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What is This?
Hyaluronic Acid Behavior in the Lamina Propria of the Larynx with Advancing Age

Anete Branco1, Sergio Augusto Rodrigues2, Alexandre Todorovic Fabro, MD, PhD3, Carlos Eduardo Fonseca-Alves4, and Regina Helena Garcia Martins, MD, PhD5

Abstract

Objectives. To analyze the hyaluronic acid behavior in the lamina propria of the larynx with advancing age.

Study Design. Prospective study.

Setting. UNESP-Univ Estadual Paulista, Botucatu Medical School, Brazil.

Subjects and Methods. Thirty vocal folds were obtained at necropsy from 10 adult males (30–50 years old) and 20 geriatric males (10: 60–75 years old; 10: over 76 years old). Midmembranous vocal fold sections were subjected to immunohistochemical reactions. Digital imaging software (ImageJ) was used to quantify the hyaluronic acid distribution over the lamina propria of vocal folds, from superficial to deep layers.

Results. Hyaluronic acid distribution was homogeneous for the larynges, at both superficial and deep layers (41.6 and 38.5, respectively). For both 30- to 50-year-old men geriatric age groups, hyaluronic acid level was lower at both layers.

Conclusion. Hyaluronic acid level in the lamina propria of the larynx of geriatric men decreases with advancing age at both layers of the lamina.

Keywords
larynx, lamina propria, vocal folds, hyaluronic acid, presbyphonia

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Introduction

Presbyphonia is the larynx aging process, during which structural and functional changes negatively affect the voice. Marked concavity at the free edges of vocal folds can be identified at the videolaryngoscopic exam due to simultaneous atrophy of the epithelium, the lamina propria, and the vocalis muscle; glottic gap during phonation is also present, making the voice breathy, feeble, and weak.1-3

The histological changes that have been described for presbyphonia include epithelial keratosis, reduced thickness of the epithelium and the mucosal glands number, lamina propria showing increased number of collagenous fibers, decreased elastic fibers, inflammatory infiltrate, edema, larger number of blood vessels, and decreased hyaluronic acid level (HA). There is also atrophy of the vocalis muscle and calcification of laryngeal cartilages.4-6 Ximenes et al6 found similarity between the senility processes of the larynx and the skin at the inguinal region of elderly cadavers; the effects of aging at both sites included atrophy of the lamina propria and the epithelium. HA is an important compound of the extracellular matrix of the larynx lamina propria, which belongs to the family of non-sulfated glycosaminoglycans. It is synthesized in the plasma membrane by an enzyme complex of several cell types, is produced by fibroblasts and macrophages, and shows a half-life of approximately 3 to 5 days.7 In the vocal folds, it plays its key functions such as mechanical stability and viscoelasticity during phonation, resistance, tissue regeneration, water content regulation in the extracellular matrix, lubrication, barrier against infections, and osmotic pressure control.8,9 On account of these important actions, HA injection into paralyzed and scarred atrophic vocal folds has resulted in significant vocal

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quality improvement, since HA extends spaces, assuring volume to the vocal folds and preventing glottic insufficiency, and preserves the viscoelastic properties of the lamina propria.9–11

The important participation of HA in the repair of sloughing tissues has been confirmed in experiments in which scarification and treatment of vocal folds with HA solution led to immediate healing process improvement and early mucosal wave recovery.12–15

Since a great part of the compounds in the lamina propria of the larynx are synthesized by fibroblasts, the hepatocyte and fibroblast growth factors have been employed for extracellular matrix repair in cases of atrophy and scars.12,13 In 2004, Hirano et al14 could demonstrate the favorable response of fibroblasts after treatment with Fibroblast Growth Factor (FGF), which resulted in higher HA level in the lamina propria of aged rats. Other authors have obtained similar results, providing great therapeutic perspectives for presbyphonia.15–18

Suehiro et al15 noted decreased expression of procollagen I and increased expression of the enzyme hyaluronan synthase (HAS) and fibronectin for rats after fibroblast treatment using varied concentrations of fibroblast growth factor (bFGF). Ohno et al16 used Transforming Growth Factor β3 (TGFβ3) in scarified folds of beagles and noted smaller quantity of granulation tissue, better vibratory properties, and favorable elastin and HA recovery.

According to Suehiro et al,17 the benefits of treating the vocal folds of aged rats with hepatocyte growth factor (HGF) included higher levels of HA and lower collagen type I. The aforementioned therapeutic measures represent a considerable advancement in phonosurgery but are still restricted to animals. Patients carrying presbyphonia may benefit from these treatments in the future; thus, it is important to determine the compromising degree HA may cause to the vocal folds of humans with advancing age.

The purpose of this study was to analyze the concentration of hyaluronic acid in the adult lamina propria of the larynx in different age groups in order to demonstrate the reduction of this component with advancing age.

**Subjects and Methods**

Human vocal folds from 30 male cadavers were removed during necropsy routine and allocated to 3 groups according to the age range: 10 larynges from control adults (30–50 years old), 10 larynges from geriatric males (60–75 years old), and 10 larynges from geriatric males older than 76 years. Excluded were larynges from patients with history of smoking, septicemia, prolonged intubation, systemic infectious diseases and remaining dermatological, autoimmune or metabolic diseases, cervical traumatism of any nature, or other causes that could compromise the mucosa of vocal folds and invalidate the immunohistochemical analysis. This information was obtained from family members and medical records.

The larynges were incised at their posterior part and macroscopically examined to rule out the presence of lesions. As a standardized procedure, the medium part of the right vocal fold was removed and immediately embedded in 10% buffered formaldehyde, where it was kept for 24 to 48 hours. Paraffin blocks were prepared for histological sections and immunohistochemical reactions. The midmembranous vocal fold sections were selected for histological analysis.

For HA immunohistochemical analyses, a secondary antibody VECTASTAIN (Abcam, Cambridge, UK) and the ABC kit (Vector Laboratories, Burlingame, California) were employed at the dilution of 1:800. The primary antibody used for HA was Ab53852 (Abcam) and the concentration used was 1:800. For antigen retrieval, 2% pepsin, pH 1.8, was added and allowed to react in an oven at 60°C for 20 minutes and at 37°C for 30 minutes. The material was allowed to react with the following substances for the respective periods, as follows: HRP secondary complex Advance (Dako, Carpinteria, California) for 30 minutes, Enzyme Advance for 30 minutes, diaminobenzidine (DAB) for 5 minutes, and Harris Hematoxylin for 20 seconds.

For blind reading of histological slides, digital image analysis (software Axion Vision, version 4.8) was done always at 40× magnification. Images were analyzed by using the program ImageJ (National Institutes of Health; http://rsb.info.nih.gov). HA measurements were performed by quantifying the antibody labeling density in the lamina propria, which was identified based on the degree of brown staining of the structures; the area was standardized as μm² and the result as percentage.18 ImageJ was used in the segmentation by standardizing the polygon as a tool to isolate the area of interest, delimiting 3 random fields. The plugin Colour Decovolution in HDAB was used to improve contrast and the icon Make Binary was employed for the segmentation itself.

This research project was approved by the Human Research Ethics Committee of Botucatu Medical School (UNESP, protocol CEP 3861-2011). For the statistical analysis of the hyaluronic acid density between age groups the Goodman test was used, considering 5% significance level.

**Results**

The Figure 1 stained in H&E show a panoramic view of whole vocal fold section of the 3 groups and the superficial and deep layers. Figure 2 of the immunohistochemical staining show that control adults (aged between 30 and 50 years) had HA homogeneously distributed over the lamina propria layers of their vocal folds. Both geriatric groups, however, had a decrease in this compound at both superficial and deep layers (Table 1, Figure 2).

**Discussion**

The quantity of HA in the lamina propria of the larynx reduced by almost 50% for geriatric men older than 60 years. This study included male larynges only; however, HA distribution over the lamina propria of vocal folds is known to differ between sexes.19 The number of female larynges in our sample was too small, which did not allow us
to make comparative analyses between sexes; thus, female larynges were excluded. According to Hammond et al, the high HA concentration in the female vocal folds represents a protection mechanism; it acts as an absorber and protector against phonotrauma. The extracellular matrix is an important cellular modulator, and HA seems to be the major substance responsible for viscosity, which also participates in the impact absorption. The HA level in the larynx may decrease under some conditions such as tissue lesions, scars, and presbyphonia. Most studies assessing HA behavior under these conditions have been conducted with animals. Thibeault et al examined rabbit vocal folds for HA behavior under the condition of acute lesions at 3, 5, 10, and 15 days after scarification and found that the level of this substance reduced at almost all studied moments. Similar results were reported by Rousseau et al for vocal folds of pigs, in which the HA decrease was followed by a collagen increase.

There was a progressive decrease in HA for the larynx of geriatric men with advancing age, further compromising the glottic gap. Atrophy of both the epithelial coverage and the vocalis muscle is known to be simultaneous to an increase in the number of collagenous fibers and a decrease in elastic fibers, making the vocal folds atrophic and tough. These structural changes directly affect the vocal features of the elderly, resulting in the major symptoms of presbyphonia such as hoarseness, high-pitched voice in men and low-pitched voice in women, phonasthenia, and difficult emission of loud sounds. A great part of these symptoms in the elderly are probably caused by the decreased HA in the vocal folds, which have this substance intensely participating in their biomechanical properties.

Table 1. Mean and Standard Deviation (SD) of Hyaluronic Acid (HA) Density, as Area Percentage (%), According to the Age Range and the Lamina Propria Layers.

<table>
<thead>
<tr>
<th>Age Range (years)</th>
<th>Superficial Layer</th>
<th>Deep Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-50</td>
<td>41.6 (10.8)*</td>
<td>38.5 (10.1)*</td>
</tr>
<tr>
<td>60-75</td>
<td>26.7 (6.1)*</td>
<td>24.6 (4.1)*</td>
</tr>
<tr>
<td>&gt;76</td>
<td>21.8 (5.7)*</td>
<td>24.1 (4.6)*</td>
</tr>
</tbody>
</table>

*Two frequencies followed by the same letter do not differ concerning to the respective age groups (lines) (p > .05). Two frequencies followed by the same letter do not differ concerning to the layers (columns) (p > .05). Goodman test.
However, HA has been proven superior. Molteni et al compared the vocal analyses in the preoperative period with those carried out at 3 and 12 months after HA injection for 38 and 27 patients, respectively, and noted evident improvement in the vocal quality for all patients. HA injection also seems to cause no histological damage since experiments have shown only a discreet inflammatory response and absence of necrosis.

Promising therapies have been developed for vocal fold atrophy based on the treatment with fibroblast and hepatocyte growth factors. The latter are highly capable of stimulating HA production, besides inducing both metalloproteinase gene expression, which contributes to lower collagen production, and hyaluronan synthase gene expression, which stimulates HA production. The new therapies have promised diagnostic analysis—collection and analysis.

References


Conclusion

Hyaluronic acid level decreases with advancing age at both layers of the lamina propria of the larynx of geriatric men.

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Author Contributions

Anete Branco, interpretation of data for the work, drafting the work, final approval of the work, agreement to be accountable for all aspects of the work; Sergio Augusto Rodrigues, contributions to the analysis and interpretation of data for the work, revising the work, final approval of the version, agreement to be accountable for all aspects of the work; Alexandre Todorovic Fabro, analysis of data for the work, drafting the work, final approval of the version, agreement to be accountable for all aspects of the work; Carlos Eduardo Fonseca-Alves, contributions to the analysis and interpretation of data for the work, revising the work, final approval of the version, agreement to be accountable for all aspects of the work; Regina Helena Garcia Martins, study design, interpretation of the data, writing and approval of the manuscript, critical revision, final approval, agreement to be accountable for all aspects of the work.

Disclosures

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References


