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Acute Exposure to Vibration is an Apoptosis-Inducing Stimulus in the Vocal Fold Epithelium

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Abstract

Clinical voice disorders pose significant communication-related challenges to patients. The purpose of this study was to quantify the rate of apoptosis and tumor necrosis factor- α (TNF- α) signaling in vocal fold epithelial cells in response to increasing time-doses and cycle-doses of vibration. 20 New Zealand white breeder rabbits were randomized to three groups of time-doses of vibration exposure (30, 60, 120 minutes) or a control group (120 minutes of vocal fold adduction and abduction). Estimated cycle-doses of vocal fold vibration were extrapolated based on mean fundamental frequency. Laryngeal tissue specimens were evaluated for apoptosis and gene transcript and protein levels of TNF- α . Results revealed that terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was significantly higher after 120 minutes of vibration compared to the control. Transmission electron microscopy (TEM) revealed no significant effect of time-dose on the mean area of epithelial cell nuclei. Extrapolated cycle-doses of vibration exposure were closely related to experimental time-dose conditions, although no significant correlations were observed with TUNEL staining or mean area of epithelial cell nuclei. TUNEL staining was positively correlated with TNF- α protein expression. Our findings suggest that apoptosis can be induced in the vocal fold epithelium after 120 minutes of modal intensity phonation. In contrast, shorter durations of vibration exposure do not result in apoptosis signaling. However, morphological features of apoptosis are not observed using TEM. Future studies are necessary to examine the contribution of abnormal apoptosis to vocal fold diseases.

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Keywords

apoptosis; epithelium; terminal deoxynucleotidyl transferase dUTP nick end labeling; transmission electron microscopy; tumor necrosis factor- α ; vocal fold

1 Introduction

Voice disorders, characterized by dysphonia or abnormal voice quality, pose a significant burden to patients, healthcare, and society (1–3). The vocal folds are a pair of membranous structures within the larynx that are involved in generating the audible sound of voice. The vocal folds approximate one another while modulating airflow from the lungs. These repeated cycles of vibration (i.e., phonation) often result in biomechanical trauma to the vocal folds. Non-keratinized, stratified squamous cell epithelium constitutes the superficial cellular surface of the vocal folds (4). Healthy epithelium is necessary to protect the underlying connective tissue by forming a barrier to divide the external environment from the multilayered lamina propria (5). Systematic investigations of the vocal fold epithelium may help to elucidate the biological mechanisms underlying vocal fold diseases of epithelial origin. These studies may also help to determine the association between vocal fold tissue changes and dysphonia.

To promote a strong barrier, rapid cell turnover is an established property of epithelial tissue (6, 7). Given that the vocal folds undergo repeated biomechanical stresses during vibration, an area of significant interest during turnover is the highly regulated mechanism of cell death. Apoptosis, the most commonly studied type of programmed cell death, is the physiological elimination of cells (8–10). Several studies have reported that apoptosis occurs in the vocal folds in response to biomechanical trauma (11–14). Following *in vivo* vocal fold vibration and vocal fold movement alone using a rabbit model, our laboratory observed morphological features of condensed chromatin and apoptotic bodies in vocal fold epithelial cells (11). Although rare, ultrastructural features of enlarged cell nuclei, which is suggestive of necrosis, were also present. To confirm that cell death occurred, we found that the vocal fold epithelium expressed an immunohistochemical marker for apoptosis using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (11). These findings are in agreement with the previously reported effects of mechanical trauma on cell death in vocal fold fibroblasts (12–14). Collectively, the literature suggests that apoptosis may be a natural biological response to vocal fold vibration and may therefore contribute to maintaining a normal epithelial barrier. Nevertheless, while vibration causes apoptosis signaling in the vocal fold epithelium, it remains unknown whether this is a static response or if increasing vibration exposure leads to greater apoptosis signaling.

The purpose of this study was to quantify the rate of apoptosis signaling in vocal fold epithelial cells in response to increasing durations and extrapolated cycles of vibration in an *in vivo* animal model. In addition, there is evidence of tumor necrosis factor- α (TNF- α) upregulation in response to biomechanical trauma in the vocal folds (15, 16). However, studies reveal inconsistencies regarding the effect of TNF- α on cell death in the vocal folds (17, 18). To examine the relationship between TNF- α and apoptosis, a secondary purpose of

this study was to determine whether gene transcript and protein levels of TNF- α are upregulated with increasing durations of vibration exposure. A prospective between subjects design was used. Animals were randomized to three groups of time-doses of vibration exposure or one control group. Laryngeal tissue specimens were evaluated for apoptosis by TUNEL method and transmission electron microscopy (TEM). Cycle-doses of vibration exposure were extrapolated based on obtained mean fundamental frequency (F_0) values and correlated with TUNEL and TEM. Additionally, TNF- α gene transcript levels and protein expression were measured.

2. Materials and Methods

2.1 Animals

Animals were 20 adult male New Zealand white breeder rabbits weighing between 2.50–3.60 kg ($M = 2.90$, $SD = 0.30$). Animals were anesthetized via intramuscular injections of ketamine (35 mg/kg), xylazine (5 mg/kg), and acepromazine (0.75 mg/kg). To maintain anesthetic effects, ketamine (17.5 mg/kg) and acepromazine (0.375 mg/kg) were subsequently administered as needed. Heart rate, respiratory rate, oxygen saturation level, and body temperature were monitored throughout the procedure to assess state of anesthesia and general well-being. This study was 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 Vanderbilt University Institutional Animal Care and Use Committee approved the animal protocol.

2.2 *In Vivo* Phonation

An *in vivo* surgical procedure was performed as described previously (19–23). Briefly, the neck was shaved from the submentum to the chest. Local anesthesia of 4 mL 0.2% lidocaine was administered at the surgical site. A midline incision was made from the hyoid bone to the sternal notch to expose the larynx and trachea. A tracheostomy was created to provide a stable airway. The trachea was transected just proximal to the sternum and the lower portion of the trachea was suspended to the sternal fascia. To supply electrical stimulation to the laryngeal musculature, stainless-steel hooked electrodes were used with a Grass S-88 stimulator (SA Instrumentation, Encinitas, CA) and constant current isolation unit (Grass Telefactor, model PSIU6; West Warwick, RI). The total train duration was 10 seconds with 3 seconds of electrical stimulation (i.e., adduction) and 7 seconds of rest (i.e., abduction).

To evoke phonation, a 3.5 cuffed endotracheal tube (Willy Rusch GmbH, Kernen, Germany) was inserted into the upper portion of the bisected trachea and positioned 2 cm below the glottal opening. The cuff of the endotracheal tube was inflated to seal off the trachea and deliver continuous humidified airflow heated at 37°C to the glottis using a Gilmont Instruments flowmeter (GF-8522, Barrington, IL) and Conch Therm III humidifier (Hudson, RCI, Temecula, CA). The simultaneous neuromuscular electrical stimulation to the laryngeal musculature and controlled airflow evoked sustained audible phonation.

For the experimental conditions, animals underwent vocal fold vibration with controlled airflow for 30 minutes ($n = 5$), 60 minutes ($n = 5$), or 120 minutes ($n = 5$). The maximum time-dose of 120 minutes was based on work by Solomon, Glaze, Arnold, and van Mersbergen (24) that revealed decreased vocal function after 120 minutes of loud speaking in humans. Subsequent work in our laboratory confirmed that 30- and 60-minute time-doses led to observations of structural and functional vocal fold tissue changes (20, 21). Modal intensity phonation was elicited with mean electrical stimulation of 1.56 mA ($SD = 0.63$) and constant airflow rate of 85.03 mL/s ($SD = 0.00$). To serve as the control condition, animals underwent vocal fold adduction and abduction with no supplied airflow for 120 minutes ($n = 5$). Vocal fold approximation with no supplied airflow was selected to examine the effect of vocal fold movement alone without vibratory collision on apoptotic cell death. This study's primary distinction between vocal fold approximation versus vibration is the variable of airflow. While the control condition causes the vocal folds to approximate without colliding at a rapid rate, the addition of airflow in the experimental conditions induces biomechanical forces such as shear, tensile, and impact stresses.

To confirm that the vocal folds were in the adducted phonatory position, endoscopic imaging was performed using a 30° 2.7-mm rigid endoscope (Karl Storz Endoscopy-America, Inc., El Segundo, CA) and Telecam-C camera (Karl Storz Endoscopy-America, Inc.). Vocal fold adduction was determined by visualization of the vocal folds approximating, although the vocal folds were not required to adduct along the entire length of the vocal folds. Acoustic signals were recorded during phonation at baseline and in 15-minute intervals using a Perception 170 Condenser Microphone (AKG, Vienna, Austria) positioned approximately 10 cm from the opening of the endoscope. Mouth-to-microphone distance and amplitude gain were held constant. Recordings were digitized at a 44 kHz sampling rate using Computerized Speech Lab Model 4500 (KayPENTAX, Lincoln Park, NJ). Acoustic samples were later edited with focus on the most representative central 1-second portions of the waveforms. Acoustic parameters of F_0 (Hz) and vocal intensity (dB sound pressure level) were averaged across three trials for each time interval. During modal intensity phonation, results revealed that mean F_0 was 538.36 Hz ($SD = 86.64$), mean intensity was 53.31 dB ($SD = 3.79$), and mean subglottal pressure was 7.30 cm/H₂O ($SD = 1.49$).

At the end of the phonation procedure, humidified airflow was discontinued. Animals were immediately sacrificed and larynges were harvested. The freshly excised larynx was dissected in half posteriorly to anteriorly. The right true vocal fold was placed in fresh 10% neutral buffered formalin solution, pH 6.8–7.2 PFNBF-1000 (Azer Scientific, Morgantown, PA) for paraffin embedding for fluorescent staining using TUNEL method and TNF- α protein expression. The left true vocal fold region was identified and dissected in the coronal plane under a microscope. The anterior to middle two-thirds portion of the membranous left vocal fold was placed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for TEM. The posterior one-third portion of the membranous left vocal fold was placed in RNA^{later}® Stabilization Solution (Ambion, Thermo Fisher Scientific, Waltham, MA) for quantitative real-time polymerase chain reaction (qRT-PCR).

2.3 Extrapolation of Cycle-Doses

Cycle-dose, defined in this study as the total number of cycles of vocal fold vibration for a given time-dose, was extrapolated from mean F_0 values during phonation. For each recorded trial of phonation, the total train duration was a total of 10 seconds, with 3 seconds of electrical stimulation (i.e., phonation) and 7 seconds of rest. To calculate the extrapolated cycle-doses per train duration, F_0 was multiplied by 3 seconds (cycle-dose train duration = mean $F_0 \times 3$). Next, to calculate cycle-doses per minute based on 60 seconds in a minute, cycle-dose train duration was multiplied by 6 (cycle-dose per minute = cycle-dose train duration $\times$ 6). Finally, to calculate all cycle-doses within a 15-minute interval, cycle-dose per minute was multiplied by 15 (cycle-dose 15-minute interval = cycle-dose per minute $\times$ 15 minutes). In summary, for each mean F_0 value per 15-minute time interval, the following formula was computed:

$$[(F_0 \text{ at } 0 \text{ minutes} \times 3) (6) (15)] + [(F_0 \text{ at } 15 \text{ minutes} \times 3) (6) (15)] + [(F_0 \text{ at } 30 \text{ minutes} \times 3) (6) (15)], \dots \text{etc.}$$

Table 1 describes the major components of the extrapolated cycle-dose calculation.

2.4 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

The right vocal fold tissue was embedded in paraffin and 5- μ m-thick sections were prepared in the coronal plane. TUNEL method fluorescent staining of formalin-fixed, paraffin-embedded tissue specimens was performed using the ApopTag® Fluorescein *In Situ* Apoptosis Detection Kit (S7110, EMD MILLIPORE International, Inc.). Post-fixed tissue sections were deparaffinized. Specimens were washed in 3 changes of xylene for 5 minutes each wash, 2 changes of 100% ethanol for 5 minutes each wash, 1 change of 95% ethanol for 3 minutes, 1 change of 70% ethanol for 3 minutes, and 1 change of phosphate buffered saline (PBS) for 5 minutes. To enhance DNA exposure, tissue sections were pretreated with a protein digesting enzyme. 100 μ L freshly diluted Proteinase K (20 μ L/mL, IHC® Specific Proteinase K, EMD MILLIPORE International, Inc.) was applied to each tissue section and incubated for 15 minutes at room temperature. Slides were drained and washed in 2 changes of PBS for 2 minutes each wash. μ 50 L equilibration buffer was applied to each tissue section and incubated for at least 10 seconds at room temperature. μ 55 L working strength terminal deoxynucleotidyl transferase (tDt) enzyme (70% reaction buffer, 30% tDt enzyme) was applied to each tissue section and incubated in a humidified chamber for 1 hour at 37°C. Slides were agitated in working strength stop/wash buffer for 15 seconds and incubated for 10 minutes at room temperature. Slides were washed in 3 changes of PBS for 1 minute each wash. Tissue sections were treated with working strength anti-digoxigenin conjugate (53% blocking solution, 47% anti-digoxigenin) and incubated in a humidified chamber for 30 minutes at room temperature while avoiding exposure to light. Slides were washed in 4 changes of PBS for 2 minutes each wash. Slides were mounted with 4',6-diamidino-2-phenylindole (DAPI) Fluoromount-G® (SouthernBiotech Associates, Inc., Birmingham, AL) under glass coverslips. For the positive control, a 5- μ m-thick paraffin embedded tissue of rabbit tonsil was used to induce cleavage of genomic DNA by deoxyribonuclease treatment. After the post-fixed tissue section was deparaffinized, the section was pretreated with deoxyribonucleic buffer consisting of 30 mM Trizma base, pH 7.2, 4 mM $MgCl_2$, and

0.1 mM dithiothreitol at room temperature for 5 minutes. 0.2 μ l deoxyribonuclease was dissolved in 2 ml deoxyribonucleic buffer and the solution was applied to the tissue section, incubated at room temperature for 10 minutes, and rinsed with distilled water. For the negative control, a true vocal fold tissue section exposed to the 120-minute phonation time-dose was used to confirm no presence of TUNEL staining. The tissue section received no tDt enzyme treatment.

Fluorescence microscopy image acquisition was performed using a 12-bit Hamamatsu Digital Camera C10600 ORCA-R² (Hamamatsu Corporation, Bridgewater, NJ) connected to a Nikon ECLIPSE 90i Microscope (Nikon Instruments, Inc., Melville, NY) on a Dell Optiplex 960 desktop computer running Windows 7 Enterprise (Microsoft Corporation, 2009). With the exception of the positive and negative controls, the experimenter was blinded to group assignment during data acquisition, quantification, and exportation. For each specimen, three distinct, non-overlapping fluorescent images were captured along the apical epithelial surface of the true vocal fold at 40x magnification at a constant exposure time (200 ms for DAPI, 500 ms for the fluorophore fluorescein isothiocyanate [FITC]). Microscopy quantification procedures were performed using Nikon Imaging Software Elements, Basic Research 3.22.00 (Nikon Instruments, Inc., Melville, NY) under Annotated Measurement Tools. To quantify the mean intensity of fluorescence for TUNEL staining, a region of interest was selected in each image to identify only the vocal fold epithelium. The region of interest was defined as the entire length of the side facing the lumen (e.g., black) with several layers of closely-packed DAPI staining cells. The epithelium ended when the primarily non-cellular lamina propria was reached, which was determined by a significant reduction in the number of DAPI-staining cells. The mean intensity of the fluorophore FITC was captured and adjusted until it appeared that all TUNEL-labeled cells were visible. The mean intensity values for FITC were exported for analysis. For each specimen, the intensity values for FITC were averaged across three distinct images as the final data point. To obtain intra-rater reliability, the experimenter repeated the quantification procedures on 10% of the images ($n = 6$) that were selected at random.

2.5 Transmission Electron Microscopy

After laryngeal excision, the left vocal fold tissue was prepared for routine TEM processing. For primary fixation, tissue specimens were immersed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and rinsed in 3 changes of 0.1 M sodium cacodylate buffer for 5 minutes each rinse. For secondary fixation, specimens were immersed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour at room temperature and rinsed in 3 changes of 0.1 M sodium cacodylate buffer for 5 minutes each rinse. An ethyl alcohol series was used for specimen dehydration. Specimens were immersed in 30%, 50%, 75%, 85%, and 95% ethyl alcohol for 15 minutes each. For final dehydration, specimens were immersed in 3 changes of 100% ethyl alcohol for 15 minutes each, propylene oxide for 5 minutes, and 100% ethanol for 5 minutes. Specimens were incubated in 2 changes of 100% propylene oxide for 15 minutes each. Stepwise infiltrations were completed with Epon resin and specimens were embedded in flat molds and allowed to polymerize for 48 hours at 60°C. Thick sections (500 nm to 1 micron) were initially cut from each block, the region of interest of the vocal fold epithelium was identified, and ultrathin sections (70 nm)

were cut and placed on 300 mesh copper grids. Grids were post-section stained with 2% uranyl acetate for 15 minutes and Reynold's lead citrate for 15 minutes.

TEM image acquisition was performed using a Philips/FEI T12 transmission electron microscope (FEI Company, Hillsboro, OR) to visualize the apical epithelial cell surface and internal cellular structures from 2,700x to 15,000x magnification. Images were captured under low-power magnification to visualize the location of the apical epithelial cell surface and high-power magnification to examine the morphology of individual epithelial cells. Microscopy quantification procedures were imported and performed using Nikon Imaging Software Elements, Basic Research 3.22.00 (Nikon Instruments, Inc., Melville, NY) under Annotated Measurement Tools. The experimenter was blinded to group assignment during data acquisition, quantification, and exportation. For each specimen, three distinct, non-overlapping TEM images at 4,400x magnification were selected for analysis. Images showed a visible apical epithelial surface and at least one identifiable epithelial cell nucleus. To measure the mean area of epithelial cell nuclei, area was defined as the number of pixels in a selected region. The outline of all nuclei were manually measured in each image. A nucleus was visually identified by showing dark contrast with evidence of chromatin or a nucleolus. Partial nuclei that were cut off, apoptotic bodies, and smaller fragmented pieces of nuclei were excluded from analysis. The number of identifiable nuclei ranged from one to six nuclei per image. For each image, the mean area values for all measured nuclei were exported for analysis. For each specimen, the area values were averaged across three distinct images as the final data point. To obtain intra-rater reliability, the experimenter repeated the quantification procedures on 10% of the images ($n = 6$) that were selected at random.

2.6 Quantitative Real-Time Polymerase Chain Reaction

After laryngeal excision, the left vocal fold tissue was prepared for qRT-PCR to measure gene expression of TNF- α . The tissue specimen was immersed in RNA $later^{\circledR}$ Stabilization Solution (Ambion, Thermo Fisher Scientific, Waltham, MA) and stored overnight at 4°C. The following day, the tissue specimen was centrifuged, RNA $later$ Stabilization Solution was removed, and the tissue was stored at -80°C. Total RNA was isolated using an RNeasy Mini Kit (QIAGEN, Valencia, CA) and treated with ribonuclease-free deoxyribonuclease I (QIAGEN) to minimize contamination from genomic DNA. The quantity of total RNA was determined using the A260/A280 ratio. Electrophoresis was used to evaluate the quality based on the appearance of the 18S and 28S ribosomal RNA bands. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using the manufacturer's recommended reaction protocol. Reactions were performed with a Biometra TGradient thermocycler (LABREPCO, Horsham, PA) using the following parameters: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and 4°C for 5 minutes.

Rabbit-specific primers for TNF- α and succinate dehydrogenase complex subunit A (SDHA) were synthesized by Integrated DNA Technologies (Coralville, IA). The specific primer sequences for TNF- α and SDHA are displayed in Table 2. The primers generated a single polymerase chain reaction (PCR) band of the expected size. DNA sequencing was used to verify the PCR products. qRT-PCR was performed using the iQ SyBR Green

Supermix Kit (BioRad, Hercules, CA) in a 20- μ L volume reaction mixture composed of 500 nM primer 1, 500 nM primer 2, 12.5 μ L iQ SyBR Green Supermix, 1 μ L template complementary DNA, and 9 μ L ribonuclease-free water. The amplification was performed under the following conditions: 95°C for 10 minutes, 40 cycles of denaturing at 95°C for 15 seconds, and annealing at 60°C for 1 minute. Fluorescence was detected with StepOnePlus (Applied Biosystems) and standard curves were used to determine the relative ratio of gene expression. Target gene ratios from the experimental and control conditions were normalized using expression ratios of the internal control gene SDHA.

2.7 TNF- α Protein Expression

Immunofluorescence staining of formalin-fixed, paraffin-embedded tissue specimens was performed using the TNF α Antibody (N-19): sc 1350 (Santa Cruz Biotechnology, Inc., Dallas, TX). Post-fixed tissue sections were heated at 55°C for 30 minutes, cooled at room temperature for 30 minutes, and deparaffinized by being washed in 3 changes of Histo-Clear for 10 minutes, 10 minutes, and 30 minutes. Specimens were rehydrated by being washed in 3 changes of 100% ethanol for 5 minutes each wash, 2 changes of 95% ethanol for 5 minutes each wash, 1 change of 75% ethanol for 5 minutes, and 1 change of distilled water for 5 minutes. For antigen retrieval, tissue sections were heated to boiling for 20 minutes in 10% Dako Target Retrieval Solution (pH 9, Agilent Technologies, Santa Clara, CA) and 90% distilled water and cooled at room temperature for 20 minutes. After briefly rinsing the slides in distilled water for several seconds, slides were washed in 1 change of PBS for 5 minutes. For antigen blocking, 100 μ L 1% BSA was applied to each tissue section. Slides were incubated in a humidified chamber at room temperature for 1 hour and 30 minutes. For primary antibody treatment, 0.5 μ L active TNF- α antibody and 100 μ L 1% BSA were applied to each specimen (dilution 1:200). Slides were stored overnight at 4°C. The following day, slides were washed in 3 changes of PBS for 5 minutes each wash. For secondary antibody treatment, 0.5 μ L secondary antibody Dnk pAb to Goat IgG conjugate (DyLight® 594, Abcam, ab96933, 0.5 mg/ml) and 100 μ L 1% BSA were applied to each specimen (dilution 1:200). Slides were incubated for 1 hour at room temperature. Slides were washed in 3 changes of PBS for 5 minutes each wash. Slides were mounted with DAPI Fluoromount-G® (SouthernBiotech Associates, Inc., Birmingham, AL) under glass coverslips. For the positive control, a 5- μ m-thick paraffin embedded tissue of rabbit tonsil was used to detect TNF- α protein expression. For the negative control, a true vocal fold tissue section exposed to the 120-minute phonation time-dose was used to confirm no presence of TNF- α staining. The tissue section received no primary TNF- α antibody treatment.

Fluorescence microscopy image acquisition was performed using a 12-bit Hamamatsu Digital Camera C10600 ORCA-R² (Hamamatsu Corporation, Bridgewater, NJ) connected to a Nikon ECLIPSE 90i Microscope (Nikon Instruments, Inc., Melville, NY) on a Dell Optiplex 960 desktop computer running Windows 7 Enterprise (Microsoft Corporation, 2009). With the exception of the positive and negative controls, the experimenter was blinded to group assignment during data acquisition, quantification, and exportation. For each specimen, three distinct, non-overlapping fluorescent images were captured along the apical epithelial surface of the true vocal fold at 40x magnification at a constant exposure

time (200 ms for DAPI, 1 s for Texas Red). Microscopy quantification procedures were performed using Nikon Imaging Software Elements, Basic Research 3.22.00 (Nikon Instruments, Inc., Melville, NY) under Annotated Measurement Tools. To quantify the mean intensity of fluorescence for TNF- α staining, a region of interest was selected in each image to identify only the vocal fold epithelium. The region of interest was defined as the entire length of the side facing the lumen (e.g., black) with several layers of closely-packed DAPI staining cells. The epithelium ended when the primarily non-cellular lamina propria was reached, which was determined by a significant reduction in the number of DAPI-staining cells. The mean intensity of the fluorophore Texas Red was captured and adjusted until it appeared that all TNF- α -labeled cells were visible. The mean intensity values for Texas Red were exported for analysis. For each specimen, the intensity values for Texas Red were averaged across three distinct images as the final data point. To obtain intra-rater reliability, the experimenter repeated the quantification procedures on 10% of the images ($n = 6$) that were selected at random.

2.8 Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows v. 23.0 (IBM Corp., 2015, Armonk, NY). Pearson product-moment correlation coefficients and single measures intraclass correlation coefficients were used to assess the intra-rater reliability for the dependent measurements of TUNEL staining, epithelial cell nuclei area, and TNF- α protein expression. Shapiro-Wilk W tests revealed that all tested variables met the assumption of normality ($p > .05$). A series of one-way between subjects analyses of variance (ANOVAs) were computed to determine the main effect of increasing time-doses of vibration exposure on the means of TUNEL staining, epithelial cell nuclei area, TNF- α gene transcript levels, and TNF- α protein expression. Significant main effects were followed up with post-hoc comparisons using the Tukey's Honestly Significant Difference test. A series of Pearson product-moment correlation coefficients were computed to investigate the correlations between total extrapolated cycle-doses of vibration exposure and the means of TUNEL staining and epithelial cell nuclei area. For all analyses, statistical significance was determined by an alpha level of $p < .05$.

3 Results

3.1 Extrapolated Cycle-Doses

To determine if there was an effect of time on F_0 (Hz) and vocal intensity (dB sound pressure level) in the experimental conditions (i.e., phonation), six separate one-way repeated measures ANOVAs were computed. Results showed no significant difference in F_0 across time interval in the 30-minute, $F(2) = 1.19$, $p = .354$, 60-minute, $F(4) = 0.49$, $p = .741$, and 120-minute phonation time-doses, $F(8) = 1.18$, $p = .342$ (Figure 1A). There was no significant difference in vocal intensity across time in the 30-minute, $F(2) = 1.87$, $p = .216$, 60-minute, $F(4) = 1.68$, $p = .204$, and 120-minute phonation time-doses, $F(8) = 0.90$, $p = .527$ (Figure 1B). Because acoustic measures were stable over time, F_0 and vocal intensity were averaged for each time-dose of 30, 60, and 120 minutes. To evaluate the effect of increasing time-dose on F_0 and vocal intensity, two separate one-way between subjects

ANOVAs were computed. Results showed no significant differences in F_0 , $F(2, 82) = 1.64$, $p = .200$ and vocal intensity, $F(2, 82) = 2.08$, $p = .131$ across conditions.

An extrapolation calculation was performed to estimate the total cycle-doses of vocal fold vibration. As expected, a between subjects one-way ANOVA revealed a significant main effect of time-dose on extrapolated cycle doses, $F(2, 12) = 301.86$, $p = .000$ (Figure 1C). Post-hoc testing showed that mean total extrapolated cycle-doses were significantly higher in the 120-minute time-dose ($M = 1,248,123.76$, $SD = 69,382.02$) compared to the 60-minute ($M = 758,364.12$, $SD = 55,773.18$; $p = .000$) and 30-minute time-doses ($M = 415,803.78$, $SD = 27,774.83$; $p = .000$). Extrapolated cycle-doses were significantly higher in the 60-minute time-dose compared to the 30-minute time-dose ($p = .000$).

3.2 Intra-Rater Reliability

Measurement 1 and measurement 2 were strongly and positively correlated for TUNEL staining, $r(4) = .82$, $r^2 = .67$, $p = .046$, epithelial cell nuclei area, $r(4) = .96$, $r^2 = .92$, $p = .002$, and TNF- α protein expression, $r(4) = .92$, $r^2 = .85$, $p = .009$. The intraclass correlation coefficient was good for TUNEL staining, ICC = .71, 95% confidence interval (.40, .95), excellent for epithelial cell nuclei area, ICC = .94, 95% confidence interval (.86, .99), and excellent for TNF- α protein expression, ICC = .91, 95% confidence interval (.81, .99). Intra-rater reliability measurements indicated acceptable repeatability.

3.3 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

Figure 2 displays representative fluorescent TUNEL-stained vocal fold tissue specimens in the control (Figure 2A), 30-minute (Figure 2B), 60-minute (Figure 2C), and 120-minute phonation time-doses (Figure 2D) acquired at 40x magnification. There was a significant main effect of time-dose on the mean intensity of TUNEL staining, $F(3, 16) = 4.41$, $p = .019$. Post-hoc testing revealed that the mean intensity of TUNEL staining in the vocal fold epithelium of animals receiving 120-minute time-doses ($M = 375.74$, $SD = 90.62$) was significantly higher than animals receiving 120 minutes of vocal fold approximation alone (control) ($M = 228.00$, $SD = 45.10$; $p = .02$). The mean intensity of TUNEL staining across control and experimental conditions is shown in Figure 2E. The positive control confirmed that the TUNEL protocol successfully induced cleavage of genomic DNA, as evidenced by extensive labeling of TUNEL in the majority of cells in a rabbit tonsil specimen (Figure 2F). The negative control confirmed that there was no presence of TUNEL staining in a true vocal fold tissue section that was exposed to the 120-minute phonation time-dose (Figure 2G). There was no significant correlation between extrapolated cycle-doses of vibration and the mean intensity of TUNEL staining, $r(13) = .51$, $r^2 = .26$, $p = .055$.

3.4 Transmission Electron Microscopy

Figure 3 displays representative TEM images of vocal fold tissue specimens in the control (Figure 3A), 30-minute (Figure 3B), 60-minute (Figure 3C), and 120-minute phonation time-doses (Figure 3D) acquired at 4400x magnification. There was no significant main effect of time-dose on the mean area of epithelial cell nuclei, $F(3, 16) = 1.56$, $p = .239$. The mean area of epithelial cell nuclei across control and experimental conditions is shown in

Figure 3E. There was no significant correlation between extrapolated cycle-doses of vibration and the mean area of epithelial cell nuclei, $r(13) = -0.40$, $r^2 = .16$, $p = .137$.

3.5 TNF- α Gene and Protein Expression

Figure 4A shows a boxplot of median mRNA expression ratios of TNF- α across control and experimental conditions. There was no significant main effect of time-dose on gene transcript levels of TNF- α , $F(3, 15) = 2.18$, $p = .133$. The mean intensity of TNF- α protein expression across control and experimental conditions is shown in Figure 4B. Results revealed no significant main effect of time-dose on TNF- α protein expression, $F(3, 16) = 0.62$, $p = .61$. Additionally, there was no significant correlation between gene transcript levels of TNF- α and the mean intensity of TNF- α protein expression, $r(17) = -.05$, $r^2 = .00$, $p = .856$. A Pearson product-moment correlation coefficient was computed to investigate the relationship between the mean intensity of TUNEL staining and the mean intensity of TNF- α protein expression. Figure 4C shows a scatterplot of TUNEL staining and TNF- α protein expression. Results revealed that the mean intensity of TUNEL staining was positively correlated with the mean intensity of TNF- α protein expression, $r(18) = .53$, $r^2 = .28$, $p = .017$. As TUNEL staining increased, TNF- α protein expression also increased.

4 Discussion

The purpose of this study was to quantify the effects of increasing time-doses and extrapolated cycle-doses of vibration exposure on the rate of apoptosis signaling in the vocal fold epithelium. Two common techniques were used to measure apoptosis. First, TUNEL method was used to detect free hydroxyl DNA ends of nuclear condensed chromatin generated by apoptotic cells. This technique enzymatically detects internucleosomal DNA cleavage, which is a hallmark characteristic of apoptosis when an endogenous endonuclease is activated (25). When DNA fragmentation occurs, TUNEL labeling indicates that these cells are committed to die via the apoptosis signaling pathway. Conversely, TEM is a method that provides high-resolution images and is considered the gold standard technique for characterizing the morphological features of apoptosis (26, 27).

TUNEL staining demonstrated that the longest studied time-dose (i.e., 120 minutes) of vibration exposure led to significantly increased intensity of immunofluorescent staining compared to vocal fold approximation alone for the same time duration. In contrast, TEM revealed no differences in the area of epithelial cell nuclei across experimental and control conditions. Rather, vocal folds that underwent approximation demonstrated the smallest epithelial cell nuclei area. These findings are in agreement with previous studies reporting the expression of TUNEL-labeled cells, condensed chromatin, and apoptotic bodies in the vocal folds after vibration and approximation (11). The present study adds to the body of literature objective quantification of TUNEL staining and the measurement of epithelial cell nuclei area. Additionally, this study provides evidence of increased TUNEL staining in vocal folds exposed to vibration for 120 minutes compared to control conditions.

Shorter time-doses of vibration exposure revealed no effects on TUNEL-labeled cells and morphological features of apoptosis using TEM. These results are interpreted to suggest that there may be a certain threshold of accumulated voicing time necessary to induce apoptosis.

Apoptosis signaling was detected in the current study at 120 minutes of vocal fold vibration exposure using the TUNEL method. The repeated biomechanical stresses accumulated over 120 minutes of vibration exposure appear to be sufficient to signal for epithelial cells to self-organize by undergoing apoptosis. The possibility that shorter durations of vibration exposure could initiate apoptosis cannot be ruled out, as time-doses between 60 and 120 minutes were not investigated in the current study. Conversely, very early stages of apoptosis may have been initiated prior to the detection of DNA fragmentation, which may suggest that there is a temporal delay in detecting apoptosis in the vocal folds.

TEM revealed no differences in the area of epithelial cell nuclei with increasing time-doses of vibration exposure. Results suggest that for the time-doses studied, vocal fold epithelial cells do not appear to shrink in size in response to vibration. Interestingly, the control condition demonstrated the smallest epithelial cell nuclei area but the least expression of TUNEL staining. It is possible that vocal fold adduction and abduction may have signaled an early sign of apoptosis in these cells. We hypothesize that the smaller area of epithelial cell nuclei may have been the result of an initial but subsequently interrupted strategy to signal cell death. Several attempts were made to perform immunohistochemical staining on vocal fold tissue specimens for caspase-3 activity. When activated by apoptosis, caspases act together as a cascade in the cleavage of proteins rather than random destruction of proteins, making caspase-3 an excellent biochemical marker of apoptosis (26). The assay stains for activation of the main caspase effector of the apoptotic cascade, caspase-3 (28). Unfortunately, the primary antibodies (i.e., Anti-ACTIVE® Caspase-3 pAb, Promega.; Anti-Caspase-3 antibody #ab2171, Abcam) and procedures that were used in this study showed nonspecific staining for caspase-3.

The second objective of this study was to determine the relationship between extrapolated cycle-doses of vibration exposure and apoptotic cell death in the true vocal fold epithelium. Extrapolated cycle-doses of vibration exposure were selected to more objectively quantify the number of cycles of vibration that the vocal folds experienced during the *in vivo* phonation procedure. In contrast to time-dose, which provides accumulated voicing time in minutes (i.e., 30, 60, 120), cycle-dose provides an estimation of the total number of vibratory cycles over time, and in this study was derived from mean F_0 . As expected, results revealed that total extrapolated cycle-doses increased as time-doses increased. Stated another way, the vocal folds were exposed to a higher number of cycles of vibration during longer periods of phonation. However, no relationships were found between cycle-doses and the outcome measures of TUNEL and TEM. These findings suggest that extrapolated cycle-doses may be less sensitive than time-doses with the current study's apoptosis measurements.

The present study indicated that neither TNF- α gene transcript levels nor TNF- α protein expression differed by time-dose. That is, TNF- α gene and protein levels did not change in response to increasing time-doses of vibration exposure. However, post-hoc testing revealed an interesting relationship between TNF- α and apoptosis, as the expression of TUNEL was positively related to TNF- α protein expression. In particular, as the intensity of fluorescent TUNEL staining increased, the intensity of fluorescent TNF- α staining also increased. This finding suggests that

TNF- α protein expression is upregulated in the vocal fold epithelium in response to vibratory-induced fragmentation of DNA. TNF- α is a cytokine that is important in initiating and directing the acute inflammatory response (29). In addition to promoting, maintaining, and regulating pro-inflammatory responses, TNF- α is largely involved in cell death, proliferation, differentiation, and communication (30, 31), and has been found to be upregulated in the vocal folds after iatrogenic injury and biomechanical trauma (32–34). Increased concentrations of TNF- α administered to cultured human vocal fold fibroblasts have been found to inhibit the rate of cell proliferation after 9 days (18). In contrast, Berchtold et al. (17) observed no evidence of cell death after 24 hours following TNF- α administration to cultured human vocal fold fibroblasts. It remains unknown whether TNF- α initiates apoptosis or whether apoptosis leads to alterations in TNF- α . It is also unclear whether TNF- α protein is being produced by the vocal fold epithelium or from cells within the lamina propria. Because TNF- α is a pro-inflammatory cytokine, it would seem logical that TNF- α was produced from cells that proliferate in response to injury such vocal fold fibroblasts (35). While the present study provides evidence of a relationship between apoptosis and TNF- α in the vocal fold epithelium, it would be ideal to more directly link apoptosis to TNF- α . Based on the current results, we are unable to conclude that TNF- α induces apoptosis in the vocal folds. Therefore, future research is necessary to further investigate TNF- α -induced apoptosis in epithelial cells of the vocal folds. Specifically, *in vitro* vocal fold epithelial cell lines would serve as an excellent model to approach this line of research. Our results revealed large variability in TNF- α mRNA expression ratios across conditions. One potential explanation for this variation is that gene expression was performed using the posterior one-third portion of the vocal fold specimens. Given that the mid-membranous portion of the vocal fold is generally believed to experience the greatest impact of collision during vibration, perhaps the evaluated tissue samples received a lessened extent of impact stresses. Thus, this might explain why TNF- α gene expression did not change systemically with time-dose.

4.1 Conclusion

Animal models are important in voice science research. The current study relied on an established *in vivo* animal model of phonation. In humans, it is impossible to control the amount of trauma that the vocal folds are exposed to during vibration. There is a large degree of variability in the etiology and maintenance of human vocal fold diseases. Fortunately, the current *in vivo* rabbit phonation model allows for systematic control over the amount of vibration exposure to the vocal folds, providing a more programmatic approach to studying tissue-level changes to the vocal folds during vibration. Despite that rabbits and humans differ in the function and frequency of phonation, many basic biochemical processes such as apoptotic cell death are similar across species. Therefore, the *in vivo* model is an appropriate initial step toward improving our understanding of apoptosis, which can eventually be translated to apoptosis in healthy and diseased human vocal folds.

The present study investigated the effects of time-doses and cycle-doses of vibration exposure on apoptosis signaling in the vocal fold. Additionally, we investigated the relationship between the pro-inflammatory cytokine TNF- α and apoptotic cell death in the vocal fold epithelium. Findings revealed evidence of apoptosis signaling in the vocal fold

epithelium after 120 minutes of vibration exposure. While this time-dose of vibration was found to signal epithelial cells in the vocal folds to die by apoptosis, it was also found that vocal fold adduction and abduction for 120 minutes in the absence of phonation resulted in an early sign of apoptosis. This morphological sign was characterized by cell shrinking, as measured in this study by a reduction in the size of epithelial cell nuclei. The current study showed that TUNEL staining was positively correlated with TNF- α protein expression. The results of this study may serve as a foundation to more accurately characterize the function of vocal fold epithelial cell death, as well as a preliminary step toward determining the potential implications of cell death in vocal fold diseases of epithelial origin.

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Abbreviations

ANOVA	analysis of variance
BSA	bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
F₀	fundamental frequency
FITC	fluorescein isothiocyanate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time polymerase chain reaction
SDHA	succinate dehydrogenase complex subunit A
tDt	terminal deoxynucleotidyl transferase
TEM	transmission electron microscopy
TNF-α	tumor necrosis factor-alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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Highlights

- The rate of apoptosis can be quantified in vocal fold epithelium after vibration.
- Apoptosis is induced after 120 minutes of modal intensity phonation.
- Shorter durations of vibration exposure do not result in apoptosis signaling.
- TUNEL staining is positively correlated with TNF- α protein expression.

Figure 1.

Extrapolated cycle-doses of *in vivo* rabbit vocal fold vibration. No differences were observed in A) mean fundamental frequency (Hz) and B) mean vocal intensity (dB) values (y-axis) across 15-minute time intervals (x-axis) in the experimental conditions. Triangles represent means for 30-minute time-doses, multiplication symbols represent means for 60-minute time-doses, and circles represent means for 120-minute time-doses. Error bars represent standard deviations of the means. C) Mean total extrapolated cycle-doses of vocal fold vibration exposure (x-axis) were significantly different across time-doses (y-axis). Mean cycle-doses were significantly higher in the 120-minute time-dose compared to the 60-minute and 30-minute time-doses, and mean cycle-doses were significantly higher in the 60-minute time-dose compared to the 30-minute time-dose. Error bars represent standard deviations of the means and *** indicates $p < .001$.

Figure 2.

Analysis of intensity of fluorescence staining in the true vocal fold epithelium using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. Panels A–D and F–G are fluorescent stained vocal fold tissue specimens in the coronal plane showing the apical cell surface on the right. Images were acquired at 40x magnification. Outlined regions of interest in red show the quantified areas of intensity of TUNEL staining in the A) control, B) 30-minute, C) 60-minute, and D) 120-minute phonation time-doses. E) Mean intensity of TUNEL staining (bits) (y-axis) differed across control and experimental conditions (x-axis). TUNEL staining was significantly increased in the 120-minute time-dose compared to the control condition. Error bars represent standard deviations of the means and * indicates $p < .05$. Fluorescent stained tissue specimens are shown of a F) paraffin section of rabbit tonsil as the positive control and G) paraffin section of rabbit true vocal fold epithelium exposed to the 120-minute time-dose as the negative control.

Figure 3.

Analysis of area of true vocal fold epithelial cell nuclei by transmission electron microscopy (TEM). Panels A–D are TEM images of the apical cell surface of the vocal fold epithelia (right). Images were acquired at 4400x magnification. Outlined regions of interest in green show the quantified areas of cell nuclei with corresponding measurements in the A) control, B) 30-minute, C) 60-minute, and D) 120-minute phonation time-doses. E) Mean area of epithelial cell nuclei (px²) (y-axis) did not significantly differ across control and experimental conditions (x-axis). Error bars represent standard deviations of the means.

Figure 4.

Analysis of gene and protein expression of tumor necrosis factor-alpha (TNF- α). A) Log-transformed mRNA expression ratios of TNF- α (y-axis) did not significantly differ across control and experimental conditions (x-axis). Thick horizontal black lines display the median, bars above the horizontal black lines display the upper quartile, and bars below the horizontal black lines display the lower quartile. Error bars display maximum and minimum values. Asterisks represent outlier values. B) Mean intensity of TNF- α protein expression (bits) (x-axis) did not significantly differ across control and experimental conditions (x-axis). Error bars represent standard deviations of the means. C) Scatterplot showing a positive correlation between mean intensity of TUNEL staining (x-axis) and mean intensity of TNF- α protein expression (y-axis). As TUNEL staining increased, protein expression of TNF- α increased.

Table 1

Cycle-Dose Extrapolation Calculations

Measurement	Calculation
Cycle-dose per train duration	Mean F0 x 3 seconds of train duration
Cycle-dose per minute	Cycle-dose per train duration x 6 trains per minute
Cycle-dose per 15-minute interval	Cycle-dose per minute x 15 minutes per interval
Total extrapolated cycle-doses	Sum of all cycle-doses per 15-minute intervals

Table 2

Primer Sequences

<u>Gene</u>		
	<u>Forward Primer</u>	<u>Reverse Primer</u>
TNF- α	TCTTCTCTTTCCTGCTCGTG	TTGTTTGGGGACTGCTCTTC
SDHA	CATTTAGCAGAACTGAAGAC	CATTTAGCAGAACTGAAGAC

Note. TNF- α = tumor necrosis factor-alpha. SDHA = succinate dehydrogenase complex subunit A