UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA

TATIANA MARIA GONÇALVES

ESTUDO DE MICROSCOPIA ELETRÔNICA DE VARREDURA E TRANSMISSÃO DE PREGAS VOCAIS DE IDOSOS

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, para obtenção do título de Mestra em Bases Gerais de Cirurgia.

Orientadora: Prof(a). Livre Docente Dr(a). Regina Helena Garcia Martins
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Palavras-chave: Idosos; Microscopia eletrônica; Pregas vocais.

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LISTA DE ABREVIATURAS E SÍMBOLOS

F – Feminino

M - Masculino

GRBASI- Scale, each letter corresponds to one analyzed vocal parameter: overall grade or severity (G), roughness of the voice (R), breathiness (B), asthenia (A), strain (S) and instability (I)

nm – nanômetro

µm - micrômetro

SEM - Scanning Electron Microscopy

TEM -Transmission Electron Microscopy

RESUMO

Introdução: denomina-se presbifonia o conjunto de alterações nos padrões vocais consequentes ao envelhecimento da laringe, podendo cursar com sintomas de disfonia, voz fraca, trêmula e baixa. Estudos histológicos e imunohistoquímicos da presbilaringe demonstram atrofia do epitélio, da lâmina própria e do músculo vocal, além de aumento de fibras colágenas e diminuição de fibras elásticas e das proteínas não fibrosas da matriz extracelular. Os estudos de microscopia eletrônica da presbilaringe são escassos e podem acrescentar detalhes ultraestruturais importantes e auxiliar na compreensão da fisiopatologia da presbifonia. **Objetivos**: descrever os achados de microscopia eletrônica de varredura e transmissão da prega vocal senil. **Casuística e métodos**: Foram removidas 16 laringes humanas durante necrópsia e distribuídas em dois grupos: controle (n-8; idade 30 - 50 anos; 6F e 2M) e idoso (n-8; idade 75- 92 anos; 6F e 2M). As porções medianas de ambas as pregas vocais foram dissecadas, fixadas em glutaraldeído 2,5% e preparadas para exames de microscopia eletrônica de varredura e transmissão. A espessura do epitélio foi medida nas fotografias de microscopia eletrônica de varredura, com aumentos semelhantes, utilizando-se o programa de morfometria digital *Scandium*. **Resultados**: ***Microscopia eletrônica de varredura****:* ***Grupo controle****:* epitélio composto por 5 a 7 camadas de células sobrepostas, raras células em descamação, e discreta ondulação. Lâmina própria com rede uniforme de fibras colágenas e elásticas, correndo paralelamente à membrana basal. ***Grupo idosos*****:** epitélio atrófico, composto por 2 a 3 células, maior número de células em descamação, junções intercelulares demarcadas por sulcos profundos. As medidas da espessura do epitélio foram menores nos grupos de idosos do que nos controles (22163.91 ± SD 14590.41nm *versus* 41783.73 ± SD 2139.314 nm, respectivamente). Lâmina própria com rede densa de fibras colágenas e elásticas formando emaranhados e com distribuição irregular. Nas camadas profundas as fibras colágenas formavam verdadeira carapaça fibrótica e rígida. ***Microscopia eletrônica de transmissão: Grupo Controle***: epitélio íntegro,

com células justapostas e desmossomos entre as junções intercelulares. Membrana basal contínua e uniforme e lâmina própria contendo fibras colágenas e elásticas em arranjo frouxo e regular, e alguns fibroblastos de diferentes formatos. ***Grupo Idosos***: células epiteliais distanciadas umas das outras e junções intercelulares alargadas. Membrana basal sem alterações, lâmina própria com predomínio de fibroblastos de formato alongado e citoplasma contendo vacúolos. Na lâmina própria havia densa rede de fibras colágenas, na qual alguns fibroblastos estavam mergulhados**. Conclusões:** neste estudo de microscopia eletrônica foram identificadas algumas alterações estruturais restritas às laringes dos idosos, tanto no epitélio como na lâmina própria, algumas delas com provável participação dos fibroblastos, o que reforça a importância de estudos adicionais voltados a essas células, tanto ultraestruturais como moleculares, por serem os principais precursores dos componentes da matriz extracelular.

Palavras chave: presbilaringe, microscopia eletrônica de varredura e transmissão, voz, presbifonia, disfonia, idoso.

ABSTRACT

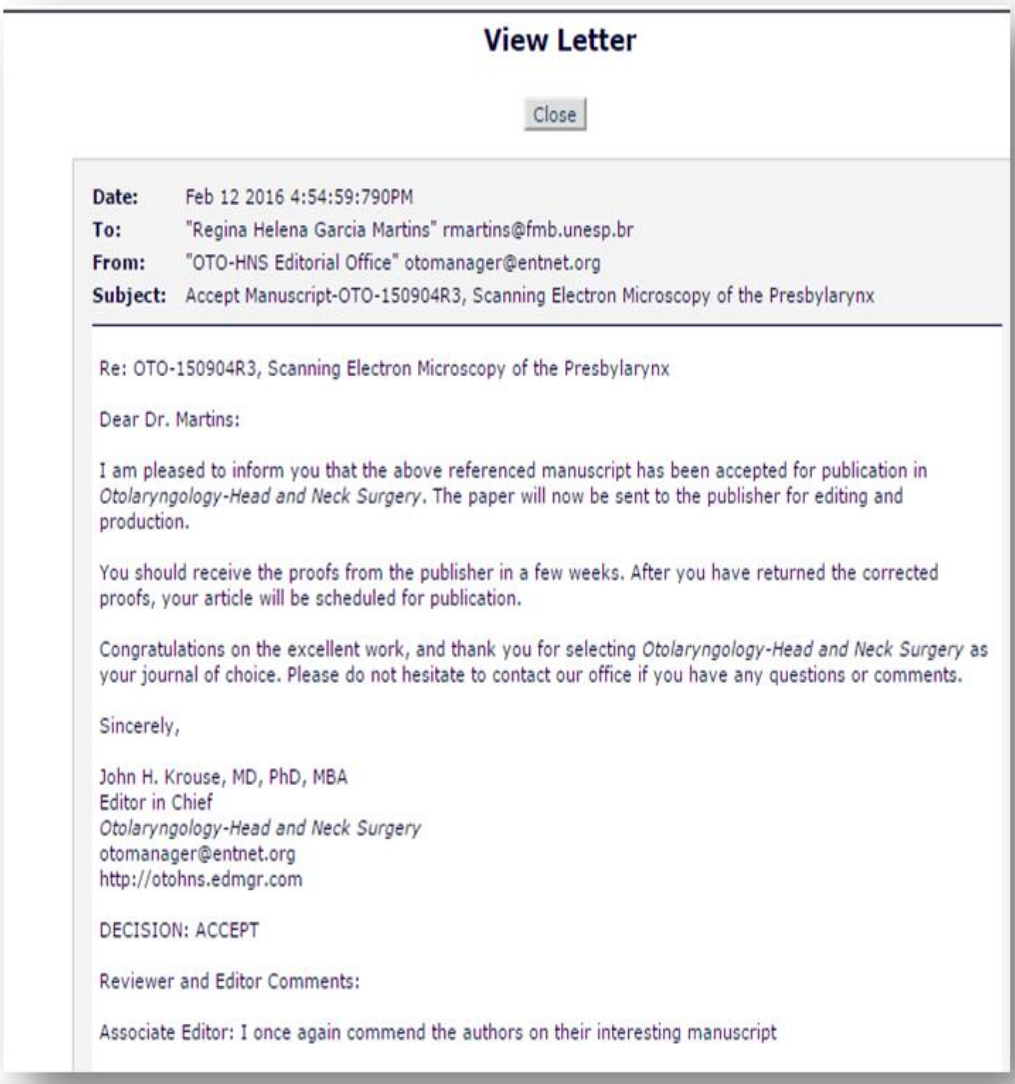
Introduction: Presbyphonia is called the set of changes in vocal patterns consequent aging of the larynx, which can present with symptoms of dysphonia, weak voice, trembling and low. Histological and immunohistochemical studies of presbylarynx show atrophy of the epithelium, lamina propria and the vocal muscle, and increase of collagen fibers and diminution of elastic fibers and non-fibrous proteins of the extracellular matrix. The studies of electron microscopy of presbylarynx are scarce and can add significant ultrastructural details contribute to further understanding of the pathophysiology of presbyphonia. **Objectives:** To describe the findings of scanning and transmission electron microscopy of senile vocal folds. Methods: 16 human larynx were removed during autopsy and distributed into two groups: control (n-8; age 30-50 years; 6F and 2M) and elderly (n-8, age 75- 92 years; 6F and 2M). The median portions of the right and left vocal folds were dissected, fixed in 2.5% glutaraldehyde and prepared for examination using scanning and transmission electron microscopy respectively. The thickness of the epithelium was analyzed in the pictures of the scanning electron microscopy with similar magnification, using the Scandium morphometric digital program. **Results:** ***Scanning electron microscopy: Control Group***: epithelium composed of 5-7 layers of overlapping cells, rare cells in flaking and slight ripple. Lamina propria with uniform network of collagen and elastic fibers running parallel to the basement membrane. ***Group Elderly***: atrophic epithelium, 2 to 3 cells, with more flaking cells and intercellular junctions marked by deep grooves. The epithelial thickness was lower in elderly than controls (22163.91 ± SD 14590.41nm *versus* 41783.73 ± SD 2139.314 nm, respectively). Lamina propria with dense network of collagen and elastic fibers forming tangles with irregular distribution. In the deep layers of collagen fibers formed shell true fibrotic and stiff. ***Transmission electron microscopy: Control Group:*** intact epithelium, with overlapping cells and desmosomes between intercellular junctions. Continuous and smooth basement

membrane and lamina propria containing collagen and elastic fibers in loose and regular arrangement, and fibroblasts of different formats. ***Group Elderly***: epithelial cells apart from one another enlarged intercellular junctions. Basement membrane unchanged lamina propria predominantly with fibroblasts with elongated shape and cytoplasm containing vacuoles. In the lamina propria was dense network of collagen fibers, in which some fibroblasts were plunged. **Conclusions:** In this study of electron microscopy identified some structural changes restricted to the larynx of the elderly in both the epithelium and lamina propria, some of them with possible participation of fibroblasts. This reinforces the importance of additional studies, both ultrastructural and molecular, facing fibroblasts, because these are the main precursors of extracellular matrix components.

Keywords: presbylarynx, scanning and transmission electron microscopy voice, presbyphonia, dysphonia, elderly.

Manuscrito 1. *Status: aceito*

Scanning Electron Microscopy of The Presbylarynx



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DECISION: ACCEPT

Reviewer and Editor Comments:

Associate Editor: I once again commend the authors on their interesting manuscript

SCANNING ELECTRON MICROSCOPY OF THE PRESBYLARYNX

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Abstract

Objectives: To describe the findings on presbylarynx under scanning electron microscopy. **Study Design**: descriptive study in cadaver study. **Setting:** Universidade Estadual Paulista, Botucatu Medical School (São Paulo, Brazil). **Subjects and Methods:** 16 vocal folds were removed during necropsies and distributed in two age groups: control (n-8; ages 30 - 50 years), and elderly (n-8; ages 75- 92 years). The median portion of the right vocal fold was dissected, fixed in glutaraldehyde 2.5%, and prepared to be examined under scanning electron microscopy. The thickness of the epithelium was measured using the Scandium morphometric digital program. **Results:** *Control group*: the epithelium had 5 to 7 overlapped cell layers, rare desquamation cells, little undulation and with protruding intercellular junctions. *Lamina propria* showed uniform network of collagen and elastic fibres in the superficial layer. A dense network of collagen was identified in the deeper layer. *Elderly group:* the epithelium was atrophic, with more desquamation cells and intercellular junctions delimited by deep sulci. *Lamina propria* with dense and irregular distribution of collagen and elastic fibres in the superficial layer. In the deep layers the collagen fibres formed a true fibrotic and rigid skeleton. **Conclusions**: The scanning electron microscopy (SEM) identified several changes in the elder larynx differentiating it from the control's. These alterations are probably related to the aging process of the vocal folds. However, the exact interpretation of these findings requires additional studies, even to molecular level, having as targets the fibroblasts.

Keywords: Presbylarynx, scanning electron microscopy, voice, presbyphonia, dysphonia, elderlies.

Introduction

Presbyphonia is the set of vocal changes secondary to aging of laryngeal structures[1]. Under such conditions, the aged voice acquires some peculiar features that denounce the age of the speaker, even on the telephone. The intensity of vocal symptoms in the elderly and the time of their appearance depend on individual factors and general health conditions[2-4]. The voice becomes lower, shaky and breathy, speaking and singing are therefore difficult, and vocal extension decreases. Men present increased fundamental frequency, rendering the voice more acute; women present a deeper voice[4, 5].

Histology and immunohistochemistry studies of senile larynges identify atrophy of the epithelium, *lamina propria,* and of the thyroarytenoid muscle, a decreased concentration of hyaluronic acid and of elastic fibres, and increased concentration of collagen fibres[6-9]. Some authors stress that such changes are not only quantitative but also qualitative[9, 10.] For Hammond *et al*[11], the aged larynx presents a decrease in reticular fibres and increased number of type I collagen fibres, but with impaired function, no longer warranting tissue resistance. The decrease in extracellular matrix glicosamines impairs further vocal folds viscoelasticity, justifying part of the vocal symptoms of the elderly.

Electron microscopy studies of the senile larynx are scarce, yet important, since they may add structural details identifiable by this method. Hirano *et al*[12], found almost twice as much collagen fibres in a study on senile larynges using scanning electron microscopy, more evident in the deeper layers of the *lamina propria.* The authors also found irregular diameters of the collagen fibres and decreased interstitial spaces that separate them from the other elements of the extracellular matrix such as glucosamine and glycosaminoglycans. These authors also noted a decrease in the amount of reticular fibres of the *lamina propria,* which correspond to a subtype of type III collagen fibres, with diameters of 50nm. The coexistence and distinction of type I and III collagen fibres in the *lamina propria* of the vocal folds were also demonstrated by Tateya *et al* [13], in a study

with adult human larynges using an interesting technique of immune-scanning electron microscopy.

The aging process of the body is complex and each study on this topic adds valuable information that enhances the understanding on the pathophysiology of the aging process of the many organs and systems. The objective of this study was to describe the findings of scanning electron microscopy of vocal folds in the elderly in order to uncover structural details of the presbylarynx.

Subjects and methods

Vocal folds removed from 16 cadavers during necropsy were distributed in two age groups: control (n-8; 30-50 years), and elderly (n-8; 75-92 years). After harvesting, the larynges were opened on their posterior portion, and examined macroscopically in order to identify lesions, in which case they were excluded.

A cross sectional fragment of approximately 0.8 to 1.0 cm of the median portion of the right vocal fold was removed (Figure 1). For scanning electron microscopy (SEM), the specimens were fixed in 2.5% glutaraldehyde for 12 hours, flushed in 0.1M phosphate buffer at pH 7.3, fixed in a 1% osmium tetroxide solution for 1 hour, flushed in phosphate buffer, dehydrated in increasing solutions of 75%-100% alcohol, and dried in a critical point device (Balzers CPD-020) with liquid carbon dioxide. The fragments of the vocal folds were assembled in a metal base with silver glue, and then covered with gold (15nm of gold) in a Balzers MED-010 device. They were visualized and photographed at increasing magnitudes under a scanning electron microscope (Quanta 200 FEG, FEI Company, Germany).

To measure the epithelium thickness was used the Scandium morphometric digital program which measures the distance between two points. A straight line was drawn from the distal surface of the epithelial to the beginning of the basal membrane. All measures were analyzed at 400x magnification, using ten

measures per picture in different sites. The mean of the values of each picture of both groups was presented in the Table 1.

The details of the epithelium and of the *lamina propria* components were presented in a descriptive way, comparing the images of the control group to the elderly's.

Exclusion criteria: sepsis, prolonged intubation, laryngeal lesions, systemic infectious diseases, dermatological diseases, autoimmune or metabolic disorders, cervical trauma, respiratory granulomatous diseases such as tuberculosis, paracoccidioidomycosis, sarcoidosis, and other causes or diseases which could affect the vocal folds mucosa and could invalidate the study. Medical records were consulted to confirm the pre-death diseases and familiars were inquired about the other comorbities.

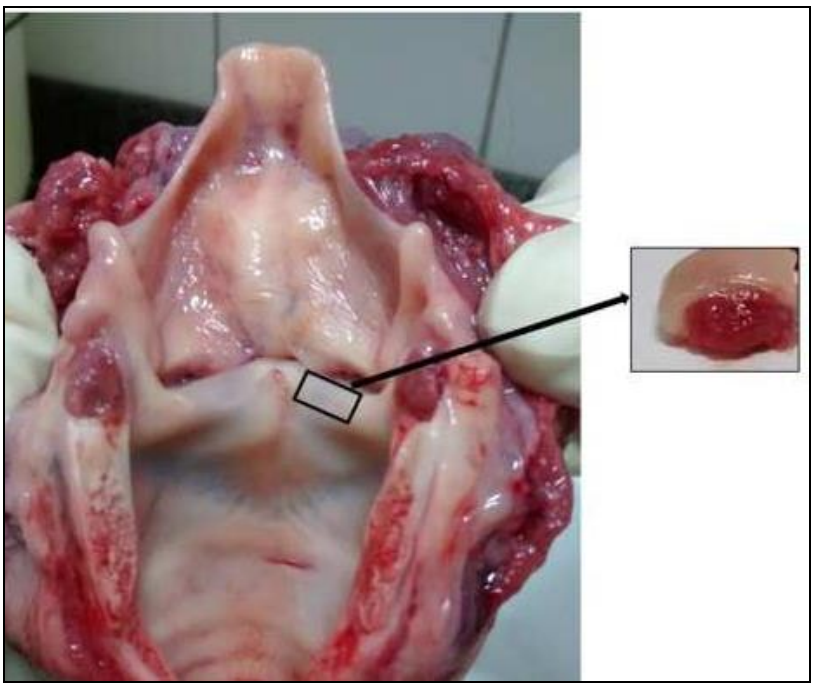


Figure 1 - Human larynx opened on the posterior portion for macroscopic examination and removal of the right vocal fold.

The research project was approved by the Human Research Ethics Committee (protocol number 13242413.1.0000.5411) and by the Pathology Department of the same institution.

Statistical analysis: The Student's t-test was used to compare the mean of the values of the thickness of the epithelium in both groups, using the 5% level of significance.

Results

All the specimens from the control group were similar at SEM. The covering mucosa had soft undulation and few desquamation cells (Figure 2). In the elderly group, most specimens had the covering mucosa more folded and with more desquamation cells (Figure 3).

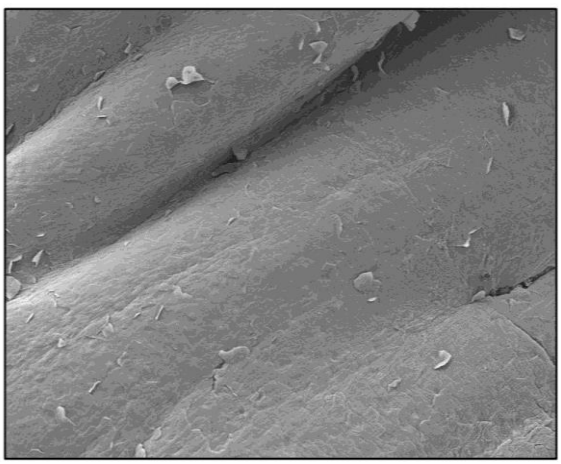


Figure 2 - Control group: surface of vocal fold with mild mucosal undulation and some desquamation cells. SEM, magnification 300X.

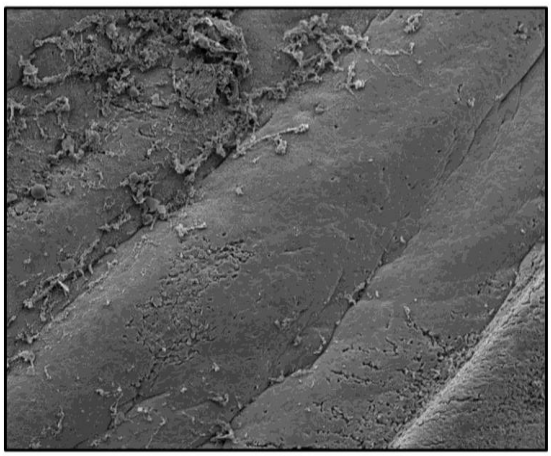


Figure 3 - Elderly group: surface of the covering mucosa of the vocal folds more folded and with more desquamation cells. SEM, magnification 300X.

In the control group micro folds drew a delicate tracery over the surface and the intercellular junctions demarcated irregular polygons on the surface (Figure 4). In six specimens of the elderly groups the intercellular junctions presented deep sulci (Figure 5) and the epithelial surface was irregular.

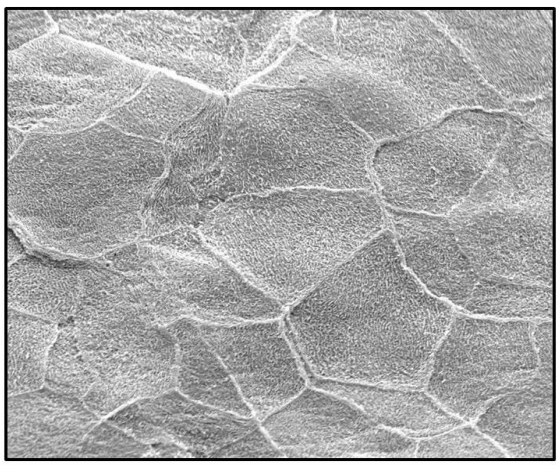


Figure 4. Control group: Epithelial surface of vocal fold highlighting the micro folds and protruding intercellular junctions. SEM, magnification 4000X.

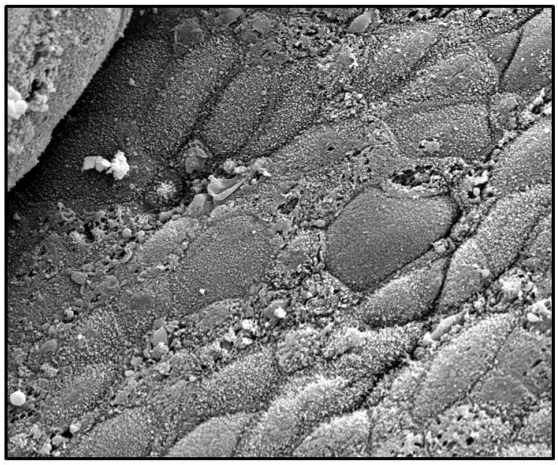


Figure 5. Elderly group: Epithelium with well-demarcated cells, separated by deep sulci. SEM, magnification 4000X.

In control group, the mucosa was formed by a juxtaposition of 5 to 7 cells that rested over the basal membrane. In the elderly groups the epithelial thickness varied, normal in two specimens and atrophic in the other six (Table 1).

Table 1. Mean and standard deviation (SD) values of the thickness of the epithelium in both groups.

Mean Values of the thickness of epithelium (nm)	Mean (SD)
Controls	41783.73 (2139.314)
Elderly	22163.91 (14590.41)
P Value	P<0.0001

In controls, in the superficial layer of the *lamina propria* a delicate network of collagen and elastic fibres was seen, running parallel to the basal membrane, just below the epithelium, (Figure 6). In elderly groups, in this site, the collagen was more abundant, and with iregular distribution (Figure 7).

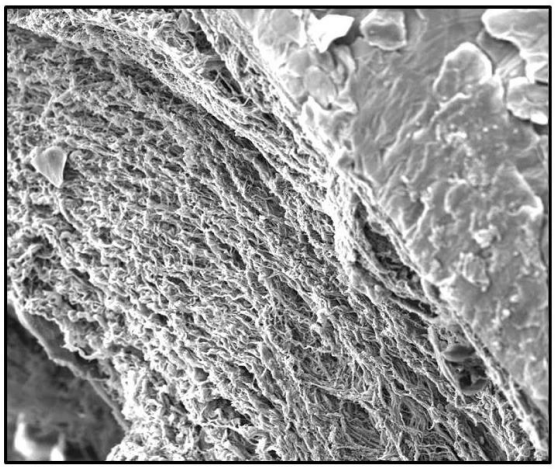


Figure 6 - Control group: detail for the lose network of collagen and elastic fibres parallel to the basal membrane in the superficial layer of the lamina propria. SEM, magnification 600X.

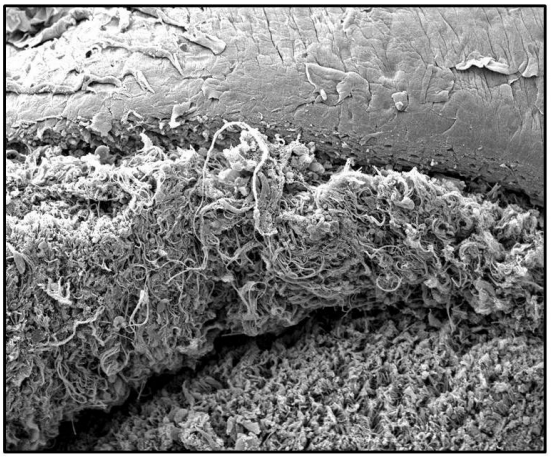


Figure 7 - Elderly group: dense and irregular network of collagen and elastic fibres in the superficial layer of the lamina propria. SEM, magnification 600X

In the deep layer of the lamina propria there was a dense network of collagen in both groups, but more intense in the elderly groups, in which a fibroticand rigid skeleton could be observed (Figures 8 and 9).

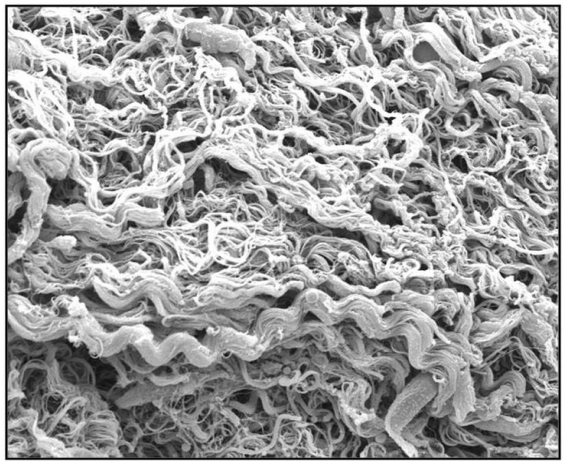


Figure 8 - Control group: detail for the dense network of collagen in the deeper layer of the lamina propria. SEM, magnification 2400X.

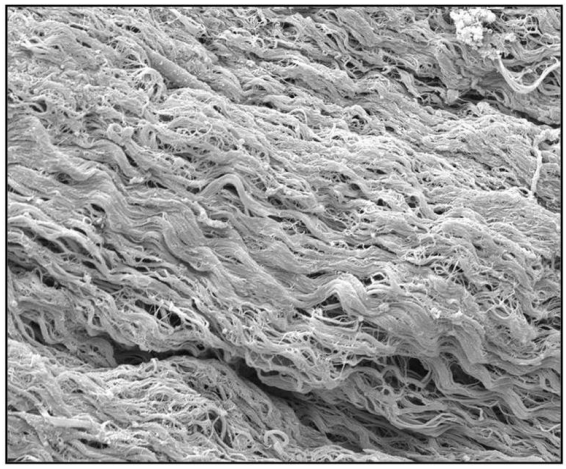


Figure 9 - Elderly group: detail for the collagen more abundant in the deeper layer of the lamina propria, forming a fibrotic and rigid skeleton.SEM, magnification 2400X.

Discussion

This study showed several changes in most vocal folds of the elderly identified by scanning electron microscopy, not reported previously. The aspect of the intercellular junctions of the epithelium of the vocal folds in the elderly, forming deep sulci, clearly demarcating the cells limits, allied to the increase of cell desquamation, and mucosal undulation, were frequent findings. Such changes may be explained by the natural aging process of the epithelium, where the cell junctions may be impaired by the decrease in the components of the extracellular matrix resulting in laxity, desquamation, and retraction[11,12].

The absence of studies with electron microscopy of the older larynx renders impossible the comparison of our descriptions to other authors. There are, nevertheless, many studies describing the skin aging process, the epithelium being similar, therefore correlations are possible. When identified in the epidermis, such changes have been attributed to the loss of fundamental substances of the extracellular matrix, causing wrinkles, epidermal retractions, loss of the proliferative capacity of the epithelium, and frailty[13,14]. The dermal *lamina propria* is described also as compact, dense, made of collagens and elastic fibres, many of which degenerating, reinforcing the hypothesis that the changes in the proteins of the *lamina propria* are not only quantitative but also qualitative.

The behaviour of the elastic fibres in the vocal folds of seniors has been discussed by authors. Some authors found important decrease in their concentration; others considered the amount unaltered, and others yet found considerable increase[11,12,13,15]. Hammond *et al*[11] analysed the concentration of elastic fibres in the *lamina propria* of 19 larynges, including children, adults, and seniors, and found a growing increase in elastin as age advances, indicating maturity of the structures of the vocal folds. The authors also observed increasing growth in collagen fibres, and underscored that this result not necessarily indicated increased production, but rather a decrease in turnover. Roberts *et al*[15], supported this same hypothesis and described the irregular distribution and larger number of

collagen fibres in the *lamina propria* of the vocal folds of the elderly, ratifying our findings, as other authors[6-9] . For those authors the collagen fibres do not finish degradation as a consequence of the impairment on the expression of the enzyme collagenase. The mature collagen fibres that are not degraded become distorted, forming irregular arrangements, and clustering in an entanglement of fibres with different diameters and in all directions, impairing tissue flexibility and elasticity. The deeper layers of the *lamina propria* receive a higher concentration of collagen fibres, as depicted in figure 8 and 9 of this study, forming a rigid skeleton. In our study, unfortunately it was not possible to differentiate the elastic fibers of the reticular collagen, requiring additional methods for this purpose

Structural changes in the fibroblasts are yet little known, and seem to be responsible for the modifications in the proteins of the extracellular matrix of the vocal folds of the elderly. Hirano *et a*l[12] consider important to study the behaviour of those cells, due to their key participation in the production of the elements of the extracellular matrix. In an interesting research those authors studied the fibroblasts of human larynges in neonates, adults, and seniors, and found that the shape of those cells changed as age progressed, going from oval in neonates to oblong and filamentous in adults and seniors. Furthermore, they emphasized that active fibroblasts had well-developed organelles, specially undulated endoplasmic reticulum and Golgi complex, which tend to decrease in the elderly.

Quan *et al*[14], in a study on fibroblast of skin of seniors, identified many fibroblasts with deletion in the mitochondrial DNA, impairing modulation of the fibroblasts and the configuration of the actin filaments of the cytoskeleton. According to those authors, such deletions might be triggered by oxidative stress and mitigated with the introduction of anti oxidants, what remains controversial among authors.

For Roberts *et al*[15], part of the changes observed in the aged vocal folds can be due to the reduction in the activity of fibroblasts, consequence of the possible decrease in blood flow. However the impairment in the vascular network of vocal folds of elderly larynxes could not be confirmed by theses authors.

Conclusion

The scanning electron microscopy identified several changes in the elder larynx differentiating it from the control's. These alterations are probably related to the natural aging process of the vocal folds. However, the exact interpretation of these findings requires additional studies, even to molecular level, having as targets the fibroblasts.

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Manuscrito 2. *Status*: *Under Review*

Transmission Electron Microscopy of The Presbylarynx

Otolaryngology - Head and Neck Surgery

Transmission electron microscopy of the presbylarynx

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Abstract:	Microscopy studies on presbylarynx have identified epithelial atrophy as well as vocal muscle atrophy, increase in collagen, and decrease in non-fibrous proteins of the extra cellular matrix. Ultrastructural studies of the presbylarynx can help us to understand the physiopathology of the presbyphonia. Objective: to describe details on the ultrastructure of the presbylarynx by transmission electron microscopy. Subjects and Methods: 16 human larynges were removed and distributed in two groups: control (n=8; 30-50 years; 6F,2M), and elderly (n=8; 75-92 years; 6F,2M). After preparation to transmission electron microscopy the specimens were examined and photographed at increasing magnitude, including epithelium and lamina propria. Results: Control Group: the epithelium was intact, with overlapped cells, and desmosomes between the intercellular junctions. The basal membrane was continuous and uniform and the lamina propria contained collagen and elastic fibers in a loose, with regular distribution, and some fibroblasts of different shape. Elderly Group: the epithelial cells were separated by enlarged intercellular junctions. The basal membrane was delicate and continuous. In the lamina propria there is a predominance of elongated fibroblasts and below the basal membrane there was a dense network of collagen fibers. Conclusions: In this study with transmission electron microscopy some structural changes peculiar to the older larynx were identified, both in the epithelium and in the lamina propria, some of them with likely participation of fibroblasts, what reinforces the importance of additional ultrastructure as well as molecular studies targeting those cells, since they are the main precursors of the extracellular matrix components.

TRANSMISSION ELECTRON MICROSCOPY OF THE PRESBYLARYNX

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Abstract

Introduction: Microscopy studies on presbylarynx have identified epithelial atrophy as well as vocal muscle atrophy, increase in collagen, and decrease in non-fibrous proteins of the extra cellular matrix. Ultrastructural studies of the presbylarynx can help us to understand the physiopathology of the presbyphonia. **Objective**: to describe details on the ultrastructure of the presbylarynx by transmission electron microscopy. **Subjects and Methods:** 16 human larynx were removed and distributed in two groups: control (n-8; 30-50 years; 6F,2M), and elderly (n-8; 75-92 years; 6F,2M). After preparation to transmission electron microscopy the specimens were examined and photographed at increasing magnitude, including epithelium and *lamina propria.* **Results:** *Control Group*: the epithelium was intact, with overlapped cells, and desmosomes between the intercellular junctions. The basal membrane was continuous and uniform and the *lamina propria* contained collagen and elastic fibers in a loose, with regular distribution, and some fibroblasts of different shape. *Elderly Group*: the epithelial cells were separated by enlarged intercellular junctions. The basal membrane was delicate and continuous. In the *lamina propria* there is a predominance of elongated fibroblasts and below the basal membrane there was a dense network of collagen fibers. **Conclusions:** In this study with transmission electron microscopy some structural changes peculiar to the older larynx were identified, both in the epithelium and in the *lamina propria,* some of them with likely participation of fibroblasts, what reinforces the importance of additional ultrastructure as well as molecular studies targeting those cells, since they are the main precursors of the extracellular matrix components.

Key words: presbylarynx, transmission electron microscopy, presbyphonia, elderly, ultrastructure, vocal folds.

Introduction

After the sixth decade of life vocal symptoms are frequently reported consequent to the voice aging process, called presbyphonia. The voice becomes hoarse, weak, low, shaky, and breathy[1-4]. With the global aging, an increase of seniors in voice clinics with those symptoms has been observed, since those symptoms impair communication and singing[4]. Preventive measures and treatment for presbyphonia intend to improve quality of life in that population, but it has been huge challenge for voice professionals, since it demands a detailed knowledge of the pathophysiology of the functional, anatomical, and ultrastructural modifications that slowly occur with aging in all phonatory apparatus, specially in the presbylarynx, many of them little understood.

Studies on the histology of the presbylarynx have demonstrated atrophic epithelium and vocal muscle, greater amount of collagen fibers in the *lamina propria,* shortness of non-fibrous proteins in the extracellular matrix, and decreased number of elastic fibers [5-9]. The studies with scanning electron microscopy (SEM) confirm some of those changes and add interesting structural details, such as a greater number of cells scaling from the epithelial surface, greater concentration of collagen cells in the *lamina propria,* with irregular distribution [5,7,10]. As a function of the important role of fibroblasts in the production of a large number of the extracellular matrix components, some authors have studied the ultrastructure of those cells in the vocal folds of elderly subjects[11-13]. Hirano *et al*.[11] compared the fibroblast in two sites of young and old larynges, the *macula flava*, and the Reinke's space, using transmission electron microscopy (TEM). The authors found structural differences between the fibroblasts of young and senior individuals, such as more elongated shape in the aged larynges in the Reinke's space, and star-shaped fibroblasts in the *macula flava,* in addition to a smaller number of organelles in the cytoplasm, suggesting decreased cell activity, most likely secondary to aging. Allah *et al*.[12] in a similar study, studied the fibroblast in the larynges of seniors, adults, and neonates, under

TEM, and noted that most fibroblasts in neonates were oval-shaped, both in the *macula flava* and in the Reinke's space; in adults, on the other hand, those cells were more elongated. The nucleus-cytoplasm rate in the fibroblasts was higher in neonates as compared to adults and seniors, although in the latter the rugose endoplasmic reticulum and the Golgi complex were moderately developed.

Each research developed along those lines adds important structural details on the better understanding of presbylarynx. The objective of this study is, therefore, to describe other findings of transmission electron microscopy in the vocal folds of seniors.

Subjects and Methods

The larynges of 16 cadavers were excised and distributed in two age groups: control (n-8; 30-50 years; 6F and 2M), and seniors (n-8; 75-92 years; 6F and 2M). The larynges were opened on their posterior portion for macroscopic examination of the vocal folds in order to identify pre existing lesions or traumatic injuries that might be acquired at the moment of the autopsy. When injuries were detected the larynxes were excluded from the study. The median portion of the left vocal fold was delicately dissected and removed in a cross sectional fashion, resulting in a fragment of about 0.8 to 1.0 cm. The right vocal fold was preserved for another study.

The fragments removed were immediately fixed in 2.5% glutaraldehyde solution and sent to the Electron Microscopy Laboratory to be processed according to its routine, as follows: the specimens were fixed in 2.5% glutaraldehyde, flushed in 0.1M phosphate buffer, pH 7.3. Subsequently, they were sectioned into 3mm x 1mm slices on pink dental wax, wetted in 2.5% glutaraldehyde fixing solution, fixed by 1% osmium acid and 0.1M phosphate buffer, pH 7.3, dehydrated in an increasing series of acetone solution (50%, 70%, 90% and 100%), and dipped in a mixture of acetone and araldite resin (Polysciences, Inc.). The fragments were removed from this mixture and

embedded in an araldite resin block in an oven at 37ºC. Semithin 0.5-μm sections were prepared with an ultramicrotome and dyed with a mixture of 1% methylene blue and 1% Azur II.

These semithin slices were examined under light microscopy and cut into ultrathin sections that were examined and photographed under a transmission electron microscope, frame by frame at increasing magnifications (model Tecnai Spirit, Co company, Czech Republic). The specimens were examined from the epithelium towards the *lamina propria*, taking digital photography in increasing magnitude. The details of the components of the epithelium and of the *lamina propria* were described, comparing the pictures of both groups.

Exclusion criteria: sepsis, intubation for more ten days, laryngeal lesions, systemic infectious diseases, dermatological diseases, autoimmune or metabolic disorders, laryngeal trauma, granulomatous diseases such as tuberculosis, paracoccidioidomycosis, sarcoidosis, and other causes of laryngeal lesions. Medical records were consulted to confirm the pre-death diseases and familiars were inquired about the other comorbities.

The project was approved by the Human Research Ethics Committee of the Botucatu School of Medicine (UNESP, protocol 13242413.1.0000.5411l), and by the Department of Pathology of the same institution.

Results

In control group, the covering epithelium of the vocal folds was intact, with overlapped cells and desmosomes between the intercellular junctions (Figure 1-a). In the elderly group, the epithelial cells were distant from one another; intercellular junctions were enlarged, impairing desmosomal contacts (Figure 1b).

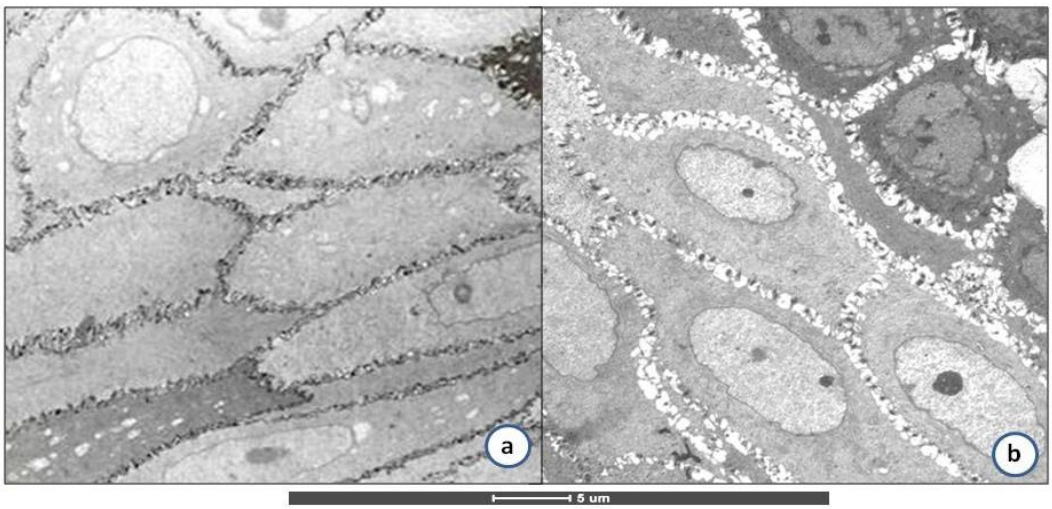


Figure 1- a) Control group: covering epithelium of the vocal folds intact, with overlapped cells and desmosomes between the intercellular junctions. b) Elderly group: epithelial cells distant from one another; intercellular junctions enlarged. Transmission Electron Microscopy.

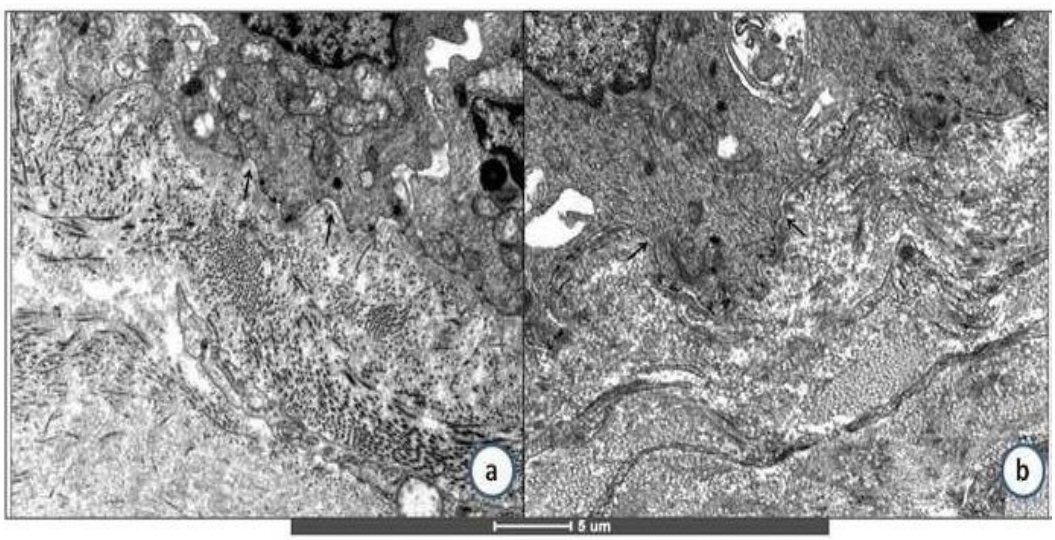


Figure 2 - a) (Control group) and B (Elderly group): basal membrane continuous and uniform (arrows). Transmission Electron Microscopy.

The basal membrane was continuous and uniform in both groups (Figures 2-a and 2-b). In controls, in the *lamina propria* the collagen and elastic fibers formed a loose, regular arrangement (Figure 3-a and 4-a). In the elderly group, in this site, the concentration of collagen fibers was higher, forming a dense network in the *lamina propria* (Figure 3-b and 4-b) in which some fibroblasts were immersed.

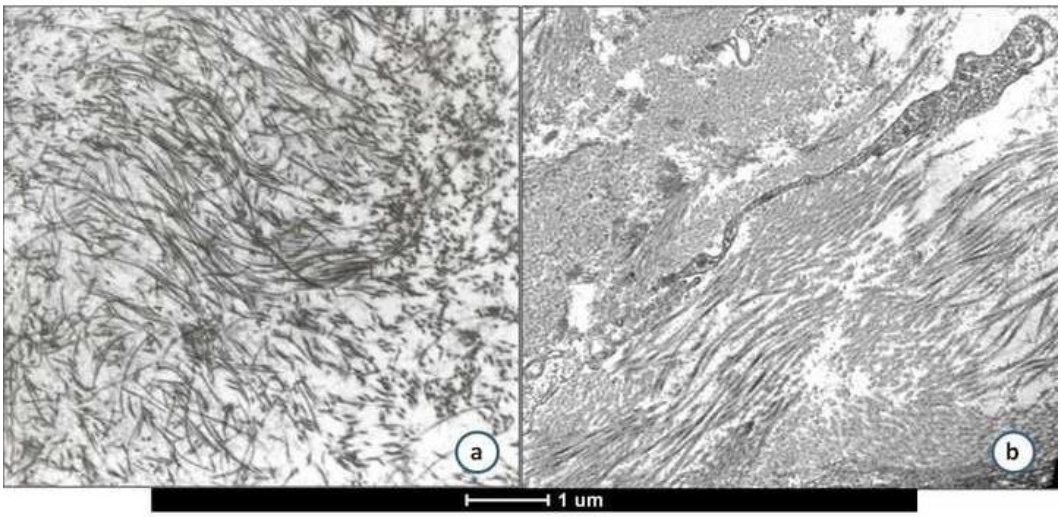


Figure 3 - a) Control group: *lamina propria* with collagen and elastic fibers formed a loose and regular arrangement. b) Elderly group: higher concentration of collagen and elastic fibers, forming a dense network in the *lamina propria*. Transmission Electron Microscopy.

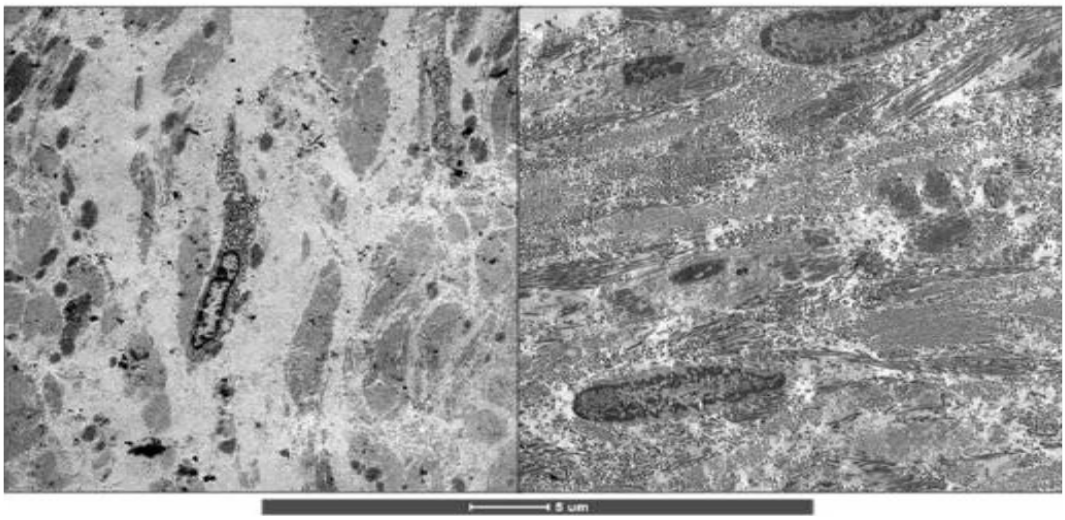


Figure 4 - a) Control group: *lamina propria* with regular arrangement of the collagen and elastic fibers. b) Elderly group: dense network of collagen and elastic in the *lamina propria* in which some fibroblasts were immersed. Transmission Electron Microscopy.

In both groups, just underneath the basal membrane, some fibroblasts were identified presenting different shapes such as elongated (figure 5), or star-like (Figure 6), and oval (Figure 7), containing large nucleus and uncondensed chromatin, abundant cytoplasm, well developed organelles in the cytoplasm, especially rough endoplasmic reticulum. In the elderly group, there was a predominance of elongated fibroblasts.

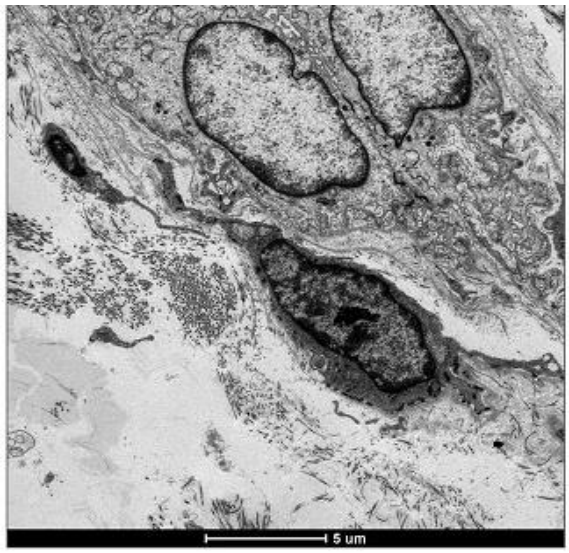


Figure 5 - Elongated fibroblast in the lamina propria of the vocal fold (Elderly group). Transmission Electron Microscopy.

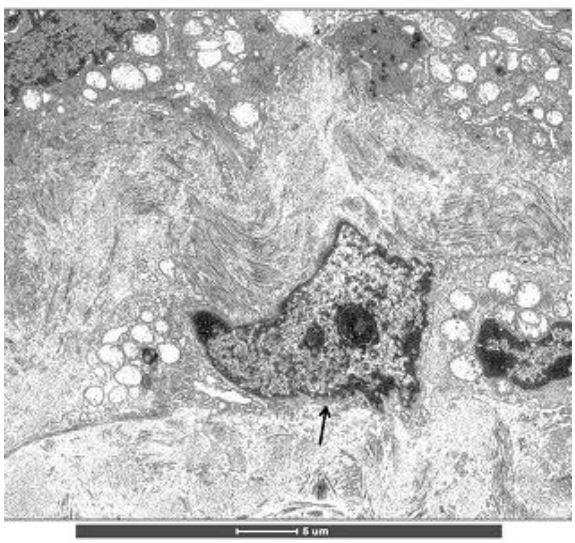


Figure 6 - Star-shaped fibroblast in the lamina propria, embedded in the collagen and elastic fibers network (Elderly group, arrow). Transmission Electron Microscopy.

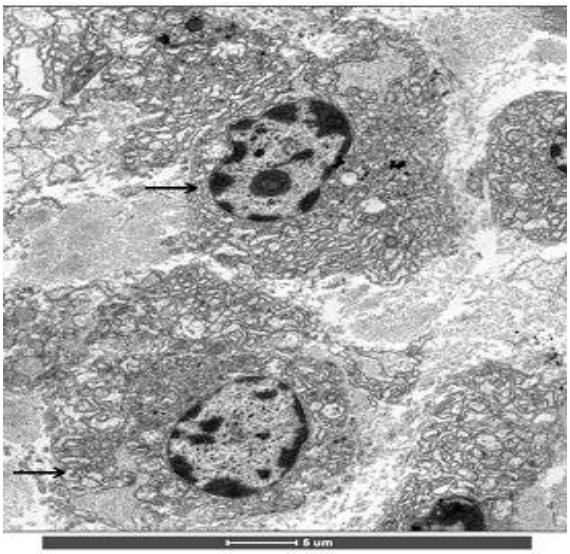


Figure 7 - Oval-shaped fibroblasts in the vocal folds lamina propria (Control group, arrows). Transmission Electron Microscopy

Discussion

In presbyphonia, functional, anatomical, and morphological changes are described in the whole pneumo-phono-articulatory system, responsible for the vocal symptoms such as difficulties in sustaining sound, decreased voice intensity and amplitude, tremor, and decreased velocity[14, 15]. Aging voice is a complex and individual process influenced by many factors such as general health, nutritional status, hormone levels, habits and vices, medications, and systemic diseases15. The influence of sex hormones over voice is one of the most studied of all those factors, since the laryngeal mucosa harbors hormone receptors, especially steroidal, including androgen, progesterone, estrogen, and corticosteroids. Such receptors are responsible for the vocal changes during pregnancy, menstrual cycle, and menopause[15].

D´haeseleer *et al.*[16] compared the perceptive voice analysis of 34 young women to the of 38 post menopausal women and observed significant difference in the G, B and S parameters of the GRBAS scale, as well as important reduction in the f0 values of the acoustic analysis. Later, those same authors analyzed the

impact of hormone replacement in the vocal quality of postmenopausal women, comparing two types of hormone replacement (estrogen or estrogen + progesterone) and they confirmed the benefits of hormone replacement in maintaining f0, regardless of the type of hormone treatment[17]. Ferraz *et al*[18] analyzed the voices of 106 women (GI: n- 43, with normal ovarian function; GII: n- 63, without normal function) and found lower f0 values in GII, around 17.4 Hz.

We know that estrogen acts in skin, bones, mucosa, and tissues extracellular matrix, acting as chemical mediator in the behavior of fibroblasts, and, as a consequence, in the production and distribution of collagen and elastic fibers[19-21]. In the skin, the absence of sex hormones renders it less elastic and frail, resulting in wrinkles, retractions, and loss of epithelial proliferative capacity, all these improving with hormone replacement [21]. As in skin, hormone replacement seems to contribute also for voice improvement in postmenopausal women[17,18].

As described above, the presence and the benefit of hormone receptors in the laryngeal mucosa are well known; nevertheless, the exact role of those hormones in the pathophysiology of the vocal changes inherent to aging is not yet entirely understood. In the elderly, besides voice impairment related to the decrease in sex hormones, the other important factor is age. The epithelium of the vocal folds in the elderly is atrophic and the epithelial cells are widely separated due to the impairment of the intercellular junctions components, produced by the fibroblasts, therefore becoming elongated and widely separated, as pointed in this study and others[22,23]. Kosztyla-Hojna *et al*[22] in a study on transmission electron microscopy on 50 larynges of senior patients demonstrated damage to the epithelial cells and to the intercellular junctions, that were filled by amorphous substance, indicating edema and inflammation, with additional increase in the blood vessels of the *lamina propria.* Some authors who examined vocal folds with edematous lesions described epithelial changes similar to senile larynges, in addition to inflammatory infiltrate[23-25].

In this study we did not identify alterations in the basal membrane, not even thickening; however, in the *lamina propria* we noted a dense network of

collagen fibers, undistinguishable from the elastic fibers by this technique. Studies in histology, immunohistochemistry, and electron microscopy confirm our findings[5,7,10,26], and demonstrate the proximity between the collagen fibers as well as their irregular distribution. In some of our specimens, fibroblasts were embedded in a dense collagen network, seeming strangled, even. Under such conditions, certainly the elasticity and flexibility of the vocal folds are impaired and explain the symptoms of presbyphonia. Comparing once again to the dermis, in the skin of seniors a compact and dense network of collagen and elastic fibers is described, many of those fibers being in degeneration[19], reinforcing the hypothesis that the alterations in the proteins of the *lamina propria* of the elderly are not only quantitative, but also qualitative, in the dermis as in the vocal folds[5,10,11].

For some authors, besides quantitative and qualitative changes of the collagen and elastic fibers, there is also a decrease in the turnover of those fibers, rendering them unable to perform their respective roles, such as flexibility and resistance[5,10,11,19]. It is believed that in this process, sex hormone decrease may have some influence, since estrogen decrease has a negative effect in the turnover of connective tissues, guided by fibroblast[5,10,27]. For those authors the collagen fibers do not finish their degradation due to impaired collagenase expression. Mature collagen fibers that do not degrade are distorted, forming irregular patterns, and cluster in fibrous entanglements of different diameters and in all directions, impairing tissue flexibility and elasticity.

Structural alterations in the fibroblast are poorly known and seem to be responsible for the modifications in the extracellular proteins of the vocal folds in the elderly. Hirano *et al*[11] consider important to study the behavior of such cells, due to their important participation in the production of the extracellular elements. In an interesting study those authors examined human laryngeal fibroblasts from neonates, adults, and seniors and found that the shape of those cells changed with aging, going from oval in neonates to elongated and filamentous in adults and seniors. Additionally, they emphasize that active fibroblasts have well developed

organelles, especially the rugose endoplasmic reticulum and Golgi complex, as we could also demonstrate.

In this study, in the control larynges we observed fibroblasts with more varied shapes, as star-shaped, oval, and elongated. In the seniors larynges the elongated fibroblasts predominated. Quan *et al* [28], in a study with skin fibroblasts of elderlies, identified many fibroblasts with deletion in their mitochondrial DNA, what hampered their shape modulation and the configuration of the actin filaments in the cytoskeleton; those changes might justify the alterations in the shape of fibroblasts and their lower activity.

Conclusions

In this study with transmission electron microscopy some structural changes peculiar to the older larynx were identified, both in the epithelium and in the *lamina propria,* some of them with likely participation of fibroblasts, what reinforces the importance of additional ultrastructure as well as molecular studies targeting those cells, since they are the main precursors of the extracellular matrix components.

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PARECER DO COMITE DE ÉTICA EM PESQUISA (CEP)

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Estudo de microscopia eletrônica de transmissão e varredura de pregas vocais de idosos

Pesquisador: Tatiana Maria Gonçalves

Área Temática:

Versão: 1

CAAE: 13242413.1.0000.5411

Instituição Proponente: Hospital das Clínicas da Faculdade de Medicina de Botucatu ((HCFMB))

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 211.190

Data da Relatoria: 04/03/2013

Situação do Parecer

Aprovado.

BOTUCATU, 05 de Março de 2013

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Assinado por: Trajano Sardenberg (Coordenador)