Transmission Electron Microscopy of the Presbylarynx in the Process of Voice Aging

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Summary: Introduction. Microscopy studies of presbylarynx have identified epithelial atrophy, as well as vocal muscle atrophy, increased collagen, and decreased nonfibrous proteins of the extracellular matrix. Studies on the ultrastructure of presbylarynx are rare and can help us understand the pathophysiology of presbyphonia.
Objective. To describe details on the ultrastructure of presbylarynx by transmission electron microscopy (TEM).
Subjects and Methods. Sixteen human larynges were removed and distributed in two groups: control (n = 8; 30–50 years; six females, two males) and seniors (n = 8; 75–92 years; six females, two males). After preparation for TEM, the specimens, including epithelium and lamina propria, were examined and photographed at increasing magnification.
Results. Control: the epithelium was intact, with overlapped cells, and with desmosomes between the intercellular junctions. The basement membrane was continuous and uniform, and the lamina propria contained collagen and elastic fibers in a regularly distributed loose arrangement, as well as some fibroblasts of different shapes. Senior: the epithelial cells were separated by enlarged intercellular junctions. The basement membrane was delicate and continuous. In the lamina propria there was predominance of elongated fibroblasts. Under the basement membrane a dense network of collagen fibers could be identified.
Conclusions. In this study with TEM some structural changes were identified, particularly in the larynx of the elderly, both in the epithelium and lamina propria, some of them with likely participation of fibroblasts, which reinforces the importance of additional ultrastructure as well as molecular studies targeting those cells, as they are the main precursors of the components of the extracellular matrix.
Key Words: Presbylarynx–Transmission electron microscopy–Elderly–Ultrastructure–Aging voice.

INTRODUCTION

After the sixth decade of life, vocal symptoms are a frequently reported consequence of the voice aging process, called presbyphonia.[1] The voice becomes hoarse, weak, low, shaky, and breathy.[1–4] As a consequence of global aging, an increased number of seniors have been seen in voice clinics with vocal symptoms, as those symptoms impair communication and singing.[4] Preventive measures and treatment for presbyphonia seek to improve quality of life in this population, but presbyphonia has been a huge challenge for voice professionals because it demands a detailed knowledge of the pathophysiology of the functional, anatomical, and ultrastructural modifications that slowly occur with aging in the entire phonatory apparatus, especially in the presbylarynx, many of which are poorly understood.

Studies on the histology of presbylarynx have demonstrated atrophic epithelium and vocal muscle, greater amount of collagen fibers in the *lamina propria,* shortness of nonfibrous proteins in the extracellular matrix, and decreased number of elastic fibers.[5–9] Studies performed using 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, and a greater concentration of collagen cells in the *lamina propria,* with irregular distribution.[5,7,10]

Because of the major 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.[10–12] Hirano et al[11] compared the fibroblasts of two sites in young and old larynges, the *macula flava* and Reinke's space, using transmission electron microscopy (TEM). The authors found structural differences between the fibroblasts of young and elderly individuals, such as a more elongated shape in the aged larynges in 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 fibroblasts 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 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 rough endoplasmic reticulum and the Golgi complex were moderately developed.

Every research developed along those lines provides further structural details for a better understanding of presbylarynx. The objective of this study is, therefore, to describe other findings of TEM in the vocal folds of seniors.

SUBJECTS AND METHODS

The larynges of 16 cadavers were excised and distributed into two age groups: control (n = 8; 30–50 years; six females, two males) and seniors (n = 8; 75–92 years; six females, two males). The larynges were opened on their posterior portion for macroscopic examination of the vocal folds to identify preexisting lesions or traumatic injuries that might have been acquired at

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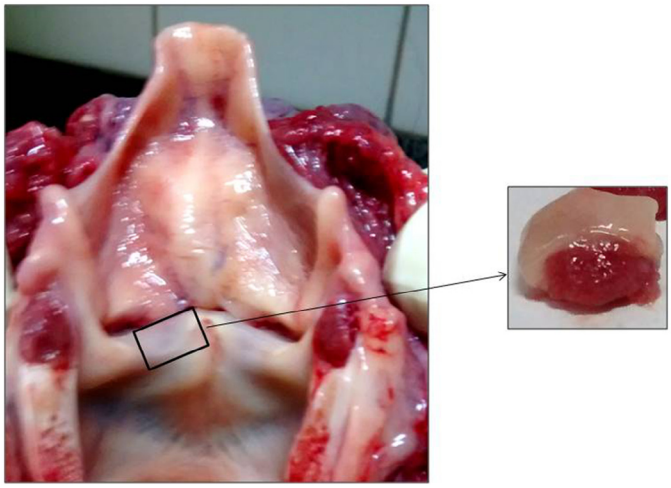

FIGURE 1. Larynx removed during necropsy and right vocal fold dissected.

the moment of the autopsy. When injuries were detected, the larynges were excluded from the study. The median portion of the right vocal fold was delicately dissected and removed in a cross-sectional fashion, resulting in a fragment of about 0.8 to 1.0 cm (Figure 1). The left 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 0.1 M phosphate buffer, pH 7.3. Subsequently, they were sectioned into 3 mm × 1 mm slices in pink dental wax, wetted in 2.5% glutaraldehyde fixing solution, fixed by 1% osmium acid and 0.1 M 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., Warrington, Pennsylvania, United States). The fragments were removed from this mixture and embedded in an araldite resin block in an oven at 37°C. Semi-thin 0.5-µm sections were prepared with an ultramicrotome and dyed with a mixture of 1% methylene blue and 1% Azur II.

These semi-thin slices were examined under light microscopy and cut into ultra-thin sections that were examined and photographed under a transmission electron microscope, frame by frame, at increasing magnifications (model Tecnai Spirit, FEI Company, Brno, Czech Republic). The specimens were examined by three researchers blinded to the groups (two authors and one researcher who is an expert in electron microscopy). The epithelium toward the *lamina propria* was evaluated by digitally photographing them at increasing magnifications. The details of the components of the epithelium and of the *lamina propria* were described, comparing the pictures of both groups.

Exclusion criteria were the following: sepsis, intubation for more than 10 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 predeath diseases, and family members were asked about other comorbidities.

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 the control group, the epithelium lining the vocal folds was intact in all specimens, with overlapped cells and desmosomes between the intercellular junctions (Figure 2A). In all the larynx of the senior group, the epithelial cells were distant from one another; the intercellular junctions were enlarged, impairing desmosomal contacts (Figure 2B).

The basement membrane was continuous and uniform in both groups, in all samples (Figure 3A,B). In all controls specimens, the collagen and elastic fibers formed a loose, regular arrangement (Figure 4A,B) in the *lamina propria*. In seven samples of the senior group, there was a higher concentration of collagen fibers forming a dense network in the *lamina propria* (Figure 5A,B), in which some fibroblasts were immersed.

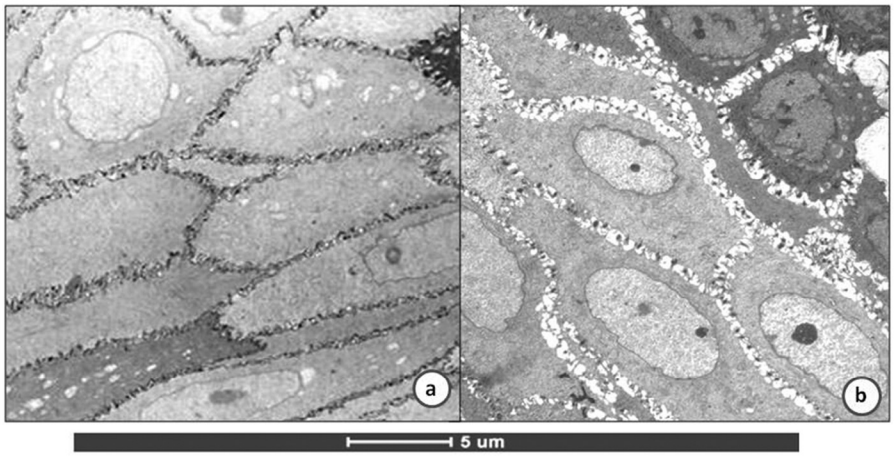

FIGURE 2. **A.** Control group: intact epithelium lining the vocal folds, with overlapped cells and desmosomes between the intercellular junctions. **B.** Senior group: epithelial cells distant from one another; intercellular junctions enlarged. Transmission electron microscopy.

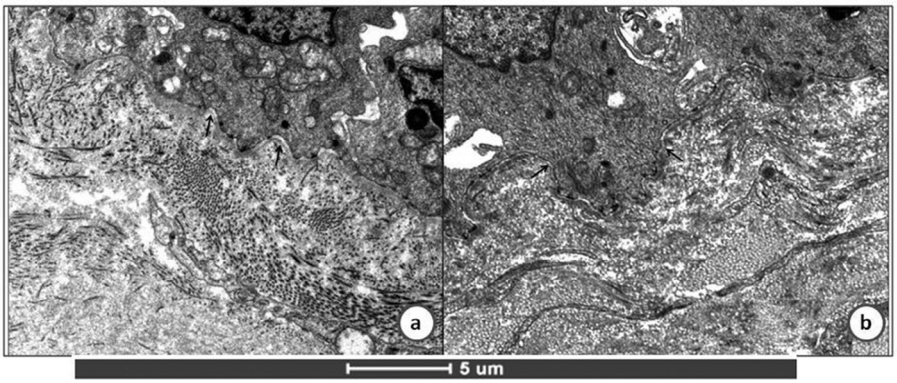

FIGURE 3. **A.** Control group and **B.** Senior group: basement membrane continuous and uniform (arrows) in both groups. Transmission electron microscopy.

In both groups, just underneath the basement membrane, some fibroblasts were identified presenting different shapes such as elongated (Figure 6A,B), or star-like (Figure 7), and oval (Figure 8), containing large nucleus and uncondensed chromatin, abundant cytoplasm, well-developed organelles in the cytoplasm, especially rough endoplasmic reticulum. In the senior group, there was predominance of elongated fibroblasts in all specimens.

DISCUSSION

In presbyphonia, functional, anatomical, and morphological changes are described in the entire pneumo-phono-articulatory system, responsible for vocal symptoms such as difficulty in sustaining sound, decreased voice intensity and amplitude, tremor, and decreased velocity.[13,14] Voice aging is a complex and individual process influenced by many factors such as general health,

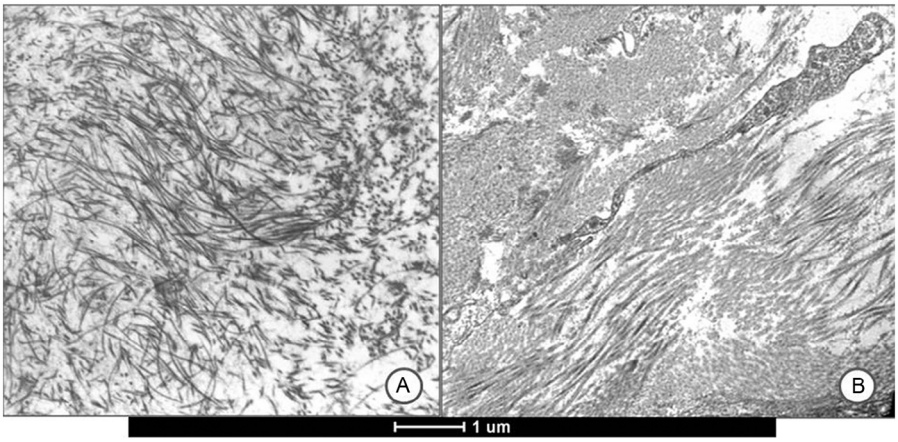

FIGURE 4. **A.** and **B.** Control group: *lamina propria* with collagen and elastic fibers forming a loose and regular arrangement.

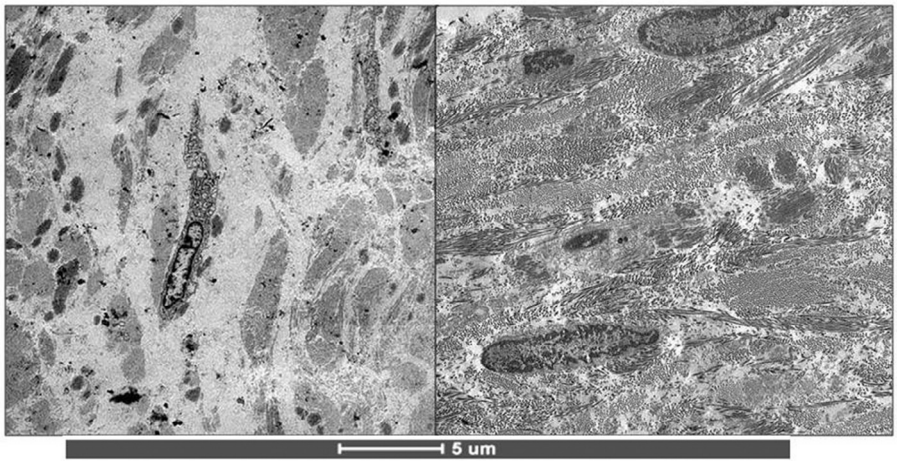

FIGURE 5. **A.** and **B.** Senior group: *lamina propria* with higher concentration of collagen and elastic fibers, forming a dense network where some cells are immersed. Transmission electron microscopy.

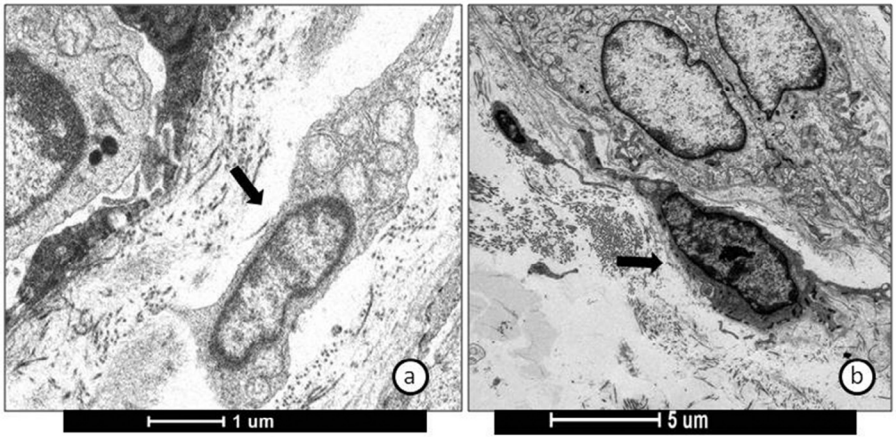

FIGURE 6. **A.** and **B.** Senior group. Elongated fibroblast in the *lamina propria* of the vocal folds (arrows). Transmission electron microscopy.

nutritional status, hormone levels, habits and vices, medications, and systemic diseases.[14]

The epithelium of the vocal folds in the elderly is atrophic and the epithelial cells are widely separated because of the impairment of the intercellular junction components, as pointed out in the present study and others.[15,16] Kosztyla-Hojna et al,[15] in a study on TEM on 50 larynges of senior patients, demonstrated damage to the epithelial cells and to the intercellular junctions that were filled with an amorphous substance, indicating edema and inflammation, with an additional increase in the blood vessels of the *lamina propria.* Some authors who examined vocal folds with edematous lesions described epithelial changes similar to the ones found in senile larynges, in addition to inflammatory infiltrate.[16–18] Hence, these findings are not specific of senile larynges.

We did not identify alterations in the basement membrane of the presbylaryges, not even thickening; however, in the *lamina propria* we noted a dense network of collagen fibers, indistinguishable from the elastic fibers by this technique. Studies in histology, immunohistochemistry, and electron microscopy confirm our finding[5,7,9,19] and demonstrate the irregular distribution of the collagen fibers.[10]

In some of our specimens of the elderly larynx, fibroblasts were dipped in a dense network of fibrous matrix. Under such conditions, the elasticity and flexibility of the vocal folds are probably impaired and could explain some symptoms of presbyphonia. We observe similar phenomena when we compare these findings with the aging process of the skin. The skin of seniors presents a compact and dense network of collagen and elastic fibers in the *lamina propria* and many of those fibers are in degeneration process. These findings support 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 well as in the vocal folds.[5,7,9,10,20] For those authors, there is also a decrease in the turnover of those fibers, rendering them unable

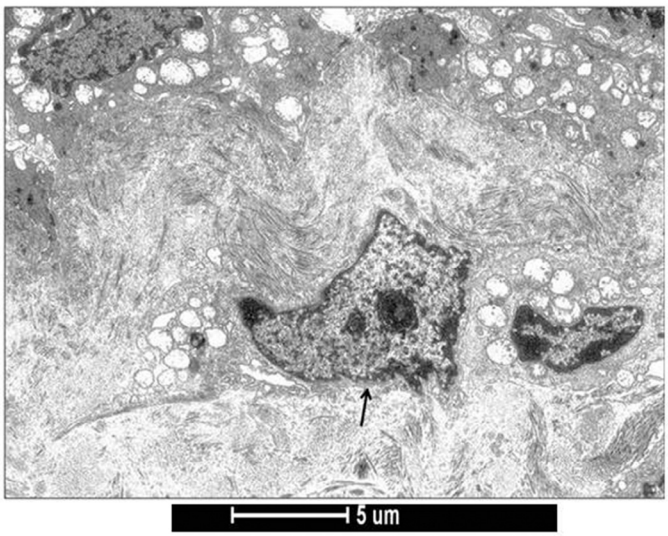

FIGURE 7. Senior group—Star-shaped fibroblast in the *lamina propria*, embedded in the network of collagen and elastic fibers (arrow). Transmission electron microscopy.

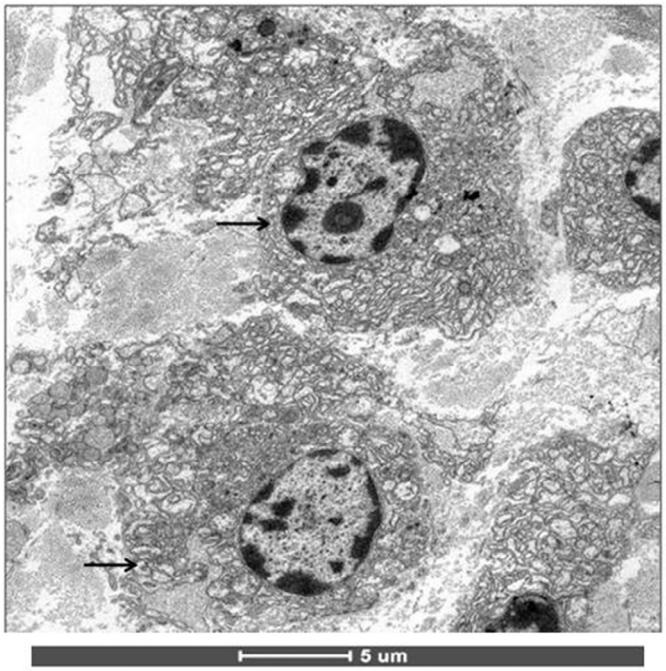

FIGURE 8. Control group—Oval-shaped fibroblasts in the *lamina propria* of the vocal folds (arrows). Transmission electron microscopy.

to perform their respective roles, such as flexibility and resistance.[20] Collagen fibers do not finish their natural degradation because of 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.[20]

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] also considered the importance of studying the behavior of such cells because of their significant participation in the production of extracellular elements. In an interesting study, the 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 rough endoplasmic reticulum and Golgi complex, as we could also demonstrate.

CONCLUSIONS

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

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