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Quantitative Study of Vibrational Symmetry of Injured Vocal Folds Via Digital Kymography in Excised Canine Larynges

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Abstract

Purpose—Digital kymography and vocal fold curve fitting are blended with detailed symmetry analysis of kymograms to provide a comprehensive characterization of the vibratory properties of injured vocal folds.

Method—Vocal fold vibration of twelve excised canine larynges was recorded under uninjured, unilaterally injured, and bilaterally injured conditions. Kymograms were created at 25%, 50%, and 75% of the vocal fold length, and vibratory parameters were compared quantitatively among conditions and studied with respect to right-left and anterior-posterior symmetries.

Results—Anterior-posterior amplitude asymmetry was found in the bilateral condition. The unilateral condition showed significant right-left amplitude asymmetry, and it showed the lowest right-left phase symmetry among the conditions. In condition comparisons, vertical phase difference did not show significant differences among conditions, while amplitudes were significantly different among conditions at all line scan positions and most vocal fold lips. Significant differences in frequency were found among the conditions at all four vocal fold lips, with the bilateral condition exhibiting the greatest frequency.

Conclusion—Digital kymography and curve fitting provide detailed information about the vibratory behavior of injured vocal folds. Awareness of vibratory properties associated with vocal fold injury may aid in diagnosis, and the quantitative abilities of digital kymography may allow for objective treatment selection.

Keywords

Digital kymography; vocal fold injury; excised larynx; anterior-posterior symmetry; right-left symmetry

INTRODUCTION

Vocal fold scarring presents a significant impediment to normal mucosal wave and voice function. It is a common vocal pathology observed in the clinical setting which can be induced by voice abuse, inflammation, trauma, radiation, neoplasia, improper healing after vocal fold surgeries, and other injuries to the vocal folds (Benninger et al., 1996). Poor voice quality and dysphonia may result from vocal fold surgery due to acute disruption of the vocal fold lamina propria (Woo, Casper, Colton, & Brewer, 1994). In scarred vocal folds,

the formation of stiff scar tissue disrupts the consistency of the mucous membrane cover, leading to asymmetrical vibration and decreased presence of mucosal wave (Benninger et al., 1996; Sataloff, Spiegel, Hawkshaw, Rosen, & Heuer, 1997). Scarring may also affect other vocal fold vibratory properties such as amplitude, frequency, vertical phase difference, and lateral phase difference. Vibratory amplitude is the mediolateral distance the vocal fold travels between its maximum opening and maximum closing. Vibratory frequency is reported in Hz, and it is defined as the number of vibratory cycles per second. Sulter, Miller, and Schutte (1996) define lateral phase difference and vertical phase difference as phase differences between the right and left vocal folds and between the caudal and cranial parts of the vocal folds, respectively. A lateral phase difference is present when the right and left vocal folds reach their respective maximum opening or closing positions at different times. A vertical phase difference is present when there is a time delay between the opening (and eventual closing) of the upper and lower vocal folds. Vertical phase difference, therefore, can be used to measure the vertically traveling mucosal wave.

The mucosal wave is a property of vocal fold vibration characterized by the alternating mediolateral motion of the upper and lower vocal fold lips. Subglottal pressure against the lower vocal fold lips causes them to open and move laterally, and when the pressure continues upward, the upper vocal fold lips open. The decrease in pressure in the subglottal space following the vertical release of air during the opening of the upper vocal fold lips pulls the lower vocal fold lips closed in the medial direction. This is followed by the closing of the upper vocal fold lips as well (Krausert et al., 2010). These successive movements of the upper and lower vocal folds are facilitated by the pliability of the mucosal epithelium and upper lamina propria, and they resemble a vertically traveling wave. Thus, this feature of vocal fold vibration is termed the mucosal wave. Accurate assessment of the mucosal wave is vital in determining the efficiency of vocal fold vibration. Videostroboscopy is currently the most popular method for the detection and measurement of the effect of scarring on vibration and mucosal wave function. Several vocal fold scarring studies using videostroboscopy have noted poor pre-treatment amplitude and symmetry of vibration, irregular glottic closure, and reduced presence of mucosal wave in cases of scarring, and they observed improvements after autologous fat injection or implantation (Hsiung, Woo, Minasian, & Schaefer Mojica, 2000; Hsiung, Kang, Pai, Su, & Lin, 2004; Neuenschwander et al., 2001; Sataloff et al., 1997). Furthermore, Rosen (2000) noted that the irregular glottic closure caused by vocal fold scarring may result in a sufficient amount of air loss and symptoms such as shortness of breath and breathy voice. Benninger et al. (1996) add that amplitude measurements with stroboscopy reveal asymmetric amplitude of vibration and a non-vibrating segment preventing mucosal wave propagation. He suggests that the high pressure necessary to sustain phonation in scarred vocal folds may contribute to the observed incomplete glottic closure.

Although these findings are generally accepted, the vibratory parameters in these studies are measured on a largely qualitative basis with human rating systems. It is valuable to know if vibratory parameters are abnormal in scarred vocal folds and whether or not they improve with treatment, but the degree to which they are injured or to which they improve can be somewhat ambiguous with these qualitative measurements. In addition, it is difficult to compare qualitative ratings of vibratory properties between studies because of varying parameter rating scales and different judges. Quantitative measurements of vibratory parameters would provide additional information about how severely the vocal folds are scarred and which treatment may be used accordingly. For example, detecting small differences in the vibratory dynamics of a patient's vocal folds before and after the development of a minimally severe scar might be easier with a specific quantitative measurement system than with a broad rating scale. The detection of this small difference

could help the clinician determine if a treatment like voice therapy might be appropriate for such a patient.

Thus far, few quantitative studies have been performed to investigate vibratory parameters in vocal fold scarring cases. Bjorck, D'Agata, and Hertegard (2002) used videostroboscopy to study the effects of collagen injection on scarred vocal folds. Amplitudes of vibration were measured in pixels, and they increased following proper collagen injection. Okamura, Yumoto, and Okamoto (1987) studied vocal fold scarring and the ensuing healing process using high speed imaging and quantitatively measured the amplitude of vibration by tracing the edge of the midpoint of the membranous sections of the vocal folds, but they did not provide the amplitude values comparing scarred and treated vocal folds. Neither of these studies reported amplitude in millimeters or a similar physical unit of measurement that would allow for comparison with other examples of scarred vocal folds outside of the immediate experimental study. Also, phase differences between the left and right vocal folds, namely, the degree of temporal symmetry of their opening and closing, in normal and scarred vocal folds were not reported.

Other techniques, such as high speed imaging and videokymography, have also been used to study a wide range of voice conditions. These techniques employ high frame rates to obtain a large amount of information about the vibratory properties of the vocal folds. Kymography may be more effective than stroboscopy for studying scarring because aperiodic vibrations may result from vocal fold injury. Stroboscopy cannot visualize aperiodic vibrations because the strobe light and camera depend on a stable frequency in order to image successive points in the glottal cycle. Svec, Schutte, and Sram (1996, 1999, 2007) used videokymography to clearly visualize vibratory properties including left-right symmetry, vertical phase difference (between upper and lower vocal fold lips), and open and closed phases. Jiang et al. (2000) and Jiang, Zhang, Kelly, Bieging, and Hoffman (2008) used kymography to quantitatively study vibratory parameters. In addition to the aforementioned reasons, kymography may be an effective method for investigating vocal fold scarring because vocal fold scarring has been suggested to alter vibration and lead to abnormal vibratory amplitude and phase (Sataloff et al., 1997), parameters that can be easily measured from kymograms.

Kymography displays the vibration of both the right and left vocal folds and can be modified to simultaneously extract several pixel lines perpendicular to the glottal axis from high speed video. These properties make it suitable for studying asymmetry. Asymmetry of vocal fold vibration often serves as a useful indicator of laryngeal abnormalities. Right-left asymmetry can be observed by evaluating the amplitude, frequency, phase differences, and axis shifts between the left and right vocal folds (Svec et al., 2007). A careful analysis of anterior-posterior asymmetry also holds notable clinical importance. With regard to the anterior-posterior dimension, a strong emphasis has been placed on evaluating phase asymmetry (Bonilha, Deliyski, & Gerlach, 2008). In this case, anterior-posterior phase is symmetrical when points equidistant from the midpoint of one vocal fold simultaneously reach maximum opening within a glottic cycle. Several techniques have been used to analyze right-left and anterior-posterior symmetries. Eysholdt, Rosanowski, and Hoppe (2003) used double-line kymography to qualitatively display a vibration frequency asymmetry between the right and left vocal folds and show the presence of anterior-posterior modes. Wittenberg, Tigges, Mergell, and Eysholdt (2000) used digital multislice kymography and juxtaposed four kymograms from various points along the same vocal folds to qualitatively observe anterior-posterior amplitude and phase asymmetry as well as right-left frequency and amplitude asymmetry. With regard to right-left asymmetry, Bonilha et al. (2008) measured phase asymmetry with digital kymography by taking a ratio of the sum of the pixel distances between the right and left glottal cycle peaks in three consecutive cycles to the total pixel distance of the sum of the periods of the cycles. Using a similar method, Qiu, Schutte, Gu,

and Yu (2003) defined the phase symmetry index to be the difference between the phase of the right and left vocal folds divided by the period of the vibratory cycle. Amplitude symmetry index, the difference between the amplitudes of the right and left vocal folds divided by the sum of the amplitudes, was also studied. Sung et al. (1999) studied right-left asymmetry with videostrobokymography by directly comparing the right and left glottic areas. These studies reach beyond observational judgment of vibrational symmetry to methodically examine anterior-posterior and right-left symmetries of the vocal folds, but they do not investigate the effects of vocal fold scarring on symmetry. We aimed to use similar principles and a similar level of detail in our analysis of symmetry in this study.

Symmetrical and organized vibration of the vocal folds is crucial to the quality of voice production. Vocal fold scarring severely interrupts this organized vibration. Most vocal fold scarring studies have used ratings to judge the level of phase (Hsiung et al., 2000; Sataloff et al., 1997) and amplitude (Sataloff et al., 1997) asymmetries, and several used two point scales (symmetry vs. asymmetry) to rate phase asymmetry which do not account for the extent of asymmetry (Biever & Bless, 1989; Haben, Kost, & Papagiannis, 2003; Sulter et al., 1996). Because these qualitative studies have suggested that vibratory asymmetry is a result of vocal fold scarring, this study aimed to model vocal fold scarring and quantitatively investigate the effects of vocal fold injury on anterior-posterior and right-left symmetries by completing a detailed symmetry analysis of amplitude and phase difference from digital kymograms. Quantitative comparisons of these parameters, along with vibratory frequency, were also performed among uninjured, unilaterally injured, and bilaterally injured vocal folds. Thus, we provide a comprehensive characterization of the vibratory properties of injured vocal folds.

METHODS

Population sample

Twelve larynges were harvested from mongrel dogs euthanized for non-research purposes. These larynges were physiologically intact, void of pathologies and trauma, and were stored in 0.9% saline solution at a temperature of -12°C until used, whereupon they were thawed.

The larynges were dissected following the procedure of Jiang and Titze (1993). The larynges were excised with about 5 cm of trachea remaining inferior to the cricoid cartilage. Along with the tissues superior to the vocal folds and a majority of extrinsic laryngeal muscles, the superior part of the thyroid cartilage was removed to the vertical level of the vocal folds to allow sufficient visualization. This also permitted two bilaterally-positioned three-pronged micrometers to be inserted in the arytenoid cartilages.

Experimental setup

Experiments were performed using the excised larynx setup in Figure 1. The excised larynx phonation apparatus was placed in a triple-walled room in order to minimize the influence of possible background noise. The trachea inferior to the larynx was secured to the air pipe with a hose clamp. Airflow was generated by an internal building source. Before reaching and eventually passing through the larynx, the air was conditioned to 35°C to 38°C and 95% to 100% relative humidity using two ConchaTherm III heater-humidifiers (Fisher and Paykel Healthcare, Inc., Laguna Hills, California) placed in series. This sequence was designed to mimic the temperature and humidity of air in the human respiratory system. The two bilaterally-positioned micrometers were used to secure the arytenoid cartilages and to fix them in an adductory posture (which allows glottic closure necessary for phonation). The vocal folds were hydrated with 0.9% saline solution throughout the experiment to minimize the effect of dehydration.

Airflow was manipulated and allowed to build up until subglottal pressure sufficient for phonation was obtained. Vocal fold vibrations were then recorded at subglottal pressures of 20 cm H₂O with a high speed digital camera (Fastcam-ultima APX, Photron USA, Inc., San Diego, California) mounted on a track system directly above the larynx. The camera recorded vibration at 4000 frames per second with a resolution of 256 × 512 pixels.

Five consecutive video recordings, with a time lapse of approximately one second between each recording, were taken during sustained phonation. Due to a pre-set partition duration on the camera, each individual recording lasted 768 frames, or 0.192 seconds. For the purpose of consistency, two representative recordings of the five were selected to be analyzed based on the degree of phonation stability. About two minutes after the first set of recordings, vocal fold scarring was simulated by injuring each larynx unilaterally at the midpoint of the right vocal fold using a soldering iron (Weller WESD51, Cooper Industries, Houston, Texas) at 850°F. The midpoint was approximated based on the anterior-posterior length of the vocal fold. The tip of the soldering iron was approximately 2 mm in length, and it was applied to the vocal fold for about one second. Following the creation of the injury on the right vocal fold, the larynx was re-mounted on the excised larynx phonation apparatus. This process was performed consistently for all larynges and lasted approximately five minutes. Vocal fold vibrations were recorded again, in the same manner as was applied to the uninjured vocal folds, at 20 cm H₂O. About two minutes after these recordings, the site of injury on the left vocal fold was selected in the same way as the right vocal fold. Then, the larynx was re-mounted and vibrations were recorded again at 20 cm H₂O. The videos from the initially uninjured vocal folds were used as the control condition to be compared with the unilaterally and bilaterally injured conditions. Figure 2 displays a photograph of the vocal folds under each condition. In previous experiments, a very similar injury-induction method was used by Zhang, Jiang, and Appel (2010) in studying the spatiotemporal vibratory characteristics of scarred canine vocal folds. The technique of creating an injury with heat may be a good model for vocal fold scar because it simulates the effect of lasers, which have been used frequently in laryngeal surgeries. The energy emitted from a CO₂ laser, for example, has the potential to create localized heat adequate to induce thermal injury and necrosis to the vocal folds, which often result in the formation of a vocal fold scar (Sataloff, Spiegel, Hawkshaw, & Jones, 1992; Reinisch & Ossoff, 1996).

The pressure 20 cm H₂O was used because it is low enough to avoid chaotic vibration, which cannot be analyzed with the curve fitting program used here. Also, 20 cm H₂O is high enough that injured vocal folds can easily phonate. The phonation threshold pressure averages (and standard deviations) for the control, unilateral, and bilateral conditions were 6.70 (3.73), 9.11 (4.21), and 12.50 (6.14) cm H₂O, respectively. Using a pressure much lower than 20 cm H₂O could jeopardize the inclusion of videos from the bilateral condition because there is a chance that the vocal folds would not phonate, and therefore it would be difficult to compare them with other larynges.

The average vocal fold length and standard deviation were 17.043 mm and 2.074 mm, respectively. The average anterior-posterior length of injury (and standard deviation) was 3.344 (1.041) mm for the left vocal fold and 3.200 (1.003) mm for the right vocal fold. The average depth of injury (and standard deviation) was 0.104 (0.038) mm for the left vocal fold and 0.093 (0.033) mm for the right vocal fold. A digital caliper was used to perform these measurements. These superficial injuries were designed to influence vibration by disrupting the vocal fold cover, which consists of the epithelium, the basement membrane zone, and the superficial lamina propria (Hirano, 1974; Ayala, Selig, Faquin, & Franco, 2007). There has been little quantitative investigation of the depth of the canine vocal fold epithelium, but Garrett, Coleman, and Reinisch (2000) report that the thickness of human epithelium is about 0.05 mm. Kurita, Nagata, and Hirano (1983) note that although the

canine superficial lamina propria is much thicker than that in humans, the mechanical properties of the epithelium don't seem to be significantly different. Therefore, the injuries may have spanned the epithelium to reach the basement membrane zone and a small portion of the superficial lamina propria.

Data analysis

Digital kymography and curve fitting were performed with a custom MATLAB (version 7.2.0.232 (R2006a), The Mathworks, Inc., Natick, Massachusetts) image edge detection program. Three pixel lines perpendicular to the glottal axis were selected to create kymograms. Line scan position was measured as a percentage of the vocal fold length. Lines selected were located at 25% (between the posterior commissure and the midpoint), 50% (at the midpoint), and 75% of the vocal fold length (between the midpoint and the anterior commissure). These kymograms could then be compared for anterior-posterior symmetry to observe the potential influence of scarring on the vibration along the length of the vocal folds.

In order to curve fit the vibration patterns in the kymogram, the glottal edges must be extracted. This has been done in the past with the threshold segmentation method to separate pixel intensities from the glottis and the surrounding vocal fold tissue (Jiang et al., 2008; Zhang & Jiang, 2005; Zhang, Jiang, Tao, Bieging, & MacCallum, 2007; Zhang, Bieging, Tsui, & Jiang, 2010). The MATLAB program used in this study, however, has the ability to manually plot points along the glottal edge in cases of poor resolution in addition to performing the original threshold technique. Compared to the original threshold technique, the manual plotting technique is better suited to differentiate between the upper and lower vocal fold lips. The original threshold technique can only separate glottis from non-glottis, and therefore, pixels with a lighter shade than the glottis do not contribute to the curve fitting trajectory for a certain vocal fold lip. This may be a problem when the upper vocal fold (which is brightly illuminated by the light source) extends further in the lateral direction while the lower vocal fold (which has a slightly darker shade than that of the upper vocal fold) appears and starts to close in the medial direction. The manual plotting technique can account for the difference between the upper and lower vocal fold lips, and therefore, it was used for most of the data analysis. The left-upper (LU), left-lower (LL), right-upper (RU), and right-lower (RL) lips of the vocal folds were segmented and distinguished to produce one curve each, allowing for the comparison of amplitude, frequency, and phase difference parameters.

With the curve fitting method, the mucosal wave motion was modeled with a sine wave where $Y_{\alpha}(t)$ is the position of vocal fold lip α at time t . $\alpha = 1, 2, 3, 4$ correspond to the left-upper, left-lower, right-upper, and right-lower vocal fold lips, respectively, as illustrated in Figure 3.

$$Y_{\alpha}(t) = A_0 + \sum_{n=1}^n A_n \sin(2\pi f t + \varphi_n)$$

where n can range from 1 to 8 and represents the order number of the vibration. A_0 , the initial offset amplitude, is a measure of the vertical displacement of a wave within the picture frame. This is calculated automatically by the MATLAB program, but it is not important for the purposes of this study. A_1 represents the sine wave amplitude as defined in physics, and it gives information about the magnitude of vibration between the open and closed phases of the glottal cycle. f , the sine wave frequency, also serves as the frequency of vibration because each repetition of a sine wave corresponds to one glottal cycle. t

represents time. ϕ , the sine wave phase number, is determined by the horizontal location (point in time) of the wave within the picture frame. Waves with the same frequency and amplitude but different phase numbers are shifted in time so that they are not superimposable, while they would be superimposable if their phase numbers were the same. Assuming that frequency is constant, the phase numbers of two waves can be subtracted to find the phase difference at a certain point in time, as shown in Figure 3. This was useful for comparison between vocal fold lips. Finding the phase difference between upper and lower lips of either the right or the left vocal fold provides information about the natural time delay involved with mucosal wave propagation. The phase difference between either the upper or the lower lips of the right and left vocal folds provides information about the right-left phase symmetry of vibration. According to the previously mentioned definitions of lateral and vertical phase difference by Sulter et al. (1996), we will define the phase differences RU – LU and RL – LL as upper and lower lateral phase difference, respectively, and RU – RL and LU – LL as right and left vertical phase difference, respectively.

Because the wave phase numbers ϕ of the individual curves could range from 0 to 2π radians, we calculated the phase difference by taking the absolute value of the difference between the wave numbers to avoid negative values. If this value was greater than π , we subtracted the value from 2π to get our final phase difference. This ensured that the phase difference would always be within the range of 0 and π radians. This simplifies the evaluation process of phase differences because, for our purposes, 0 radians and 2π radians represent the same phase shift. Waves with phase differences of $0 + 2\pi n$ ($n = 0, 1, 2, \dots$) are considered to show constructive interference (crests of one wave line up with crests of the other wave, and troughs line up with troughs), and waves with phase differences of $\pi + 2\pi n$ ($n = 0, 1, 2, \dots$) show destructive interference (crests of one wave line up with troughs of the other wave). Rather than determining the proximity of the phase difference to π from either greater or lower than π on a scale from 0 to 2π , the scale was halved so that 0 radians represents one extreme and π radians represents the other. Therefore, for lateral phase difference, maximum asymmetry occurs when the right-upper and left-upper or right-lower and left-lower lips have a phase difference of 0 radians because the trough of the right vocal fold would line up with the trough of the left vocal fold, or their crests would line up (Figure 4). This would indicate that at the time of the maximum opening position of one vocal fold, the other vocal fold would be at its maximum closing. A lateral phase difference of π radians indicates maximum symmetrical vibration because at glottal closure, the trough of the left vocal fold lines up with the crest of the right vocal fold, and vice versa for maximum glottal opening, as illustrated in Figure 4. The extremes of the 0 to π radians scale for vertical phase difference have different meanings than those for lateral phase difference. Zero radians corresponds to the smallest possible phase difference between the upper and lower vocal folds (crests match up with crests, and troughs with troughs), and π radians corresponds to the largest possible phase difference. Figure 3 provides a good example of a small vertical phase difference.

The MATLAB software program used in this study ideally fitted a sine wave to its respective vocal fold lip so that the crest and trough corresponded to the points of maximum glottal opening and closing, depending on which vocal fold is being considered. The troughs of the right vocal fold correspond to maximum glottal opening and its crests correspond to maximum glottal closing, and vice versa for the left vocal fold. Because the amplitude of a sine wave is half of the vertical distance between the crest and the trough (the peak-to-peak amplitude), this value must be doubled in order to relate to the vibratory amplitude, which is the maximum lateral displacement of the vocal fold lip from its medial position. The doubling of the amplitude value obtained from the sine wave allowed it to be transformed into a physiologically relevant parameter. This MATLAB program also allowed for calibration from pixels to millimeters. The vocal folds were measured in millimeters with a

digital caliper before the experiment. A function in the program allows the user to draw a line to mark the pixel length of the known distance and enter the actual distance in millimeters. Therefore, a line was drawn to mark the distance between the anterior and posterior ends of the vocal fold, and the millimeter measurement from the digital caliper was entered to allow the program to create a proportion to assign pixels to a certain length in millimeters.

Statistical analysis

Paired t-tests (and Wilcoxon Signed Rank Tests in cases of failure of the Shapiro-Wilk test for normality ($P < 0.050$)) were used for right-left amplitude symmetry analysis. For right-left amplitude symmetry analysis, the vibratory parameter amplitude was the dependent variable and vocal fold lip was the independent variable. One-way repeated measures analysis of variance (and Friedman repeated measures analysis of variance on ranks in cases of failure of the Shapiro-Wilk test for normality ($P < 0.050$)) was used in anterior-posterior amplitude symmetry analysis, right-left phase symmetry analysis, and condition comparisons. For anterior-posterior amplitude symmetry analysis, the vibratory parameter amplitude was the dependent variable, and line scan position was the independent variable. This analysis was performed for the unilaterally and bilaterally injured conditions. For right-left phase symmetry analysis, the vibratory parameter lateral phase difference was the dependent variable, and condition was the independent variable. For condition comparisons, the vibratory parameters amplitude, vertical phase difference, and frequency were the dependent variables, and condition was the independent variable. A significance level of 0.05 was used for all tests.

Box plots were used to graphically display the data from these analyses. The box boundaries represent the 25th and 75th percentiles, the error bars represent the 10th and 90th percentiles, the line through the box represents the median, and the dots represent the outlying points.

RESULTS

To examine measurement precision, the reliability of the manual plotting technique applied to the kymographic glottal edges was tested by performing twenty curve fitting trials on the same five cycles of vibration from the same video recording at a constant line scan position. Table 1 reports the calculated mean and standard deviation of the amplitude and the frequency of all four vocal fold lips (LU, LL, RU, RL), the right and left vertical phase difference (RU – RL and LU – LL), and the upper and lower lateral phase difference (RU – LU and RL – LL).

Figure 3 shows an example of a typical curve-fitted kymogram. The colors blue, green, red, and teal correspond to the left-upper, left-lower, right-upper, and right-lower vocal fold lips, respectively. This example shows slight right-left asymmetry in amplitude, with the left vocal fold lips exhibiting larger amplitudes than those of the right vocal fold lips. The right and left vocal folds are symmetrical with respect to phase difference. The lateral phase differences (RU – LU and RL – LL) are approximately π radians because on both the upper and lower vocal fold lips, the crests of the left line up with the troughs of the right. The vertical phase differences (RU – RL and LU – LL) are just above 0 radians because the crests and troughs of the upper and lower curves on both the right and left vocal folds line up approximately in phase.

Figure 5 displays the results of the anterior-posterior symmetry analysis where the vibratory parameter amplitude was compared at the 25%, 50%, and 75% line scan positions for all four vocal fold lips of the unilaterally and bilaterally injured conditions. The condition averages, standard deviations, and *p*-values for each test are listed in Table 2. In the

unilaterally injured condition, significant differences in amplitude were found for the left-upper ($P < 0.001$), right-upper ($P = 0.002$), and right-lower ($P = 0.013$) vocal fold lips with the 50% line scan position having the largest amplitude, followed by the 75% position and the 25% position, in descending order. In the bilaterally injured condition, significant differences in amplitude were found for the left-upper ($P = 0.012$), left-lower ($P = 0.004$), and right-upper ($P = 0.006$) vocal fold lips with the 75% line scan position typically having the largest amplitude, followed by the 50% position and the 25% position, in descending order.

Figures 6 and 7 display the results of right-left symmetry analysis in amplitude and lateral phase difference. Table 3 lists the condition averages, standard deviations, and p -values for each test. With the exception of the lower vocal fold lips of the bilaterally injured condition at the 75% line scan position ($P = 0.005$ with left-lower lip exhibiting greater amplitude than right-lower lip), no significant differences in amplitude were found between the right and left vocal folds on either the upper or lower lip of the control and bilaterally injured conditions. The unilaterally injured condition displayed significant differences in amplitude between the right and left vocal folds on the upper lips at the 25% ($P = 0.016$), 50% ($P < 0.001$), and 75% ($P < 0.001$) line scan positions and on the lower lips at the 25% ($P < 0.001$), 50% ($P = 0.002$), and 75% ($P = 0.012$) line scan positions. In each of these cases, the amplitude of the left vocal fold was greater than that of the right. Significant differences in the upper lateral phase difference (RU – LU) among the three conditions were found at the 50% ($P = 0.032$) and 75% ($P = 0.031$) line scan positions where the bilaterally injured condition exhibited the greatest lateral phase difference, followed by the control condition and the unilaterally injured condition, in descending order respectively.

Comparisons of amplitude, vertical phase difference, and frequency between the conditions are displayed in Figures 8-10. Table 4 lists the condition averages, standard deviations, and p -values for each test. Significant differences in amplitude among the three conditions were found for all four vocal fold lips at all line scan positions except the left-lower ($P = 0.386$) vocal fold lip at the 75% line scan position. In all cases of significant difference, the control condition showed the largest amplitude, followed by the unilaterally and bilaterally injured conditions, in descending order, respectively. No significant differences were found among the three conditions in vertical phase difference. Significant differences in frequency among the three conditions were found for all vocal fold lips at the 50% line scan position. All four vocal fold lips had p -values of < 0.001 .

DISCUSSION

In the analysis of anterior-posterior symmetry, amplitude asymmetry among the 25%, 50%, and 75% line scan positions was found in the bilaterally injured condition. By observing the high speed videos, it is apparent that the bilaterally injured vocal folds were partially immobilized at the midpoint by the injuries on both vocal folds, and, in most cases, the vocal fold segments anterior to the injuries seemed to vibrate while those posterior to them did not. This could be an effect of dampening by the arytenoid cartilages (Jiang et al., 2008) in which the cartilage reduces the vibration in the nearby posterior segment of the vocal folds while the anterior segment remains elastic and subject to mediolateral displacement. The experimental setup and the fixation of the arytenoid cartilages in an adductory posture may also influence the greater vibratory amplitude of the 75% line scan position compared with the 50% and 25% positions.

The unilaterally injured condition also exhibited a significant difference in amplitude among the 25%, 50%, and 75% line scan positions, but in this case, the 50% position had the largest amplitude, rather than the 75% position like in the bilaterally injured condition. Therefore, it is difficult to determine whether anterior-posterior amplitude asymmetry exists in the

unilaterally injured condition, especially considering that Jiang et al. (2008) found results with normal vocal folds that were similar to the results reported here with unilaterally injured vocal folds: that the 50% position has the largest amplitude, followed by the 75% position and 25% position in descending order. To better determine whether anterior-posterior amplitude asymmetry is present in unilaterally injured vocal folds, future studies should use a system that analyzes additional pixel lines along the anterior-posterior length of the vocal folds.

The lack of a significant difference in amplitude among the 25%, 50%, and 75% line scan positions in the left-lower vocal fold lip of the unilaterally injured condition and the right-lower vocal fold lip of the bilaterally injured condition may be due to the difficulty in visualizing the lower vocal fold lips compared to the upper vocal fold lips. Because the camera records vocal fold vibration from above the larynx, it is more challenging to accurately observe the full lateral displacement of the lower vocal fold lips. Anterior-posterior amplitude symmetry analysis was not performed on the control condition because this study focuses on the symmetry of injured vocal folds and because the ANOVA results comparing the amplitude at the 25%, 50%, and 75% line scan positions in normal vocal folds were previously reported by Jiang et al. (2008).

Although not reported in this study, future studies should develop a method for objectively analyzing anterior-posterior phase symmetry. Wittenberg et al. (2000) created kymograms from several line scan positions on the same recording of vocal fold vibration, stacked the kymograms so the time frames lined up, and qualitatively compared the mediolateral displacement of the vocal folds at each line scan position at a specific point in time. This method could be extended to calculate the phase delay between the maximum opening points of the different line scan positions within the same glottal cycle. If this type of analysis is introduced, it may be valuable for systematically studying vocal fold injury and other vocal disorders that may involve inconsistent vibration along the anterior-posterior length of the vocal folds.

In the analysis of right-left symmetry, significant differences in amplitude were not found between the right and left vocal folds for either the upper lips or the lower lips at any line scan positions in the control and bilaterally injured conditions. The lower vocal fold lips of the bilaterally injured condition were an exception to this finding, as the amplitude of the left-lower vocal fold lip was significantly greater than that of the right-lower vocal fold lip at the 75% line scan position. This unexpected result is likely related to the previously mentioned suggestion that the lower vocal fold lips are more difficult to visualize than the upper vocal fold lips. The absence of significant differences between the right and left vocal folds in all other cases was expected because in the control and bilaterally injured conditions, both the right and left vocal folds are either uninjured or injured, leading to symmetrical lateral vibration. On the other hand, the unilaterally injured condition exhibited significantly lower amplitudes for the right vocal fold than for the left in both the upper and lower lips at all three line scan positions. This reduction in amplitude is most likely due to the effect of the stiff injured tissue on the right vocal fold (Benninger et al., 1996; Sataloff et al., 1991). The injured tissue on the right vocal fold may also alter the timing of the typically synchronous vibration of the right and left vocal folds, leading to a right-left phase asymmetry in unilaterally scarred vocal folds. Evidence for this is seen in the significantly lower (and therefore less symmetrical) lateral phase difference in the upper vocal fold lips of the unilaterally injured condition compared to the control and bilaterally injured conditions. The upper vocal fold lips are generally easier to curve fit than the lower lips because they are visible throughout the whole glottal cycle of vibration. This may explain why the lower lateral phase difference may be more difficult to detect and why significant changes among conditions were not observed.

Comparison of the three conditions showed that there are significant differences among conditions in the amplitude for all four vocal fold lips and all line scan positions except the left-lower vocal fold lip at the 75% line scan position. This exception is likely due to the inability to visualize the full vibratory cycle of the lower vocal fold lips. For the left vocal fold, the average amplitudes of the unilaterally injured condition are closer to those of the control condition than those of the bilaterally injured condition because like the control condition, the unilaterally injured condition has an uninjured left vocal fold. Because right vocal fold of the unilaterally injured condition is injured, its average right vocal fold amplitudes are closer to those of the bilaterally injured condition than those of the control condition. These findings may be explained by previously mentioned reasoning for the reduced amplitude of the injured vocal folds. The unilaterally injured condition consistently exhibits larger average amplitude values on the right-upper and right-lower vocal fold lips than the bilaterally injured condition (Table 4). This may suggest that although both conditions are injured on the right vocal fold, the injury status (injured or uninjured) of the left vocal fold may impact the vibration of the right, with larynges having uninjured left vocal folds showing greater right vocal fold amplitudes. The quantitatively determined significant differences between the amplitudes of control and injured conditions may be more reliable and methodologically consistent than, and therefore may have additional clinical value when compared with, the qualitative ratings of the effect of scars on amplitude that are provided in most studies.

Significant differences among conditions were not observed for vertical phase difference on either the left or right vocal fold at any of the three line scan positions. The injured conditions were expected to exhibit reduced vertical phase difference on their respective injured vocal folds (the right vocal fold for the unilaterally injured condition and both vocal folds for the bilaterally injured condition) due to an increase in stiffness, but this was not supported by the statistical results. It is possible that the distinctions between control and injured conditions based on vertical phase difference were blurred because, of all the parameters used in this study, vertical phase difference had the lowest reliability with the curve fitting method (Table 1).

The 25% and 75% line scan positions were not tested for differences in frequency among conditions due to the strong evidence supporting constant frequency along the anterior-posterior direction of the vocal folds (Jiang et al., 2008). It is likely that the results for these line scan positions would have been extremely similar to those of the 50% line scan position. The significant difference in frequency among conditions for all vocal fold lips seems to suggest that frequency may increase when the vocal folds are injured. This increase in frequency may be caused by an increase in stiffness of the vocal fold cover (Chhetri, Berke, Lotfizadeh, & Goodyer, 2009). It is possible that the extent of change in frequency among conditions in these ex vivo experiments may be further magnified in an in vivo larynx because subglottal pressure would not be held constant in that situation, as it was in these experiments. Changes in subglottal pressure lead to frequency changes (Jiang & Titze 1993; Jiang et al., 2008; Shipp & McGlone, 1971). In an in vivo larynx, additional subglottal pressure (increased effort) would most likely be employed to overcome glottal insufficiency and to achieve phonation in unilaterally and especially bilaterally injured vocal folds (Benninger et al., 1996; Neuenschwander et al., 2001; Sataloff et al., 1997). Thus, there would be a potential increase in frequency due to the subglottal pressure increase.

Although our MATLAB digital kymography program has the ability to curve fit high order vibrations such as period doubling and tripling, only first order periodic vibrations were analyzed in this study. It is difficult to compare vibratory parameters of curves with different orders, and although it is outside the scope of this study, a method should be developed for this comparison in future studies. Due to its ability to convert vibratory amplitude values

from pixels to millimeters, the calibration function in our MATLAB program holds clinical and scientific significance. Whereas pixel measurements of amplitude are only useful for relative comparisons between samples in one study, measurements in millimeters allow findings, such as ours, to be compared with those of other studies that use physical measurements. Calibrating to a physical measurement increases reliability of results within studies as well. For example, if the distance between the camera and the vocal folds changes between samples, the area of the picture frame taken up by the vocal folds, and consequently the perceived amplitude in pixels, will change, even if the actual amplitude stays constant. This is relevant to the clinic as well, because with a calibration function in their software, clinicians can record video of their patients' vocal fold vibration during different visits and compare the amplitude values without worrying about whether the endoscope is positioned consistently each time. Although it would be difficult for clinicians to measure the vocal fold length with a method similar to that used in this study, a laser can be attached to the endoscope, and parallel beams that are a known distance apart can be projected onto the vocal folds (Hanson, Jiang, D'Agostino, & Herzon, 1995). This distance is visible in the high speed images and can be used to create a proportion that allows for calibration of other distances (such as vibratory amplitude) from pixels to a physical unit of measurement. In addition, the data in our study and other current and future quantitative studies may allow physicians to consult the literature to compare their patients' vibratory properties with reported values. It would be especially valuable when the clinician observes a change in a patient's vocal fold vibration and can compare it with studies that report quantitative changes under varying vocal fold conditions, such as injury in this study.

The present study is limited by the use of ex vivo larynges because there is a lack of physiological scar response in the tissue after injury. Injury to in vivo larynges would result in the production and deposition of collagen, a decrease in elastin, and the disorganization of collagen fibrils and elastin fibers (Benninger et al., 1996; Thibeault, Gray, Bless, Chan, & Ford, 2002; Hansen & Thibeault, 2006). These events would lead to a loss of normal tissue elasticity and an increase in stiffness. Although there may have been an increase in stiffness due to necrosis, the full effects of vocal fold scarring on vibration could not be observed because post-injury extracellular matrix activity does not occur in the vocal fold injury model used in this study. For this reason, future quantitative study of vibratory parameters of in vivo scarred vocal folds may be useful.

Eysholdt et al. (2003) noted that each type of asymmetry may be related to a group of vocal pathologies. They suggested that right-left asymmetry is related to cysts, unilateral vocal fold paralysis, polyps or Reinke's edema. Anterior-posterior asymmetry is thought to be related to abnormal conditions induced by heterogeneous distribution of muscular tension, or stiffness. Because a scar's impact on vocal fold stiffness may resemble the disabling effects of paralysis as well as a heterogeneous distribution of tension and stiffness along the anterior-posterior direction of the vocal folds, the study of both types of symmetry may be appropriate for scarring. This may also support the use of anterior-posterior and right-left symmetry analyses to study vocal fold injury in this experiment.

Translation of the quantitative kymographic analysis techniques of this study to the clinic and the ability to collect detailed information about the vibratory properties of scarred vocal folds could lead to an objective way to detect and determine the severity of vocal fold scarring. Awareness of the severity of scarring could be useful in the selection of a treatment method. For example, minimally severe scarring cases with only slightly abnormal vibratory parameters may only require voice therapy, whereas severe cases might only show significant improvement with a vocal fold injection intended to reduce glottal insufficiency and restore pliability. Kymography may be even more useful in cases where it could differentiate unscarred vocal folds from slightly scarred vocal folds, which would require

voice therapy. It is possible that qualitative techniques using videostroboscopy would be less effective than kymography in cases like this where fine details about vibratory function must be gathered. The results from this kymography study may also be useful because vibratory parameters are reported at three line scan positions. Many previous vocal fold scarring studies rated symmetry without respect to specific positions on the vocal folds. The additional spatial information reported in this study may help clinicians recognize vibratory dynamics of vocal fold scarring more effectively, even if they use other techniques such as stroboscopy.

Future studies should extend this study's method of quantitative measurement of vibratory parameters in injured vocal folds to the quantitative study of treatments for vocal fold injury. While most current studies of vocal fold scarring treatments use ratings to determine whether treatment resulted in an improvement in vibratory function, quantitative treatment studies could provide a measure of the degree of improvement by comparing pre and post treatment vibratory parameter measurements. Then, various injection and implantation techniques and materials could be compared based on their effectiveness in restoring normal vibratory parameters. Improvement in these parameters reflects more organized vibration and better mucosal wave function which result in a higher quality of voice production.

CONCLUSION

The vibratory characteristics of unilaterally and bilaterally injured conditions were quantitatively investigated in comparison with a control condition with normal vocal folds. The control condition did not show statistically significant differences in either phase or amplitude between the right and left vocal folds. The unilaterally injured condition exhibited right-left amplitude and phase asymmetry, and the bilaterally injured condition exhibited anterior-posterior amplitude asymmetry. Segments with reduced amplitude were observed at the midpoint of bilaterally injured vocal folds, and the anterior end showed much greater vibratory activity than the posterior end. Among the three conditions, statistically significant differences were found in the amplitude of vibration at all three line scan positions and in vibratory frequency at the 50% line scan position. Detailed knowledge of the characteristics of vibration in injured vocal folds may aid clinicians in diagnosis. The quantitative abilities of digital kymography may potentially allow for objective and consistent treatment selection.

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Figure 1.
The excised larynx experimental setup.

Figure 2. Photographs of (a) uninjured control, (b) unilaterally injured, and (c) bilaterally injured vocal folds.

Figure 3. Kymogram showing curves fitted to vocal fold lips. Vertical and lateral phase differences are displayed. VA stands for vibratory amplitude, and SWA stands for sine wave amplitude.

Figure 4.

Kymogram of vibration with lateral phase asymmetry. Hypothetical positioning of curves to achieve maximum and minimum lateral phase symmetry is displayed.

Anterior-posterior symmetry - Amplitude - Unilateral

Anterior-posterior symmetry - Amplitude - Bilateral

Figure 5.

Comparison of amplitude at the 25%, 50%, and 75% positions for all four vocal fold lips of the a) unilateral and b) bilateral conditions.

Figure 6. Comparison of the amplitude of the left and right (a) upper vocal fold lips and (b) lower vocal fold lips at each line scan position for the three conditions.

Figure 7. Comparison of the (a) upper and (b) lower lateral phase difference of the three conditions at each line scan position.

Group comparison - Amplitude- 25%

Group comparison - Amplitude - 50%

Figure 8. Comparison of the amplitude of the three conditions for each vocal fold lip at the (a) 25%, (b) 50%, and (c) 75% line scan positions.

Figure 9.

Comparison of the (a) left and (b) right vertical phase difference of the three conditions at each line scan position.

Figure 10. Comparison of the frequency of the three conditions for each vocal fold lip at the 50% line scan position.

Table 1

Curve fitting reliability		
Parameter	Mean	Standard Deviation
Amplitude (mm)		
LU	2.49	0.03
LL	1.89	0.02
RU	1.57	0.01
RL	1.76	0.03
Vertical Phase Difference (radians)		
LU-LL	0.35	0.05
RU-RL	0.16	0.06
Lateral Phase Difference (radians)		
RU-LU	3.11	0.02
RL-LL	2.95	0.05
Frequency (Hz)		
LU	364.07	0.66
LL	363.93	0.65
RU	365.82	0.67
RL	363.00	0.62

Mean and standard deviation of amplitude, vertical phase difference, lateral phase difference, and frequency from twenty trials of manual plotting of the kymographic glottal edge and curve fitting of the same five cycles of vibration at the same line scan position in the same larynx

Table 2

Anterior-posterior symmetry				
Amplitude (mm)	25%	50%	75%	P
Unilateral				
LU	0.87 (0.46)	1.25 (0.60)	1.15 (0.54)	* <.001
LL	0.89 (0.52)	1.04 (0.53)	0.89 (0.39)	0.226
RU	0.69 (0.47)	0.82 (0.51)	0.74 (0.49)	* 0.002
RL	0.52 (0.34)	0.80 (0.58)	0.69 (0.39)	* 0.013
Bilateral				
LU	0.55 (0.50)	0.70 (0.59)	0.81 (0.46)	* 0.012
LL	0.45 (0.35)	0.73 (0.61)	0.90 (0.54)	* 0.004
RU	0.39 (0.22)	0.67 (0.55)	0.66 (0.33)	* 0.006
RL	0.43 (0.33)	0.56 (0.48)	0.60 (0.39)	0.338

Mean and standard deviations of amplitude in unilateral and bilateral conditions under different line scan positions

* Significant *P*-value

Table 3

Right-left symmetry						
Amplitude (mm)	LU	RU	LL	RL	P upper	P lower
Control						
25%	1.17 (0.82)	1.11 (0.63)	0.92 (0.50)	1.03 (0.69)	0.835	0.225
50%	1.45 (0.78)	1.45 (0.72)	1.09 (0.51)	1.23 (0.65)	0.986	0.245
75%	1.20 (0.50)	1.15 (0.60)	0.81 (0.37)	0.93 (0.39)	0.417	0.899
Unilateral						
25%	0.87 (0.46)	0.69 (0.47)	0.89 (0.52)	0.52 (0.34)	* 0.016	* <0.001
50%	1.25 (0.60)	0.82 (0.51)	1.04 (0.53)	0.80 (0.58)	* <0.001	* 0.002
75%	1.15 (0.54)	0.74 (0.49)	0.89 (0.39)	0.69 (0.39)	* <0.001	* 0.012
Bilateral						
25%	0.55 (0.50)	0.39 (0.22)	0.45 (0.35)	0.43 (0.33)	0.091	0.527
50%	0.70 (0.59)	0.67 (0.55)	0.73 (0.61)	0.56 (0.48)	0.426	0.078
75%	0.81 (0.46)	0.66 (0.33)	0.90 (0.54)	0.60 (0.39)	0.067	* 0.005

Lateral phase difference (radians)					P
Control					
Unilateral					
Bilateral					
25%					
RU – LU					0.338
RL – LL					0.155

B

Lateral phase difference (radians)		Control	Unilateral	Bilateral	P
50%	RU – LU	2.36 (0.41)	2.08 (0.61)	2.53 (0.30)	* 0.032
	RL – LL	2.69 (0.24)	2.38 (0.59)	2.44 (0.46)	0.107
75%	RU – LU	2.33 (0.43)	1.82 (0.75)	2.43 (0.45)	* 0.031
	RL – LL	2.63 (0.27)	2.23 (0.84)	2.26 (0.55)	0.181

Mean and standard deviations of amplitude and phase difference in control, unilaterally injured, and bilaterally injured conditions under different line-scan positions

* Significant *P*-value

Table 4

Group comparison				
Amplitude (mm)	Control	Unilateral	Bilateral	P
25%				
LU	1.17 (0.82)	0.87 (0.46)	0.55 (0.50)	* <0.001
LL	0.92 (0.50)	0.89 (0.52)	0.45 (0.35)	* <0.001
RU	1.11 (0.63)	0.69 (0.47)	0.39 (0.22)	* <0.001
RL	1.03 (0.69)	0.52 (0.34)	0.43 (0.33)	* 0.001
50%				
LU	1.45 (0.78)	1.25 (0.60)	0.70 (0.59)	* <0.001
LL	1.09 (0.51)	1.04 (0.53)	0.73 (0.61)	* 0.001
RU	1.45 (0.72)	0.82 (0.51)	0.67 (0.55)	* <0.001
RL	1.23 (0.65)	0.80 (0.58)	0.56 (0.48)	* <0.001
75%				
LU	1.20 (0.50)	1.15 (0.54)	0.81 (0.46)	* <0.001
LL	0.81 (0.37)	0.89 (0.39)	0.90 (0.54)	0.386
RU	1.15 (0.60)	0.74 (0.49)	0.66 (0.33)	* 0.008
RL	0.93 (0.39)	0.69 (0.39)	0.60 (0.39)	* 0.002
Vertical Phase Difference (radians)	Control	Unilateral	Bilateral	P
25%				
LU - LL	0.64 (0.53)	0.64 (0.70)	0.50 (0.48)	0.472
RU - RL	0.79 (0.80)	0.66 (0.78)	0.87 (0.71)	0.338
50%				
LU - LL	0.59 (0.47)	0.73 (0.42)	0.57 (0.81)	0.890
RU - RL	0.53 (0.47)	0.67 (0.88)	0.43 (0.41)	0.105
75%				
LU - LL	0.90 (0.62)	0.88 (0.69)	0.35 (0.63)	0.069
RU - RL	0.56 (0.45)	0.67 (0.73)	0.37 (0.42)	0.120
Frequency (Hz)	Control	Unilateral	Bilateral	P
50%				
LU	258.99 (96.24)	270.60 (73.28)	304.06 (86.14)	* <0.001
LL	259.27 (96.29)	270.79 (73.64)	305.23 (87.44)	* <0.001
RU	258.93 (95.96)	270.59 (74.26)	305.87 (89.56)	* <0.001
RL	260.20 (97.63)	271.11 (75.10)	305.53 (89.43)	* <0.001

Mean and standard deviations of amplitude, phase difference, and frequency in control, unilaterally injured, and bilaterally injured conditions under different line scan positions

* Significant *P*-value