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Collagen Microstructure in the Vocal Ligament: Initial Results on the Potential Effects of Smoking

Jordan E. Kelleher¹, Thomas Siegmund¹, and Roger W. Chan^{2,3}

¹ Mechanical Engineering, Purdue University, 585 Purdue Mall, West Lafayette, IN 47907

² Otolaryngology–Head and Neck Surgery, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390

³ Biomedical Engineering, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390

Abstract

Objective—This investigation quantitatively characterizes the collagenous microstructure of human vocal ligament specimens excised postmortem from non-smokers and smokers.

Study Design—Retrospective Cohort Study

Methods—Second harmonic generation (SHG) imaging was performed at three anatomical locations of vocal ligament specimens: anterior, mid-membranous, and posterior regions. Two microstructural parameters were extracted from the SHG images: (1) normalized fiber density, and (2) fiber dispersion coefficient, quantifying the degree of collagen fiber dispersion about a preferred direction.

Results—For both the non-smoker and smoker subjects, the fiber dispersion coefficient was heterogeneous. Differences in the collagenous structure of non-smokers and smoker subjects were pronounced at the mid-membranous location. However, the directionality of the heterogeneity in the smoker subjects was opposite to that in the non-smoker subjects. Specifically, the fiber dispersion coefficient in the non-smoker subjects was lower in the midmembranous region (indicating more fiber alignment) than at the anterior/posterior regions, but for the smoker subjects the fiber dispersion coefficient was higher at the mid-membranous region. The normalized fiber density was near constant in the non-smoker subjects, but the smoker subjects had fewer fibers in the mid-membranous region than at the anterior/posterior regions.

Conclusion—Spatial microstructural variations may exist in the vocal fold ligament both in non-smokers and smokers. Smoking appears to influence the degree and direction of microstructure heterogeneity in the vocal fold ligament.

Keywords

Vocal Fold; Microarchitecture; Fiber dispersion; Fiber density; Smoking; Heterogeneity

Corresponding Author: Thomas Siegmund, School of Mechanical Engineering, Purdue University, 585 Purdue Mall, West Lafayette, IN 47907, U.S.A. siegmund@purdue.edu; Phone: (765) 494 9766; Fax: (765) 494-0539..

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INTRODUCTION

The air that we breathe passes through the larynx and vocal folds before reaching the lungs to oxygenate the blood; thus, the quality of the air being breathed is likely to have an influence on the structural characteristics of the vocal folds. The vocal fold microstructure can be classified using a three-layer scheme as: vocal fold cover (epithelium and superficial layer of the lamina propria), vocal ligament (intermediate and deep layers of the lamina propria), and muscle. The superficial, intermediate, and deep layers are collectively referred to as the lamina propria. The particular interest of this investigation is the effects smoking may have upon the histology (i.e. microstructure) of the vocal ligament. According to the Centers for Disease Control and Prevention, approximately 20% of U.S. adults smoke cigarettes as of 2009.¹ From a clinical/acoustical perspective of the voice, smoking has been shown to lower the fundamental frequency,²⁻⁴ increase tremor,⁵ increase jitter and shimmer,⁶ and lead to a higher incidence of hoarseness.⁷ Concerning the vocal fold structure, smoking leads to chronic irritation and possibly an increase in vocal fold mass,³ thickening of the vocal fold epithelium,⁸ and cellular aberrations and carcinomas in the epithelium.^{9,10} Most of the studies above only focused on the epithelium, but less is known about the effect of smoking on the vocal ligament.

The fibrous (e.g. collagen and elastin) and interstitial (e.g. proteoglycans) proteins of the extracellular matrix are primarily responsible for the elastic and viscous properties of the vocal fold lamina propria. In a recent study it was demonstrated that smoking may alter the spatial heterogeneity in the elastic modulus of the vocal folds.¹¹ Since the macroscopic elastic properties are a reflection of the tissue's microstructure (e.g. elastin and collagen), it is hypothesized that smoking would alter the microstructural heterogeneity in vocal fold tissue. Furthermore it is hypothesized that a measure of the collagen fiber orientation distribution would provide a quantitative description of the microstructure, and would thus allow for a quantitative assessment of differences in the degree of tissue heterogeneity between non-smoker subjects and smoker subjects. Since this study focuses on the collagenous microstructure, only the vocal ligament was examined as it has been reported to have more longitudinally aligned collagen fibers than the vocal fold cover.¹²

The fibrous proteins of the vocal ligament have been recognized to be aligned primarily in the anterior-posterior direction.¹²⁻¹⁴ Two parameters quantitatively characterize the microstructure: (1) a normalized fiber density and (2) a fiber dispersion coefficient. The two parameters were extracted from second harmonic generation (SHG) images of fibrous proteins in the vocal ligament as obtained by multi-photon microscopy. Also, the spatial variations of these two parameters were investigated in the anterior-posterior direction of the ligament. Multi-photon microscopy has been illustrated to effectively image a tissue's collagen fiber architecture without using exogenous stains.^{15,16}

As smoking has been documented to cause potentially destructive consequences to the histology and repair of biological tissues,¹⁷ the present study would aid in the understanding of the underlying causes of acoustical changes to the voice of smokers compared to non-smokers.

MATERIALS AND METHODS

Vocal Fold Tissue Specimens

Vocal ligament specimens were isolated from the excised larynges of eight human cadaveric subjects, Table 1. Five non-smoker subjects and three smoker subjects were included, all of whom were Caucasian, except subject D who was African American. The smoking history of the three smoker subjects was not available in the information provided with the tissue. All larynges were procured from the Willed Body Program of the University of Texas Southwestern Medical Center and gross examination of the vocal folds revealed no abnormalities. The Institutional Review Board of University of Texas Southwestern Medical Center approved the sample preparation and testing protocols. Following the protocol of Chan et al.,¹⁸ the vocal ligament specimens with portions of the thyroid and arytenoid cartilages to maintain the natural anterior and posterior attachments were dissected with instruments for phono-microsurgery, separated from the underlying vocalis muscle and immediately placed in phosphate buffered saline (PBS).

Microstructure images, following a protocol described in the subsequent section, were taken in an *anterior* region (close to the anterior commissure), the *middle* region (midmembranous), and a *posterior* region (close to the arytenoid cartilage) of the samples. The anatomy is shown in Figure 1. This process was followed for all samples, except for subjects A and D. The images at the posterior location of subject A and the anterior position of subject D could not be obtained due to the sample preparation difficulties.

Microscopy and Image Processing

Specimens were embedded in optimal cutting temperature compound (Tissue-Tek, Sakura Finetek Inc.) and placed in a -20°C freezer until sectioning. In order to maintain proper specimen orientation the medial-lateral (*m-l*) and anterior-posterior (*a-p*) directions were labeled. The orientation of the coordinate axes relative to the anatomy is shown in Figure 1. Frozen specimens were transferred to a cryostat (i.e. a microtome in an enclosure maintained near -20°C) and sectioned into 10 µm thick sagittal slices, which were then placed onto a glass slide. The sagittal sections were spanned by the *a-p* and *i-s* coordinates.

Second harmonic generation (SHG) images were acquired using a Zeiss laser scanning confocal microscope (510 META) using an Achroplan water immersion objective with 40× magnification, 0.8 numerical aperture, and a working distance of 3.6 mm. Microscopy was performed at the Live Cell Imaging Facility at University of Texas Southwestern Medical Center. Excitation was achieved using a tunable (705-980 nm) coherent Chameleon Ti:Sapphire pulsed near-infrared laser at an average power of 1.3 W. The excitation wavelength in this study was 900 nm. 900 nm was used in this study because it was noticed to produce a strong SHG signal of the collagen structures. Other excitation wavelengths could have been selected, though a compromise between SHG signal strength and excitation power must be achieved. The SHG emission wavelengths were detected using a bandpass filter of 390-465 nm (which is roughly double the frequency of the excitation light). While both backward (i.e. reflected light) and forward (i.e. transmitted light) SHG signals were simultaneously detected, the reflected images were not considered further as these provided

a weaker signal than the transmitted images. The effect of the laser's polarization on the SHG signal was not assessed, because the SHG intensity has been shown to possess minimal dependence on the polarization angle.¹⁹ The resulting 512×512 pixel image had a field of view of $230 \times 230 \mu\text{m}$. Images were contrast enhanced such that 1% of data was saturated at low and high intensities of the original image, and then padded with the mean intensity scale value increasing the image size to 1024×1024 pixels. To enhance visual contrast microstructure images such as Figure 1(b) are displayed on a greenscale rather than the grayscale used in the image analysis. The raw SHG images were loaded into Matlab® (version 7.10) and analyzed using automated, custom-programmed scripting to determine microstructure characteristic parameters.

The normalized collagen fiber density FD was obtained as a measure of collagen content. A threshold was applied to the images using Otsu's method,²⁰ which has been proven to be effective for biological samples.²¹ Otsu's method seeks to separate the image's gray-level histogram into two classes (i.e. white and black pixels) such that the optimal threshold is determined to be the one that minimizes the intra-class variance of the binarized image. The normalized fiber density FD is calculated as the ratio of the sum of all white pixels (attributed to the collagen fibers) to the total number of pixels in the image.

To determine the collagen fiber orientation a spectral analysis was employed. Applying a two-dimensional Hann window reduced edge effects in the spectral analysis. The two-dimensional discrete Fourier Transform was implemented and the power spectrum was displayed.^{22,23} The digital image processing technique utilized to calculate the collagen fiber dispersion is documented in Kelleher et al.²⁴, therefore, the details are omitted in this report. Following an approach suggested in Gasser et al.,²⁵ the fiber dispersion coefficient around the preferred anterior-posterior direction, κ , is:

$$\kappa = \frac{1}{4} \int_0^\pi \rho(\Theta) \sin^3 \Theta d\Theta. \quad (1)$$

Values of κ range from $\kappa=0$ for perfectly aligned collagen fibers to $\kappa=1/3$ for randomly distributed fibers.

To assess the degree of the heterogeneity of the collagen microstructure in fiber dispersion and in the normalized fiber density the parameters FD and κ are defined:

$$\Delta FD = FD(\text{middle}) - \text{average}[FD(\text{anterior}), FD(\text{posterior})] \quad (2)$$

$$\Delta \kappa = \text{average}[\kappa(\text{anterior}), \kappa(\text{posterior})] - \kappa(\text{middle}). \quad (3)$$

RESULTS

Typical collagenous microstructures obtained at the anterior, middle, and posterior positions are displayed in Figure 2(a-c) for one non-smoker (subject C) and in Figure 3(a-c) for one smoker (subject G). Figures 2(d) and 3(d) depict the von Mises distributions characterizing the fiber dispersions. In the non-smoker subject, the collagen fibers at the mid-membranous

region exhibit a stronger alignment than at the anterior or posterior regions. Conversely, the collagen fibers at the mid-membranous region in the smoker subject exhibit a greater amount of dispersion than at the anterior or posterior regions. This finding was not only limited to these two specific subjects but was present in all specimens. Table 2 summarizes the values of the two microstructural parameters (FD and κ) at three locations along the anterior-posterior axis for all subjects. The fiber dispersion coefficient of the middle region was generally lower (indicating more alignment) in the non-smokers than in the smokers. The normalized fiber density of the middle region was generally higher in the non-smokers than in the smokers.

Samples were grouped into smokers and non-smokers. Averages of normalized fiber density for the two groups at the anterior, middle, and posterior regions are displayed in Figure 4. For the non-smoker subjects the normalized fiber density was found to increase monotonically from the anterior to posterior regions. For the smoker subjects the normalized fiber density was found to exhibit a minimum in the middle region when compared to the anterior and posterior regions. Averages of the fiber dispersion coefficients for the two groups at the anterior, middle, and posterior regions are depicted in Figure 5. For non-smoker subjects the collagen fiber orientation was found to be less pronounced at the anterior and posterior regions, but for the middle region collagen was found to be strongly aligned. The opposite trend was discovered in the smoker subjects. There, in the middle region collagen fibers were found to be less aligned than in the anterior and posterior regions.

Microstructure heterogeneity measures, (FD and κ) are listed for each specimen individually in Table 2. The group (non-smoker and smoker) means of FD and κ were compared to those of a reference homogeneous tissue microstructure ($FD=0$ and $\kappa=0$) using a one sample Student's t-distribution ($p^*<0.05$). The null hypothesis was that the means of FD and κ for the non-smoker and smoker groups subjects were equal to zero (signifying a homogeneous microstructure). Following from Figures 4 and 5 as well as motivated by prior investigation of the heterogeneity in mechanical properties of the vocal fold tissue,¹¹ the alternative hypotheses were that FD and κ of the non-smokers ($n = 5$) were greater than zero, and FD and κ of the smokers ($n = 3$) were less than zero. Since half of the distributions are of concern (i.e. if κ and FD were greater than zero for the non-smokers or less than zero for the smokers), one-tailed tests were performed. The fiber dispersion for the non-smoker subjects and for the smoker subjects was found to be heterogeneous with statistical significance ($p=0.036$ and $p=0.021$, respectively). However, the heterogeneity in the normalized fiber density was not significant for the non-smoker subjects nor for the smoker subjects ($p=0.368$ and $p=0.100$, respectively).

Furthermore, differences in microstructure parameters FD and κ between the non-smoker and smoker groups were investigated. A two sample Student's t-distribution was employed considering the null hypothesis that the mean FD of the non-smoker subjects was equal to that of the smoker subjects. The alternative hypothesis was that the mean value of FD of the non-smokers was greater than that of the smokers. For the middle segment, values of FD of the non-smokers was much greater than that of the smokers, with near statistical significance ($p=0.054$). The FD s of the anterior and posterior regions were similarly compared between

the non-smoker subjects and the smoker subjects. The calculated p -values were $p=0.431$ and $p=0.425$ for the anterior and posterior regions, respectively, and no substantial difference in FD between the non-smoker subjects and the smoker subjects at the anterior and posterior regions was present.

A two sample Student's t -distribution was employed considering the null hypothesis that the mean of κ from the non-smoker subjects was equal to that of the smokers and the alternative hypothesis that the mean of κ from the smoker subjects was greater than that of the non-smokers. It was found that for the middle location the mean κ of the smoker group was greater than that of the non-smoker group with near statistical significance ($p=0.062$). Likewise, κ from the anterior and posterior regions were tested to see if there was a statistical difference between the non-smokers and smokers. The resulting p -values were $p=0.104$ and $p=0.143$ for the anterior and posterior regions, respectively, indicating only minor differences in the fiber dispersion at the anterior and posterior locations between the non-smoker subjects and the smoker subjects.

DISCUSSION

Clinical studies have concluded that smoking can cause acoustic changes in the voice, including the lowering of vocal fundamental frequency.^{3,4} Smoking has also been qualitatively shown to potentially induce histological damage in vocal fold tissues,^{8,26,27} as well as other tissues.^{28,29} This study is a step towards understanding these microstructural changes and their implications to the biomechanics of vocal fold tissue.

The tensile deformation of the vocal fold cover and vocal ligament has been shown to be heterogeneous, with the mid-membranous region stretching less than the anterior and posterior regions.¹¹ This leads to heterogeneity in the biomechanical properties (i.e. longitudinal elastic modulus) with the mid-membranous region being approximately 8-10 times stiffer than the anterior and posterior regions.^{11,30} Interestingly, this heterogeneous deformation was noticed to not be as strong in the vocal fold cover and vocal ligament specimens of smokers; thus, the smoker subjects had a more homogeneous modulus distribution.³⁰ The present investigation demonstrated that there may be differences in the microstructure of the vocal ligament between non-smoker subjects and smoker subjects, particularly at the mid-membranous location. The non-smoker subjects revealed more aligned collagen fibers at the mid-membranous region than at the anterior or posterior regions, and a more uniform fiber density. This would logically lead to the kind of heterogeneous tensile deformation documented in Kelleher et al.,³⁰ where the midmembranous region was observed to stretch less than the anterior and posterior regions. Contrary to the non-smoker subjects, the smoker subjects displayed less collagen fiber alignment and lower collagen fiber density at the mid-membranous region than at the anterior or posterior regions. This may provide microstructural support to the observations in Kelleher et al.³⁰ that the specimens from smokers had a nearly homogeneous distribution of the longitudinal elastic modulus.

One limitation of this investigation was the absence of vocal fold cover specimens. The vocal folds are comprised of a layered structure where the vocal fold cover and the vocal

ligament have distinct histological compositions and functional biomechanical differences. Quantitatively studying the fiber dispersion coefficient and normalized fiber density in vocal fold cover specimens from non-smoker subjects and smoker subjects should be explored in further studies. Another limitation of this study was that the dependence of collagenous microarchitecture on the depths into the tissue was not analyzed to explore if heterogeneities exist in the medial-lateral direction. Only the microstructural variations in the anterior-posterior direction were analyzed. Finally, this investigation focused solely on the collagenous microstructure of the vocal folds since collagen fibers are considered to be the predominant load-bearing structural protein in soft tissues. Other extracellular matrix proteins (e.g. elastin, proteoglycans) contribute to the tissue's elasticity and could be imaged by conventional histological staining and then analyzed.

The authors recognize that there exists a plethora of subject-specific characteristics (e.g. age, gender, genetic makeup) that could potentially contribute to the present findings. However, given the small sample size of this initial study, it was not viable to separate the individual contributions of each of these factors. In addition to smoking, the histological structure of vocal fold tissues is also affected by aging. It has been documented that the arrangement of collagen fibers are less organized in geriatric vocal folds.¹² However, in related studies, there was shown to be no significant differences in the collagen fiber content between adult (age 22-54 years) and geriatric (age 65-82 years) vocal folds,³¹ and that most age-dependent changes of the macro-scale vocal fold biomechanical properties could occur below age 60.³² Since all of the subjects (except subject A) of the present investigation are in the geriatric category, age-related effects may not be a significant confounding factor. Nevertheless, in order to provide insight beyond the initial study level, the protocol employed here will need to be applied to a larger number of subjects, and to clearly define the study population (e.g. the smoking history for the subjects). The present initial study was not able to take such factors into account due to the small number of subjects available.

CONCLUSION

The collagen structure of the vocal ligament was investigated in subjects with both a non-smoking and a smoking history. Strong differences in the collagenous microstructure between the non-smoking and the smoking subjects were found: (1) the collagen fibers are more dispersed at the mid-membranous location in the smoker subjects than in the non-smoker subjects, and (2) there is lower collagen fiber density at the mid-membranous location in the smoker subjects than in the non-smoker subjects. The heterogeneity in the fiber dispersion was found to be statistically significant for both the non-smoker and the smoker subjects. However, the directionality of this heterogeneity was opposite for the non-smokers and the smokers. The collagen alignment was stronger in the middle region than at the anterior and posterior regions in the non-smoker group, but collagen was more randomly oriented at the middle region for the smoker group.

The microstructural heterogeneity in vocal fold tissue could be responsible for the macroscopic heterogeneity in the elastic characteristics of the vocal fold cover and vocal ligament reported in previous studies.^{11,30} Since smoking is found to effect the

microstructure of the vocal ligament, the resulting microstructural variations are likely to impact the vibratory and acoustical characteristics of phonation.

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FIGURE 1.

(a) Superior view of transverse section of the larynx indicating the imaging locations at the level of the vocal folds: (1) anterior, (2) middle, and (3) posterior locations; as well as the coordinate system: *a-p* anterior-posterior, *m-l* medial-lateral, and *i-s* inferior-superior directions, respectively. (b) Lateral view of a vocal ligament specimen showing an image orientation relative to the coordinate system.

FIGURE 2.

SHG images of the vocal ligament specimen from subject C (a non-smoker) at the (a) anterior region, (b) mid-membranous region, and (c) posterior region. (d) The corresponding von Mises distribution $\rho(\Theta)$ fits for the three image locations.

FIGURE 3. SHG images of the vocal ligament specimen from subject G (a smoker) at the (a) anterior region, (b) mid-membranous region, and (c) posterior region. (d) The corresponding von Mises distribution $\rho(\theta)$ fits for the three image locations.

FIGURE 4.

Means $\pm$ standard deviations of the normalized fiber density *FD* at three locations along the anterior-posterior axis of the vocal ligament comparing the non-smokers and the smokers.

The number of images contributing to each mean is noted in the plot.

FIGURE 5.

Means $\pm$ standard deviations of the fiber dispersion coefficient κ at three locations along the anterior-posterior axis of the vocal ligament comparing the non-smokers and the smokers.

The number of images contributing to each mean is noted in the plot.

TABLE 1

Details on the human vocal ligament specimens analyzed in this study.

Subject	Sex	Age	Postmortem Hours
A	Male	45	18
B	Male	64	19
C	Female	73	76
D	Female	70	65
E	Female	97	59
F (smoker)	Male	78	48
G (smoker)	Female	79	28
H (smoker)	Male	86	41

TABLE 2

Microstructural parameters (normalized fiber density FD , fiber dispersion coefficient κ) at three locations for the non-smokers and smokers. Measures of the microstructural heterogeneity, FD and κ , are calculated by Eqns. (2) and (3), respectively.

	Anterior image		Middle image		Posterior image			
Subject	FD	κ	FD	κ	FD	κ	ΔFD	κ
A	0.163	0.190	0.144	0.144	--	--	-0.019	0.045
B	0.078	0.192	0.164	0.170	0.336	0.286	-0.043	0.070
C	0.165	0.191	0.172	0.139	0.076	0.234	0.052	0.074
D	--	--	0.163	0.137	0.136	0.195	0.027	0.058
E	0.109	0.240	0.167	0.259	0.198	0.226	0.013	-0.026
F (smoker)	0.094	0.078	0.135	0.310	0.168	0.164	0.004	-0.189
G (smoker)	0.133	0.188	0.048	0.333	0.167	0.237	-0.102	-0.120
H (smoker)	0.176	0.152	0.091	0.188	0.190	0.031	-0.092	-0.096