to us due to their involvement in a variety of cellular activities, such as cell proliferation, differentiation, apoptosis, inflammation, and wound healing. Also of interest were multifunctional polypeptide TGF β 1, and the multifunctional extracellular matrix glycoprotein, fibronectin, with their respective contributions to cell adhesion, growth, migration, and differentiation. These epithelial and mesenchymal factors play an important role in preserving cellular integrity and tissue function ⁴.

Results of the present study revealed a significant upregulation in gene expression of the tight junction protein ZO-1, in response to a 120 minute time dose of modal intensity phonation, compared to control. We interpret this finding as suggesting that there is sufficient enough disruption to the tight junction complex at this time dose to initiate transcriptional signaling and subsequent upregulation of ZO-1 to preserve the cell to cell adhesion properties of the epithelium. This notion is supported by studies conducted in the cornea, in which investigators have hypothesized that disruption of the basement membrane initiates an upregulation of basement membrane structural proteins, among other proteins, to preserve basement membrane integrity ²². We provide further evidence for this idea through our structural electron microscopic investigations, which reveal significantly decreased epithelial cell depth and disruption of the basement membrane and exposure of microfilaments of the actin cytoskeleton after prolonged vibration exposure ³.

In contrast, ZO-1 gene expression was significantly downregulated following a 120 minute time dose of raised intensity phonation, compared to modal intensity phonation of the same duration. These findings suggest that there may be differential cellular responses to the regulation of the vocal fold epithelial barrier in response to acute phonotrauma. An

alternative explanation for the downregulated ZO-1 and occludin gene expression observed after raised intensity phonation could be greater disruption of the epithelium, thereby reducing the amount of viable tissue required for proper signaling between or among intercellular junctional complexes and the nucleus. Whereas, the significant upregulation of ZO-1 gene expression after 120 minutes of modal intensity phonation, may be a more normal or expected epithelial barrier response to simple disruption of the junctional complex. In both cases, because GAPDH, a key enzyme involved in energy metabolism is still transcribed by the cell, we suspect that the cell is not completely dead.

While junctional complex proteins have traditionally been considered primarily responsible for assembling epithelial adhesive structures, contemporary thinking provides a notion that the roles of individual junctional complex proteins may be more intricate than previously thought. For example, the overexpression of occludin, a transmembrane constituent of the tight junction complex, leads to functional changes in epithelial barrier integrity²³. Further, when the occludin gene is silenced, there is increased permeability to divalent organic cations, and decreased ability of occludin clones to extrude apoptotic cells from the renal epithelium.²⁴ These findings implicate the failure of adjoining cells to sense the presence of these apoptotic cells and/or a decreased functional capacity to coordinate the compulsory cytoskeletal activity required to extrude these cells²⁴. These studies point to the importance of occludin's function as a key barrier protein, contributing to basic homeostatic mechanisms crucial for cell viability and function. Occludin has also been found to be intimately associated with tissue inflammatory processes. For example, knocking out occludin in mice, leads to inflammation and hyperplasia in the gastric epithelium²⁵ and has been linked to TNF mediated deterioration of the epithelial barrier during epithelial-to-mesenchymal transition²⁶. Thus, it appears that occludin's function is not only heavily protective, contributing to maintaining epithelial barrier integrity and function, but it is also closely linked to fundamental tissue inflammatory mechanisms. There is still much more to be learned regarding the relationship between tight junction proteins and epithelial barrier function of the vocal fold. Of particular interest are the specific contributions and bond between ZO-1 and occludin. ZO-1, a cytoplasmic scaffolding protein for occludin, is critical in the formation of tight junctions, signaling between paracellular pathways, and proper establishment of intercellular junctional structures²⁷⁻²⁹. Suzuki et al have nicely demonstrated that cell polarity directs the biogenesis of tight junctions by first allowing adherens junctional proteins to join with their corresponding counterparts, with ZO-1 localizing to the junction thereafter. Occludin follows by binding to ZO-1 and this attachment subsequently leads to the tight junctions unlinking from the adherens junction and localizing to the apical most portion of the epithelial layer³⁰. We highlight this unique property of ZO-1 because the sequential upregulation of the junctional complex constituents will depend on the integrity of ZO-1 upregulation and signaling following phonotrauma and initial establishment of adherens junctions. Thus, we suspect that both adherens junction proteins (e.g., E-cadherin) and tight junction proteins (e.g., occludin) are necessary for proper ZO-1 signaling and the eventual re-establishment of a functional vocal fold epithelial barrier.

No gene transcript level changes in the adherens junction protein β -catenin were detected in the present study, irrespective of time-dose or magnitude-dose. Interestingly, these results

are consistent with recent studies that have demonstrated an inverse relationship between β -catenin and E-cadherin. This is not surprising, considering the interactions between β -catenin and E-cadherin in the junctional complex. E-cadherin, a calcium-dependent transmembrane protein extends outward from the cell surface, binding to its neighboring cadherins in order to establish adherens junctions³¹. Recent studies have highlighted the role of E-cadherin in fundamentally regulating levels of β -catenin signaling by phosphorylating the n-terminal of β -catenin, rendering β -catenin inactive when loss of cadherin-based adhesion occurs³². Results of the present study revealed that E-cadherin gene transcript levels are downregulated following 120 minutes of raised intensity phonation compared to modal intensity phonation and control. Further, a significant time dose was detected, inasmuch as E-cadherin gene transcript levels were further downregulated at 120 minutes of raised intensity phonation, compared to 60 minutes of phonation of the same magnitude dose (i.e., raised intensity phonation). However, as mentioned above, an alternative explanation for the downregulation of E-cadherin at 120 minutes of raised intensity phonation (similar to the downregulation of Occludin and ZO-1 at the same time dose), could be greater damage to the epithelium, resulting in diminished epithelial cell viability and signaling.

This relationship between the coordinated and dynamic nature of β -catenin and E-cadherin interactions in the regulation of cell-cell adhesion and transcriptional activation in the vocal fold deserves much more attention. The literature also raises an interesting notion that the timing of these interactions is likely to be important, as theories have recently surfaced that the release of catenins from their adherens junctions may occur prior to their nuclear translocation for gene regulation, suggesting that the upregulation of E-cadherin or β -catenin may very well depend on time after injury³².

No gene transcript level changes in the inflammatory mediators IL-1 β and COX-2, the multifunctional peptide TGF β 1, or the multifunctional extracellular matrix glycoprotein Fibronectin were detected in the present study, irrespective of time-dose or magnitude-dose. Transcription of these factors resides primarily in the lamina propria³³, and thus, we suspect that disruption of the epithelial barrier may eventually lead to the signaling of cells in the extracellular matrix to initiate tissue repair functions, such as cell adhesion, growth, cell migration, and cell differentiation. Thus, we offer an alternative explanation to phonotraumatic injury that disrupts the vocal fold epithelial barrier although unavoidable, may actually be necessary to drive the underlying mesenchymal tissues into repair mode, and that the development of pathology may be related to faulty tissue reconstitution. While for some this is detrimental, yet for others, the routine homeostatic mechanisms necessary for optimal tissue recovery from phonotrauma are sound, thus leading to proper reconstitution of tissues and normal patterns of recovery. Findings in the present study are consistent with our previous reports. That is, transient durations of modal intensity phonation result in no significant changes in inflammatory gene transcript levels, and albeit not significant in this study, a general observation was made of increased transcript levels of the inflammatory mediators IL-1 β and COX-2 with increasing magnitude doses of vibration exposure¹⁹. In light of our structural electron microscopy findings that the epithelial barrier remains intact in tissues exposed to modal intensity phonation, we hypothesize that when damage does not completely breach the epithelial barrier, the inflammatory cascade is

mutated. Stated differently, in these conditions, we suspect that the mesenchyme does not initiate a full blown inflammatory response. This suspicion seems to be supported by the general observation of increased inflammatory gene transcript levels, albeit insignificant, with increasing magnitude doses of vibration exposure, particularly in the context of the exposed actin cytoskeleton and basement membrane observed under electron microscopy.

Epithelial barrier function was measured using tissue electrophysiology (EP), which provides a measure of transepithelial resistance across epithelia. Transepithelial resistance is a measure used to reflect the capacity of the epithelium to resist the passage of solutes and solvents across tissues. Higher resistance values are therefore reflective of a tight and protective epithelial barrier. On the contrary, lower resistance values may be suggestive of an increase in leakiness across the epithelial barrier¹³. Electrophysiology is now a commonly accepted technique for measuring changes in epithelial permeability, and has proven to provide a useful approach to assessing the response of the vocal fold epithelium to a variety of challenges^{14,15}. Given the well-preserved stratified squamous epithelia, paracellular spaces, and intact basement membrane observed in tissues exposed to modal intensity phonation of short durations, it is not surprising that there are no functional changes noted in normalized transepithelial resistance between modal intensity phonation and control, and modal intensity phonation and normal. It is worth noting however, that albeit not significant, a general observation of lower normalized transepithelial resistance measures was made in tissues exposed to raised intensity phonation, where extensive desquamation of the stratified squamous epithelium, disruption of the basement membrane, and exposure of the actin cytoskeleton was found (suggestive of a disrupted epithelial barrier) compared to modal intensity phonation, control, and normal.

Conclusion

These data provide the first account of vocal fold tissue responses to varying time and magnitude doses of vibration exposure using gene transcript, tissue ultrastructure, and functional measurement outcomes. We extend a hypothesis to be tested in future systematic studies that disruption of the vocal fold epithelial barrier from phonotraumatic injury although unavoidable, may be necessary to drive the underlying mesenchymal tissues into repair mode. Faulty reconstitution of tissue may therefore be related to the disruption of normal homeostatic mechanisms. This may provide an explanation for why some speakers will eventually develop benign vocal fold pathology, while others appear to benefit from the normal tissue homeostatic mechanisms in place for optimal tissue recovery.

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Figure 1.

Gene transcript level changes in the tight junction proteins occludin and ZO-1 in response to increasing time doses (30 minutes, 60 minutes, or 120 minutes) and magnitude doses of vibration exposure, control (abbreviated as C), modal intensity (abbreviated as M), and raised intensity (abbreviated as R). Filled circle (•) symbols correspond to outliers. Asterisk (*) symbols correspond to P values < 0.167.

Figure 2.

Gene transcript level changes in the adherens junction proteins E-cadherin and β -catenin in response to increasing time doses (30 minutes, 60 minutes, or 120 minutes) and magnitude doses of vibration exposure, control (abbreviated as C), modal intensity (abbreviated as M), and raised intensity (abbreviated as R). Filled circle (•) symbols correspond to outliers. Asterisk (*) symbols correspond to P values <0.167.

Figure 3.

Gene transcript level changes in the inflammatory mediators Interleukin-1 β (IL-1 β) and Cyclooxygenase-2 (COX-2), the multifunctional peptide Transforming Growth Factor β 1 (TGF β 1), and the multifunctional extracellular matrix glycoprotein Fibronectin in response to increasing time doses (30 minutes, 60 minutes, or 120 minutes) and magnitude doses of vibration exposure, control (abbreviated as C), modal intensity (abbreviated as M), and raised intensity (abbreviated as R). Filled circle (•) symbols correspond to outliers.

Figure 4.

Transepithelial resistance of the normal rabbit vocal fold epithelium (abbreviated as N), and in response to increasing time doses (30 minutes, 60 minutes, or 120 minutes) and magnitude doses of vibration exposure, control (abbreviated as C), modal intensity (abbreviated as M), and raised intensity (abbreviated as R). The y-axis displays transepithelial resistance values normalized to normal rabbit vocal fold epithelia (i.e., normalized trans-epithelial resistance). Filled circle (•) symbols correspond to outliers.

Figure 5. Transmission Electron Microscopy of the vocal fold in response to increasing time doses (30 minutes, 60 minutes, or 120 minutes) and magnitude doses of vibration exposure (control, modal intensity, and raised intensity).

Table 1
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'
β -catenin	Forward	5'-ATG TGG ATT TGG AAC CCA AG-3'
	Reverse	5'-CCA AAG GGA GGC TTC CTA GT-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'
GAPDH	Forward	5'-TCG GCA TTG TGG AGG GGC TC-3'
	Reverse	5'-TCC CGT TCA GCT CGG GGA TG-3'

ZO-1: zonula occludens-1

IL-1 β : interleukin 1 β

COX-2: cy- clooxygenase-2

TGF β 1: transforming growth factor β -1

GAPDH: glyceraldehyde-3-phosphate dehydrogenase.