Goodyer et al., J Otol Rhinol 2016, 5:2
http://dx.doi.org/10.4172/2324-8785.1000275

Journal of Otology & Rhinology

Review Article

A SCITECHNOL JOURNAL

The Anisotropic Elasticity of the Human Vocal Fold

Goodyer EN[1], Gunderson ML[2], Tateya C[3], Tateya T[4,5] and Dailey SH[6]

Abstract

Objective: To verify the anisotropic nature of the vocal folds by reproducing an experiment led by Rholfs that measured vocal fold elasticity in the transverse and longitudinal directions. To present a physiological explanation of the measured phenomena using immunochemical results.

Methods: 6 cadaveric human excised larynges were hemi-sectioned in the mid-sagittal plane exposing the vocal folds, and orthogonal measurements of tension made at 3 equally spaced points. Immunohistochemistry carried out by Ichiro Tateya was used to visualise collagens and elastins in the deep layer of the lamina propria.

Results: The LSR results indicate that the measured elasticity of the vocal folds are highly anisotropic. The immunohistochemistry results show that there is a strong alignment of collagens and elastins along the longitudinal axis of human vocal folds.

Conclusion: The measured anisotropic behaviour is due to the alignment of collagens & elastins in the lamina propria.

Keywords

Elasticity; Vocal folds

Introduction

Understanding if vocal fold viscoelasticity is isotropic or anisotropic is essential, as it brings to bear on multiple evolving approaches related to restoring or reconstructing the vocal fold lamina propria. Mitchell & Tojeira present methodologies for the construction of augmentation materials using scaffolds which are inherently anisotropic [1], and the need for anisotropic structures are found in other fields, such as musculoskeletal tissue engineering [2], regenerative cardiac tissue engineering [3], and nerves [4]. As vocal folds are required to perform mechanically, it is reasonable to extend the need to deploy anisotropic engineered tissue to phonosurgery. We therefore undertook a rheologic evaluation of human cadaveric vocal folds to confirm the results presented by Rohlfs [5], which demonstrated that the vocal folds inherently exhibit an anisotropic nature using the Linear Skin Rheometer [6-9]; and present immunochemical results obtained by Tateya et al., which show that there is an alignment of elastins and collagens in the longitudinal axis of the vocal folds [10]

Materials and Methods

With University of Wisconsin School of Medicine and Public Health Institutional Review Board and the Ethics Board of De Montfort University (DMU) approval, six human larynges were used. Visual inspection revealed normal appearing vocal folds in 5 out of 6 larynges, obtained from autopsy cases, frozen within 24 hours after death using liquid nitrogen, and stored at -80°C. The larynges were thawed overnight at 4°C and gradually warmed to 37°C. Each was sharply divided in the mid-sagittal plane into two hemi-larynges and affixed to a Styrofoam base with pins to prevent movement (Figure 1).

The LSR was attached using a 2mm internal diameter bore cannula, with a vacuum pressure of approximately 50 millibar. No epithelial violation or tear was noted. A cyclical shear force of 1g was applied in both the transverse and longitudinal directions, and the resultant displacements logged. Five readings were taken from the mid-membranous site in both directions and averaged, the intention being to limit the tissue under test to be the free edge of the LP layer. The units used for all shear modulus results are Pascals.

The linear skin rheometer

The LSR applies a sinusoidal force of ± 1 g tangentially to the tissue, thus applying a shear force. The resultant strain is measured. A linear regression is applied to the force and displacement cycles, from which we derive the Dynamic Spring Rate (DSR) in units of force applied to achieve a unit of linear displacement. DSR is also known as stiffness or rigidity, and is measured in units of g/mm.

DSR measures the stiffness of the whole body under test. The shear modulus is a property of the material itself, which is derived by a mathematical transformation [6] of stiffness using the geometry of the material under test. As our interest is the ratio of modulus with respect to direction (i.e. the degree of anisotropic behaviour), the ratio is the same whether we use DSR or modulus.

Immunohistochemistry for collagen and elastin

Vocal folds were cut with sharp blades into 10-15 mm thick sections, soaked in embedding medium (O.C.T. compound, Tissue-Tek, Kyoto, Japan), frozen quickly with a combination of acetone and dry ice, and kept at -80°C. The samples were sectioned coronally

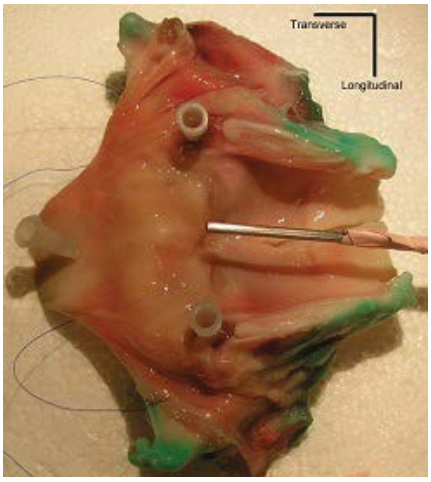

Figure 1: Typical experimental set up.

***Corresponding author:** Eric Goodyer Professor of Instrumentation, The Faculty of Technology, De Montfort University, The Gateway, Leicester, LE1 9BH, UK, Tel: 44 (0) 1289 309784; E-mail: eg@dmu.ac.uk

Received: March 03, 2016 **Accepted:** April 12, 2016 **Published:** April 17, 2016

or horizontally. Frozen sections of 30 µm thickness were made from each sample, and mounted on slides (Surperfrost / Plus Microscope Slides, Fisher Scientific, Pittsburgh, PA), air-dried and stored at -20°C until use.

Collagen types I, III, and elastin were identified by immunohistochemistry. The slides were washed 3 times with PBS and fixed for 2 minutes at room temperature in 4% paraformaldehyde, followed by washing in PBS 3 times. After blocking for 2 hours at room temperature, sections were incubated in primary antibody solution overnight at 4°C. Working dilutions and sources of antibodies used in this study included mouse Monoclonal Anti-Collagen Type I Clone COL-1 (Sigma, Birmingham, AL) at 1:1000, mouse Monoclonal Anti-Collagen Type III Clone FH-7A (Sigma, Birmingham, AL) at 1:2000, and mouse Anti Elastin Monoclonal antibody (Chemicon, Temecula, CA) at 1:100. These anti collagen antibodies had no or negligible cross-reactivity with the other types of collagen.

Samples were washed and incubated for 2 hours at room temperature with the second antibody solution. TOTO-3 (200 nM, Molecular probes, Eugene, OR) was also mixed in this procedure for nuclear staining. Finally, samples were washed 3 times in PBS and mounted on coverslips in Vectashield (Vector Labs, Burlingame, CA) for observation under a Laser Scanning Confocal Imaging System (BIO-RAD MRC-1024, Hercules, CA). The images of serial optical sections were collected every 1.5 µm in depth for three-dimensional reconstruction. Omission of the primary antibody served as a negative control. Working dilutions and sources of second antibodies used in the immunofluorescence technique included Texas-Red conjugated anti-mouse IgG (Jackson immunoresearch, PA) diluted to 1:200, and Texas-red anti-rabbit IgG (Jackson immunoresearch, PA) diluted to 1:400. As a blocking solution, 5% normal goat serum in 0.1% Triton-X with PBS was used. One percent normal goat serum in 0.1% Triton-X with PBS was used in the primary and second antibody reactions.

Results

Biomechanical results

Table 1 shows the results for all six larynges being a mean ratio of 2.48, meaning that vocal fold tension in the longitudinal direction is 2.48 times higher than the transverse direction. The mean coefficient of variance for all the data used is 12%.

Confidence determination in the data was obtained by comparison of left side data versus right side data, resulting in a correlation coefficient (CC) of 0.86, where 1.0 is a perfect match.

The value of 0.86 is acceptable, but a larger sample needs to be taken to increase our confidence in the conclusion.

Immunohistochemistry results

The distribution patterns of collagen subtypes and elastin in vocal folds detected by immunofluorescence technique were the same as previously described by Tateya et al. [10]. In the deep layer of the lamina propria (DLLP), collagen type I, collagen type III and elastin constructed fibers and were well-organized in the direction vertical to the vocal fold edge of the coronal plane and parallel to the vocal fold edge of the horizontal plane. Representative images are shown in Figure 2 and scale bars are 10 µm.

Figure 2A Collagen Type 1 48 year old Female 60x: This is the DLLP of the vocal fold sectioned parallel to the vocal fold edge of the horizontal plane. Magenta is collagen type I and green is nuclei. Type I fibers were thinner than most of type III fibers

Figure 2B Collagen Type 3 48 year old female x60x: This is the DLLP of the vocal fold sectioned parallel to the vocal fold edge of the horizontal plane. Magenta is collagen type III and green is nuclei. There were various diameters of collagen type III fibers. Most of them were thick and wavy and some were thin.

Figure 2C Collagens type 1 & 3 55 year old male 120x: This is the DLLP of the vocal fold sectioned vertical to the vocal fold edge of the coronal plane. Magenta is collagen type I and green is collagen type III.

Figure 2D Elastin 48 year old female 60x: This is the DLLP of the vocal fold sectioned parallel to the vocal fold edge of the horizontal plane. Magenta is elastin and green is nuclei. Elastin fibers were less wavy than collagen type I and type III.

Figure 2E Elastin and Collagen Type 3 55 year old male 120x: This is the DLLP of the vocal fold sectioned vertical to the vocal fold edge of the coronal plane. Magenta is elastin and green is collagen type III.

Discussion

Quantifying the extent of the anisotropic nature of vocal folds has only been recently presented in the published literature. This is due to the nature of available apparatus, which is often based on techniques such as parallel plate rheometry that cannot resolve out strain with respect to the direction of applied stress. The LSR has enabled measurement of the stiffness of the LP to be measured without the need to dissect it our its anatomical context, and the because of the linear nature of the applied force it is now possible to be obtain strain data with respect to the direction of the applied stress. The motivation for this study was to confirm the findings of the team led by Rohlfs that the vocal folds present a highly anisotropic nature, and to determine if the cause of the measurable anisotropic behaviour of the LP is due to the alignment of collagens and elastins.

This was achieved by bringing together the methods used in two previous unrelated studies, into the biomechanical and the immunohistochemical properties of the vocal folds. By repeating and fusing these studies we conclude that the vocal folds are inherently anisotropic, and the underlying cause being that the collagens and elastins have a strong directional alignment, as presented in Figures 2A-2E.

Table 1: Shear modulus taken in the Transverse & Longitudinal directions.

Hemi-section	Age	Sex	Shear Modulus Pascals Transverse	Shear Modulus Pascals Longitudinal	Ratio Longitudinal to Transverse
1 Left	60	M	1683	2119	1.26
1 Right	60	M	1890	2673	1.41
2 Left	37	F	2635	1919	0.73
2 Right	37	F	1951	damaged	
3 Left	64	F	1486	7080	4.76
3 Right	64	F	1238	5393	4.36
4 Left	65	M	1675	4499	2.69
4 Right	65	M	1729	3971	2.31
5 Left	??	F	4860	7320	1.51
5 Right	??	F	1921	5284	2.75
6 Left	41	F	2675	5741	2.15
6 Right	41	F	1949	6457	3.31

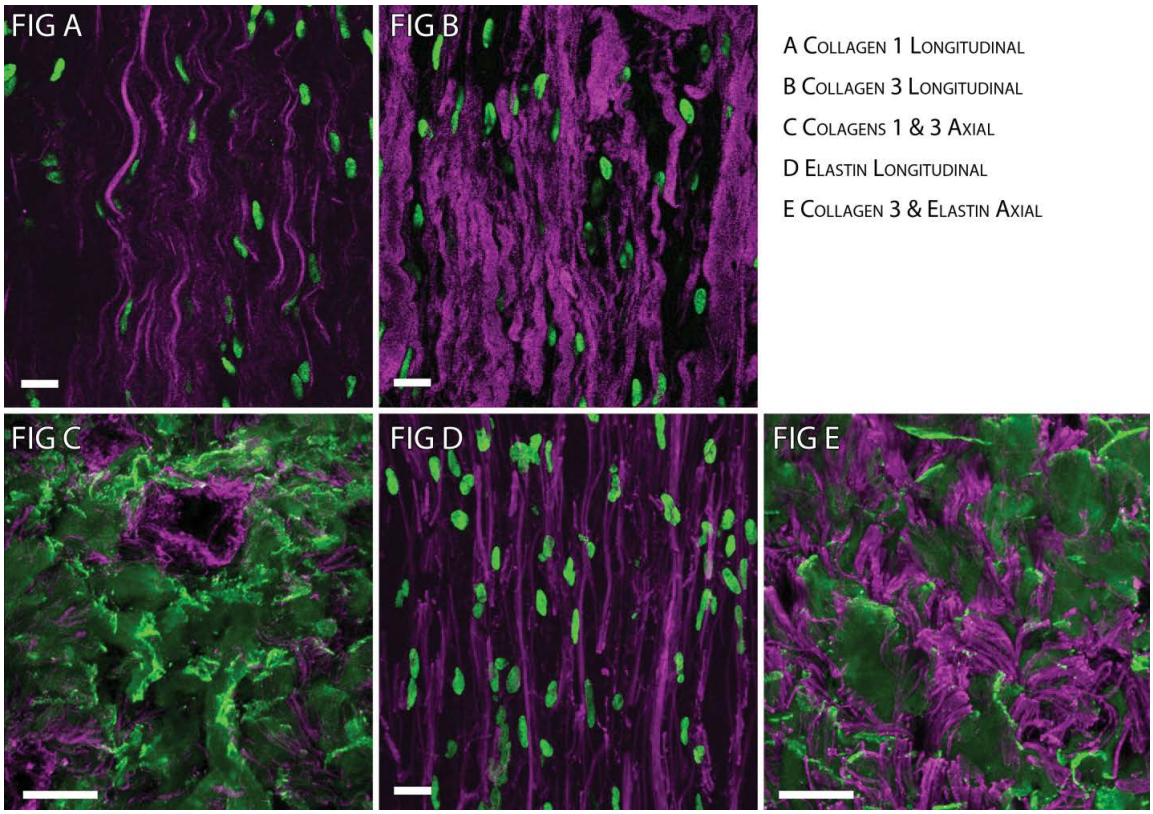

Figure 2: Stains showing Collagen and Elastin Alignments in the Deep Layer of the Lamina Propria. Figures A,B,D are 60 times magnification. Figures C and E are 120 times magnification. The scale bar is 10 μm.

Acknowledgements

This work was carried out with the support of the Engineering Physics and Science Research Council (EPSRC) of the United Kingdom

Conflicts of Interest: None

References

1. Mitchell GR, Tojeira A (2013) Role of Anisotropy in Tissue Engineering. 3rd International Conference on Tissue Engineering 59: 117-125.
2. Li WJ, Mauck RL, Cooper JA, Yuan X, Tuan RS (2007) Engineering controllable anisotropy in electrospun biodegradable nanofibrous scaffolds for musculoskeletal tissue engineering. J Biomech 40: 1686-1693.
3. Senel Ayaz HG, Perets A, Ayaz H, Gilroy KD, Govindaraj M, et al. (2014) Textile-templated electrospun anisotropic scaffolds for regenerative cardiac tissue engineering. Biomaterials 35: 8540-8552.
4. Phillips J, Rosemary D (2012) Novel technology for simple assembly of aligned 3D cellular collagen materials for tissue engineering. Tissue Engineering and Regenerative Medicine International Society- World Congress, Vienna, Austria.
5. Rohlfs AK, Goodyer E, Clauditz T, Hess M, Kob M, et al. (2013) The anisotropic nature of the human vocal fold: an ex vivo study. Eur Arch Otorhinolaryngol 270: 1885-1895.
6. Goodyer E, Hemmerich S, Müller F, Kobler JB, Hess M (2007) The shear modulus of the human vocal fold, preliminary results from 20 larynges. Eur Arch Otorhinolaryngol 264: 45-50.
7. Goodyer E, Welham NV, Choi SH, Yamashita M, Dailey SH (2009) The shear modulus of the human vocal fold in a transverse direction. J Voice 23: 151-155.
8. Goodyer E, Gunderson M, Dailey SH (2010) Gradation of stiffness of the mucosa inferior to the vocal fold. J Voice 24: 359-362.
9. Goodyer EN, Gunter H, Masaki A, Kobler J (2003) Mapping the viscoelastic Properties of the Vocal Fold, AQL, Hamburg.
10. Tateya T, Tateya I, Bless DM (2006) Collagen subtypes in human vocal folds. Ann Otol Rhinol Laryngol 115: 469-476.

Author Affiliations

Top

[1]*De Montfort University, Bio-Informatics Research Group, UK*

[2]*University of Wisconsin Madison, School of Veterinary Medicine, USA*

[3]*Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Japan*

[4]*Institute for Virus Research, Kyoto University, Japan*

[5]*The Hakubi Center, Kyoto University, Japan*

[6]*University of Wisconsin School of Medicine and Public Health, Department of Surgery, Division of Otolaryngology-Head and Neck Surgery, USA*