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Fibroblasts in the human vocal fold mucosa: an ultrastructural study of different age groups

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ABSTRACT

Introduction: An investigation was carried out to determine the morphological characteristics of fibroblasts in two portions of the vocal fold (VF) mucosa, the macula flava (MF) and Reinke's space (RS), of three different age groups: newborns, adults and geriatrics.

Methods: Normal human VF obtained from autopsy cases were included in this study: four from mature newborns; four from middle-aged adults; and four from geriatric cases. Fibroblasts in RS and MF were investigated by transmission electron microscopy.

Results: The fibroblasts of the MF in both adults and newborns tended to be stellate in shape, with a small nucleus/cytoplasm (N/C) ratio and a well-developed rough endoplasmic reticulum (rER) and Golgi apparatus (GA). Most of the fibroblasts present in RS were oval in newborns and spindle-shaped in adults, with a large N/C ratio and less developed rER and GA. The majority of fibroblasts of the geriatric MF were stellate in shape; while in geriatric RS, the majority of fibroblasts were spindle-shaped with an N/C ratio of 0.5 to 2.0 as in the case of adults. However, the development of rER and GA was less marked in geriatrics than in adults.

Conclusion: Histological changes of fibroblasts in the VF mucosa are one of the important causes of the change in voice quality with ageing. Furthermore, geriatric changes in the vocal ligament can be attributed to the activities and the presence of ageing processes in fibroblasts of geriatric VF mucosa.

Keywords: fibroblasts, human vocal fold mucosa, macula flava, Reinke's space

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INTRODUCTION

The vibratory movement of human vocal folds (VF) plays an important role in the normal function of phonation.[1,2] Furthermore, human adults, in contrast to musical instruments that require multiple sound generators, can produce a great variety of sounds through the use of a single pair of vocal folds.[3,4] This great versatility is attributed, at least partially, to the unique structure of human VF.[5] The VF of the human adult consists of five layers: epithelium; superficial, intermediate and deep layers of the lamina propria (LP); and the vocalis muscle. The most important part of the vibrating structure of the VF is the epithelial layer and the superficial layer of the LP. The superficial layer of the LP consists chiefly of a very phable amorphous substance referred to as Reinke's space (RS).[6] In human newborns, the LP of the VF edge is structurally uniform, consisting mainly of an amorphous substance and resembling the RS of adult vocal folds.[7]The structure consisting of the intermediate layer (composed chiefly of elastic fibres) and the deep layer (composed chiefly of collagenous fibres) is called the vocal ligament.[6]

The vocal ligament exists only in human adults. It is not present in young children or animals. The vocal ligament develops as the child grows and its development is completed at the end of puberty.[7,8] The viscoelastic properties of the human VF mucosa are very important for the vibratory behaviour.[9,10] Again, the newborn VF mucosa lacks not only a vocal ligament but also adequate viscoelasticity for vibration.[11] Elastic and collagenous fibres of the LP of the vocal ligament are synthesised by fibroblasts.[12] The maculae flavae (MF) of the VF control this synthesisation.[13] The MF is a conspicuous mucosal bulge visible through the mucosa as a whitish yellow mass,[13] is elliptical in shape,[14] and located at the anterior and posterior ends of the membranous portion of the bilateral VF.[13] The vibratory portion of the VF is connected to the thyroid cartilage anteriorly via the anterior MF and anterior commissure tendon, and posteriorly to the arytenoid cartilage via the posterior MF. The vocal ligament runs between the anterior and

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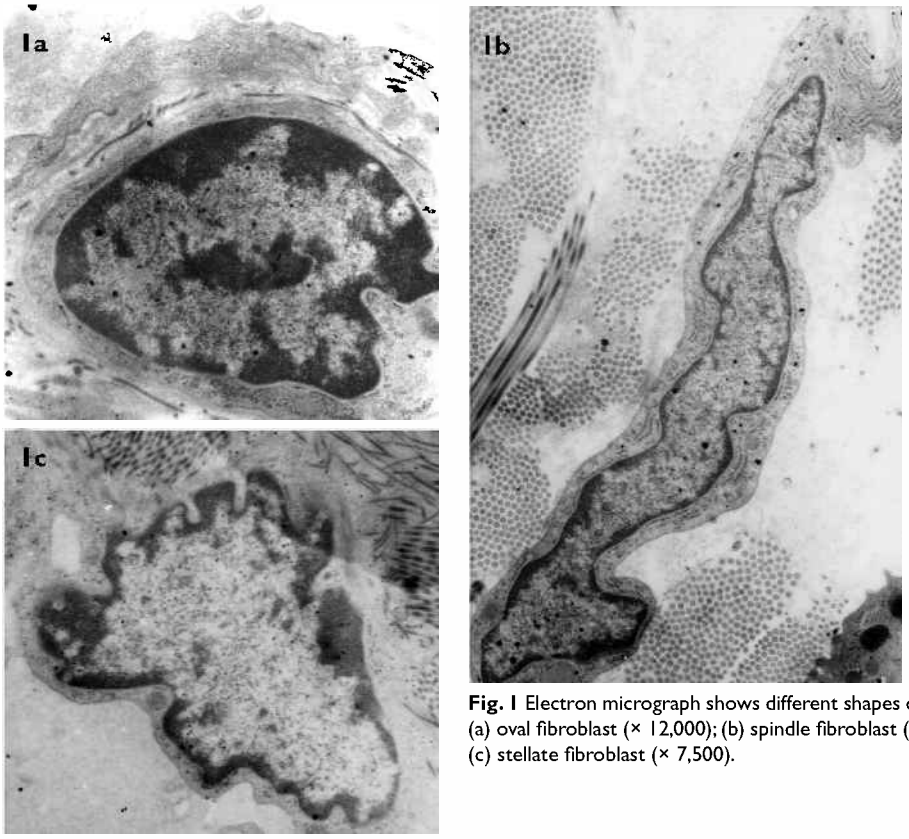


Fig. 1 Electron micrograph shows different shapes of fibroblasts: (a) oval fibroblast (× 12,000); (b) spindle fibroblast (× 7,500); and (c) stellate fibroblast (× 7,500).

posterior MF.[15,16] The MF in newborn VF, although immature,[7,15] appears to be an important structure and essential to the growth and development of the vocal ligament.[17] Changes in the VF are supposed to be related to the structure and function of fibroblasts. The purpose of this study was to investigate the histological structure and age-related changes of fibroblasts in the human VF mucosa in three different age groups: newborns, adults and geriatrics.

METHODS

Normal human VF obtained from autopsy cases were included in this study: four from mature newborns (two males and two females), four from middle-aged adults (two males and two females with an age range of 32–44 years), and four from geriatric cases (two males and two females with an age range of 70–78 years). All participants were from among the attendees at Al Azhar University Hospital, Cairo, Egypt. The approval of the hospital's ethics and research committee was obtained prior to the commencement of the study. Informed consent was obtained from the relative of each case recruited into the study. Fibroblasts in RS and MF were investigated by transmission electron microscopy (TEM). For TEM examination, the specimens were fixed in 2.5% glutaraldehyde at 4°C for two hours, rinsed with cacodylate buffer solution and postfixed in 2% osmium tetroxide with cacodylate buffer solution at 4°C for two hours. The specimens were then dehydrated in graded concentrations of ethanol and embedded in epoxy resin. Semithin sections were prepared with an ultramicrotome. They were stained with 1% toluidine blue for light microscopic examination and stained with uranyl acetate and lead citrate, as well as tannic acid for TEM examinations.

A total of 40 fibroblasts in RS and 40 fibroblasts in MF from each age group (ten fibroblasts from each RS specimen and ten fibroblasts from each MF specimen) were evaluated in terms of cellular shape, nucleus/cytoplasm (N/C) ratio and intracellular organella. Cellular shapes were classified into three types: oval, spindle and stellate. The N/C ratio was divided into three groups: < 0.5 (i.e. the nucleus accounted for less than one-third of the cell); 0.5–2.0 (i.e. the nucleus occupied one- to two-thirds of the cell); and > 2.0 (i.e. the nucleus accounted for more than two-thirds of the cell). Evaluations of intracellular organelles included: the degree of development of rough endoplasmic reticulum (rER) and Golgi apparatus (GA), presence of lipid granules, existence of glycogen particles (GP), and number of free ribosomes and abnormal

Table I. Shapes of fibroblasts present in newborns, adults and geriatrics.

Shape	Newborn		Adult		Geriatric	
	RS (%)	MF (%)	RS (%)	MF (%)	RS (%)	MF (%)
Oval	75	45	20	0	25	10
Spindle	25	7.5	80	27.5	75	22.5
Stellate	0	47.5	0	72.5	0	67.5

Percentage of 40 fibrocytes (ten from each specimen, n = 4) in Reinke's space (RS) and the same in macula flava (MF).

Table II. Nucleus/cytoplasm (N/C) ratios in newborns, adults and geriatrics.

N/C ratio	Newborn		Adult		Geriatric	
	RS (%)	MF (%)	RS (%)	MF (%)	RS (%)	MF (%)
< 0.5	5	10	30	25	15	30
0.5–2.0	17.5	80	65	75	80	60
> 2.0	77.5	10	5	0	5	10

N/C ratio in % of 40 fibrocytes (ten from each specimen, n = 4) in Reinke's space (RS) and the same in macula flava (MF).

mitochondria. The degrees of development of rER and GA were classified into three types: undeveloped (i.e. there was no rER and/or GA appearing in the cell section); moderately developed (i.e. 1–5 rER and/or GA appeared in the cell section); and well-developed (i.e. ≥ 6 rER and/or GA appeared in the cell section).

RESULTS

Electron microscopic observations clarified that the fibroblasts were found to be oval (Fig. 1a), spindle (Fig. 1b) and stellate (Fig. 1c) in shape. Oval-shaped fibroblasts were more abundant (75%) in newborns' RS compared to their presence in the MF, observed in nearly half of the cells studied (45%) while stellate fibroblasts were observed in the other half (47.5%). In adults, the majority of fibroblasts in RS were spindle-shaped (80%), while in MF stellate fibroblasts accounted for the majority of the cells studied (72.5%). In geriatrics, the majority of fibroblasts in RS were spindle-shaped (75%), whereas in MF most fibroblasts were stellate in shape (67.5%). Stellate-shaped fibroblasts were not observed in the RS of the three groups (Table I).

The N/C ratio was < 0.5 (Fig. 2a), 0.5–2.0 (Fig. 2b) and > 2.0 (Fig. 2c). The majority of fibroblasts of RS in newborns appeared with an N/C ratio > 0.2 (77.5%), and it was about 0.5–2.0 in the fibroblasts of MF. In adults and geriatrics, the N/C ratio was 0.5–2.0 in most fibroblasts in both RS and MF (65% in RS and 75% in MF of adults, and 80% in RS and 60% in MF of geriatrics). Meanwhile, fibroblasts with an N/C ratio < 0.5 were more frequent in adults (30% in RS and 25% in MF) and geriatrics (15% in RS and 30% in MF) than in newborns (5% in RS and 10% in MF). N/C ratios > 2.0 were rare in both RS and MF in both adults (5% in RS and 0% in MF) and geriatrics (5% in RS and 10% in MF) and no fibroblast in MF in adults had an N/C ratio > 2.0 (Table II).

Fibrocytes with undeveloped (Fig. 3a), moderately developed (Fig. 3b) and well-developed (Fig. 3c) rER and GA were observed. In newborns, the majority of fibroblasts in RS had undeveloped rER and GA (75%), whereas in MF, fibroblasts with moderately developed rER and GA were most frequently observed. In adults, fibroblasts with well-developed rER and GA were more frequently observed in MF (60%) than in RS (20%). Moreover, the majority of fibroblasts in RS had undeveloped rER and GA (50%). In geriatrics, the majority of fibroblasts in RS had moderately developed rER and GA (80%), whereas in MF, fibroblasts with moderately developed rER and GA were most frequently observed (65%). Therefore, the degree of development of rER and GA in fibroblasts present in the RS of VF was greater in geriatrics than in adults and least in newborns. In MF, fibroblasts with undeveloped rER and GA were more common in geriatrics (30%) than in adults (5%) and/or newborns (5%), whereas those with well-developed rER and GA were less frequent in geriatric MF (5%) than in adult MF (60%) and/or newborn MF (40%). The development of rER and GA in fibroblasts present in the MF of VF was, therefore, greatest in adult MF, followed by geriatric MF and least in newborn MF (Table III).

GPs had not been found in any fibroblasts of newborns and/or adults. In geriatrics, GP had been noted in MF. They were observed in 22 out of 40 fibroblasts that were stellate in shape. The N/C ratio of these fibroblasts was < 0.5 in nine cells and between 0.5–2.0 in 13 cells. The rER and GA were undeveloped in seven cells, moderately developed in 13 cells and well-developed in two cells. This data suggests that there was no relationship between

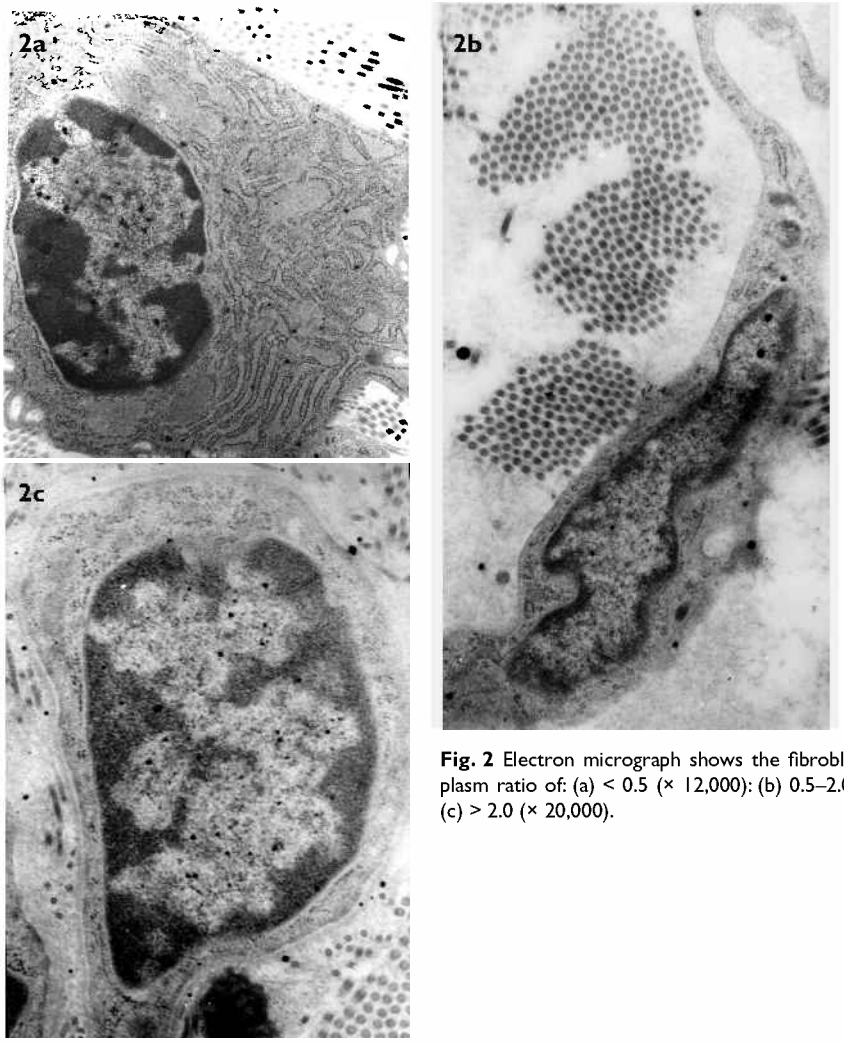


Fig. 2 Electron micrograph shows the fibroblast nucleus/cytoplasm ratio of: (a) < 0.5 (× 12,000): (b) 0.5–2.0 (× 10,000); and (c) > 2.0 (× 20,000).

the presence of GP and the development of rER and GA. Free ribosomes were noted much more abundantly in fibroblasts of newborn MF. Swollen mitochondria were noted only in one fibroblast in geriatric RS and in two fibroblasts in geriatric MF. Lipid droplets were not observed in fibroblasts of newborns. They were observed in 12 fibroblasts in adult RS and in 16 fibroblasts in adult MF. In geriatric VF, lipid droplets were noted in ten fibroblasts in RS and in eight fibroblasts in MF. The existence of lipid droplets did not appear to have any relationship to the shape of fibroblasts, N/C ratio and/or development of rER and GA.

The oval-shaped fibroblasts appeared to have a larger N/C ratio, i.e. with minimal cytoplasm. Meanwhile, spindle- or stellate-shaped fibroblasts appeared to have a small N/C ratio (i.e. they had more abundant cytoplasm) (Table IV). Stellate-shaped fibroblasts tended to have well-developed rER and GA, spindle-shaped fibroblasts appeared to have less developed rER and GA, while oval-shaped fibroblasts were associated with the least developed rER and GA (Table V). In the majority of fibroblasts, the N/C ratio was 0.5–2.0. The N/C ratio was < 2.0 in all fibroblasts with well-developed and moderately developed rER and/or GA. The N/C ratio varied in fibroblasts with undeveloped rER and/or GA. In other words, fibroblasts with more cytoplasm tended to have more rER and GA (Table VI).

DISCUSSION

The most important function of fibroblasts is to produce elastic and collagenous fibres as well as intercellular substances including collagen, glycosaminoglycan, elastin and fibronectin.[8,12,18,19] In the active phase, fibroblasts have well-developed rER and GA, which play an important role in producing intercellular substances and fibres. In the proliferative phase, fibroblasts have less developed rER and GA, and many free ribosomes.[20] Previous cytological investigations of fibroblasts in the superficial layer of the LP and MF indicated some morphological changes with ageing.[11,21,22] The

Table III. Development of rough endoplasmic reticulum and Golgi apparatus.

Development	Newborn		Adult		Geriatric	
	RS (%)	MF (%)	RS (%)	MF (%)	RS (%)	MF (%)
Undeveloped	75	5	50	5	5	30
Moderately developed	25	55	30	35	80	65
Well developed	0	40	20	60	15	5

Percentage of rough endoplasmic reticulum and Golgi apparatus of 40 fibrocytes (ten from each specimen, n = 4) in Reinke's space (RS) and the same in macula flava (MF).

Table IV. Relationship between shape and nucleus/cytoplasm (N/C) ratio.

N/C ratio	Shape		
	Oval (%)	Spindle (%)	Stellate (%)
< 0.5	7	29.5	18.5
0.5–2.0	36	59	81.5
> 2.0	57	11.5	0

Relationship between shape and N/C ratio of 40 fibrocytes (ten from each specimen, n = 4).

Table V. Relationship between shape and development of rough endoplasmic reticulum and Golgi apparatus.

Development	Shape		
	Oval (%)	Spindle (%)	Stellate (%)
Undeveloped	53	50	3
Moderately developed	41	34	32
Well developed	6	16	65

Relationship between shape and development of rough endoplasmic reticulum and Golgi apparatus in 40 fibrocytes (ten from each specimen, n = 4).

Table VI. Relationship between nucleus/cytoplasm (N/C) ratio and development of rough endoplasmic reticulum and Golgi apparatus.

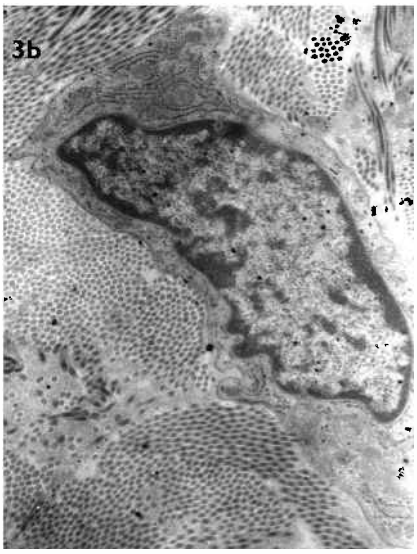
Development	N/C ratio		
	< 0.5 (%)	0.5–2.0 (%)	> 2.0 (%)
Undeveloped	26	42	33
Moderately developed	17	42	41
Well developed	76.5	32.5	0

Relationship between N/C ratio and development of rough endoplasmic reticulum and Golgi apparatus in 40 fibrocytes (ten from each specimen, n = 4).

distribution of fibroblasts differs according to the site in the VF mucosa.[12] The cells appear dense in MF and sparse in RS.[8] Moreover, the density of fibroblasts has been reported to be much greater in newborns and young children than in adults in both RS[8,12] and MF.[17] Again, the density of fibroblasts is lower in geriatric MF than in adult MF, while in RS there is no difference between the two age groups.[8,21] This study showed similar results to the previously reported data.

The results of this study indicated that fibroblasts in MF were much more active than those in RS in both newborns and adults; while in geriatric MF, fibroblasts appeared to be less active than those in RS. Furthermore, fibroblasts appeared to be slightly more active in geriatric RS than in adults and newborns. In addition, the frequency of active fibroblasts was found to be greater in adult MF than in newborn MF. However, the activity of MF fibroblasts in adults was reduced in the geriatric group. This decrease in activity of fibroblasts was supposed to cause the structural changes in the vocal ligaments. Again, inactive fibroblasts (i.e. those with no rER and GA) were more frequently observed in newborns, where no vocal ligament has developed, than in adults. In geriatric RS, fibroblasts were more active than in adults. Therefore, on the basis of these results and previously reported results,[5,7,8,11-13,17,21,23-26] it is postulated that fibroblasts in MF of newborns and young children produce elastic and collagenous fibres to develop the immature vocal ligament. In infants, fibroblasts in MF are activated to become stellate in shape and synthesise extracellular matrices. Meanwhile, fibroblasts in the adult MF produce elastic and collagenous fibres for the metabolism of the mature vocal ligament which should be consistently renewed. Geriatric changes in the fine structure of the LP of the VF are supposed to be one of the essential causes of voice changes with ageing. In geriatrics, the elastic fibres tend to become atrophic and the collagenous fibres tend to increase their density and run in various directions. These changes, which are related to the function of fibroblasts in the vocal ligament, increase the stiffness of the VF mucosa. This in turn influences the viscoelasticity and stiffness of vibrating tissue and contribute to the ageing of the voice.

It has been stated that in an aged VF, a decrease in the thickness of the LP, especially the superficial layer, causes bowing and incomplete glottic closure.[27] Active fibroblasts participate in the production of intercellular substances of the MF and possibly of the RS.[12] Fibroblasts also produce mucopolysaccarides for the ground substance. The fibre components and the ground substance influence the viscoelasticity and stiffness of the VF and therefore voice quality and ageing of the voice.[13] However, newborn fibroblasts in some



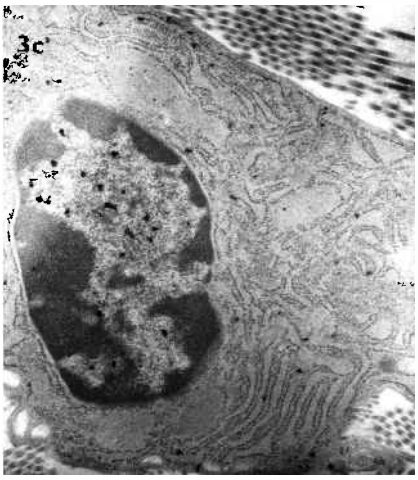


Fig. 3 Electron micrograph shows the development of rER and GA in fibroblasts, as (a) undeveloped rER and GA (× 20,000); (b) moderately developed rER and GA (× 18;000); and (c) well-developed rER and GA (× 12,000).

cases have rER and GA in their cytoplasm, like those in adults, but in many fibroblasts, free ribosomes are well-developed in the cytoplasm while rER and GA are not fully developed.[17] In geriatrics, GA and rER were fewer than in younger adults.[21] Basal bodies were also seen and such fibroblasts are still immature.[17] The GP tends to accumulate in atrophic cells, although it was reported by Sato et al[24] that GPs are present in the LP of newborns and decrease with the increase in fibrous components in the RS. Their existence was not related to the development of rER and GA.[8] The existence of fibroblasts with GP in the geriatric group in this study was a sign of senescence, as previously presumed by Hirano et al.[8] In addition, the presence of lipid droplets in fibroblasts may be related to vitamin A storing cells which produce reticular fibres or type 3 collagen, as suggested by Kajikawa.[20] The high density of free ribosomes in newborn fibroblasts indicates that these fibroblasts are proliferating, as suggested by Hirano et al.[12] Furthermore, in this study, and as previously reported in the study by Hirano et al,[8] swollen mitochondria could only be determined in a few fibroblasts of geriatric VF.

In conclusion, histological changes of fibroblasts in the VF mucosa are one of the important causes of the change in voice quality with ageing. Furthermore, geriatric changes in the vocal ligament can be attributed to the activities and presence of ageing processes in fibroblasts of geriatric VF mucosa.

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