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Original Article

Organization of the fibrous connective tissue of the fallopian tubes in fertile and climacteric periods: a scanning electron microscopic and stereologic study

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Abstract: The extracellular matrix (ECM) of the fallopian tubes is subject to several changes due to hormonal influences and aging. However, there is a lack of studies regarding its arrangement in older women. We aimed to analyze the organization of ECMcomponents, including collagen and elastic fibers, in the fallopian tube's ampulla from young and old women by means of scanning electron microscopical and stereological methods. Twenty-one samples were analyzed: 12 from female cadavers in a fertile age (G1) and 9 from the climacteric period (G2). Masson's trichrome stain was used to observe collagen and smooth muscle, while Weigert's Fuchsin-Resorcin was employed to observe elastic fibers. Statistical analysis was performed by the Wilcoxon-Mann-Whitney test with the aid ofthe R© software. The tissue was also fixed for scanning electron microscopic analysis in a modified Karnovsky solution and the three-dimensional organization of fibrous connective tissue was observed and compared. Statistically significant differences ($P < 0.01$) were found in all stereologic comparisons of the extracellular matrix between the groups, which revealed a higher volumetric density of the fibrous tissue in the climacteric group. Scanning electron microscopy showed degenerative alterations of extracellular matrix. According to our results, aging caused significant changes to the elements of the extracellular matrix and the smooth muscle of the fallopian tubes.

Keywords: Human fallopian tube, ampulla, extracellular matrix, scanning electron microscopy, stereology

Introduction

Ectopic pregnancy is a relevant gynaecologic and obstetric complication that occurs in approximately 2% of all pregnancies, usually in the ampulla of the Fallopian tubes (FT) [1, 2]. Moreover, a great number of diseases can afflict the FT and spread to other organs, such as high-grade serous ovarian carcinoma (HGSOC), which was believed to have an epithelial origin in the ovary itself, although later its origins were the FT cells [3].

It is known that women older than 35 years old have less fecundity compared to their younger cohort and that pregnancies after 45 years old are considered dangerous, as they present a great risk for the woman's and her fetus' health [4]. This is due to the "depletion" of the ovaries that happens during the climacteric period: near the age of 45 years, only a few primordial follicles continue to be stimulated by LH and FSH. As the follicles atrophy, estrogen production by the ovaries reduces substantially. During menopause, the female hormones decline to very low levels, and the menstrual cycle ceases [5].

The fallopian tubes are divided into three portions-isthmus, ampulla, and fimbriae-that differ histologically and physiologically [6], and, although their anatomic and histologic structures are well-known, little is said about the structure of the extracellular matrix (ECM) of the FT [7].

The ECM in general has a role that goes beyond the simple structural support of the cells-it is directly related to corporal homeostasis by regulating processes such as cellular migration, growth, differentiation, and even structural morphogenesis. Thus, the ECM plays a key

role in regulating reproductive function in the FT [8-10]. Several types of collagen, glycosaminoglycans (GAGs), and glycoproteins compose the ECM and allow the maintenance of all their functions, especially keeping the tissues united, preventing them from tearing, and untangling one from another [11].

The main purpose of this study is to observe the ECM arrangement in the FT of fertile-aged and climacteric women through histologic, stereologic, and scanning electron microscopy (SEM) methods.

Material and methods

Ethics procedures

This study complies with the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh, 2000). Our Internal Review Board approved study guidelines. Also, the protocol (No. 89868318.6.0000.5243) received approval from the Ethics Committee on Human Research of the Federal Fluminense University.

Sample

Fragments of 3×3 cm^2 of the FT in the middle shaft (*ampulla*) were obtained from 21 women during autopsies. The cause of death of all women was non-related to the urogenital tract. The first group (G1) was composed of female cadavers in fertile age (n = 12), while the second group (G2) was comprised of female cadavers in the climacteric period (n = 9).

Histologic procedures and stereological method

The fragments were divided by isotropic and random orthogonal triplet probe sections ('orthrip method') [12], which consists of making the material uniformly isotropic by dividing the fragment three times consecutively. The first section is random, the second is orthogonal to the first, and the third orthogonal to the second. This allows random sections to be obtained that are uniformly isotropic [12].

After the section, the material was immediately placed in a fixative solution. The time elapsed between death and fixation of the samples was < 6 h. Part of the tissue specimens was routinely processed for paraffin embedding, and 5-µm thick sections were stained with hematoxylin/eosin [13] and examined by a pathologist not involved in the study to detect and exclude foci of carcinoma, metaplasia, hydrosalpinx, or any disease that could admittedly interfere with the analysis of the morphologic structure.

For the stereological analysis, 5 µm sections were stained with Weigert's Fuscin-Resorcin to observe the elastic system fibers (stained dark violet) and Masson's trichrome stain to detect collagen (blue-stained) with intense counterstain to the smooth muscle (red-stained) [13].

The analyzed fields were then digitized to a final magnification of ×400 using a video camera coupled to a light microscope (BH-2 Olympus®). The selected histologic areas were then quantified by applying a test-grid system (M42) on the digitized fields on the screen of a color monitor (Sony®).

From stereological principles in an isotropic tissue, the area distribution of a given structure-as determined on a two-dimensional section of the structure-is proportional to the volume distribution of the structure. The volume density (Vv) of the histological components was calculated as Vv = Pp/Pt, where Vv was the volume density, p was the tissue component under consideration, Pp was the number of test points associated with p, and Pt was the number of points in the test system. The stereology methods as a whole was already described in the literature [14].

Statistical analysis

The statistical treatment of the data was performed using the R® Software [15]. Wilcoxon-Mann-Whitney statistical test was used for comparing the averages obtained in both groups (G1 and G2). It was stipulated a level of reliability of 99%, and a *p*-value < 0.01 was considered significant.

Scanning electron microscopy (SEM)

Part of the sample was fixed with a modified Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium phosphate buffer, pH 7) for 48 h at 4°C.

For each woman, about three fixed FT fragments (1×1 cm) were washed in PBS for 2 h at 4°C and then incubated in 40 mL of 2M NaOH at room temperature for 8 days. The samples

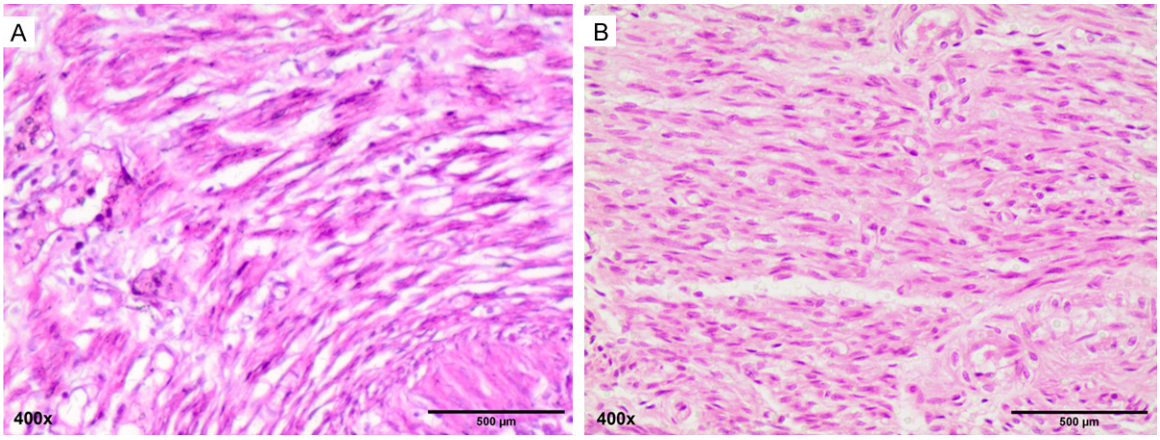

Figure 1. The ampulla from the G1 (A) and G2 (B). The organization and visual aspect of ampulla's cells and tissue organization is shown. Muscle fibrosis and a reduced vascular number were some of the characteristics observed by routine hematoxylin-eosin stain. H&E, 100×.

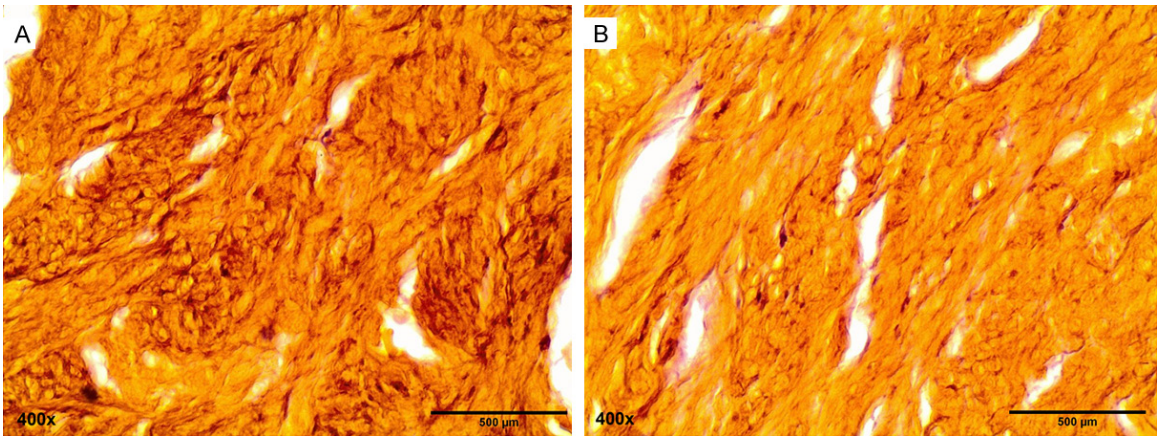

Figure 2. The ampulla from the G1 (A) and G2 (B). The elastic fibers (stained dark) are shown in a greater amount in younger women (G1) in comparison to older women (G2). Weigert's resorcin fuchsin, 400×.

were then rinsed in three changes of 24 h each in 40 mL of distilled water at room temperature until they were pale and transparent. The processing of materials for high-vacuum SEM followed standard procedures [16, 17]. Accordingly, decellularization preparations were first treated with 1% tannic acid in PBS for 2 h and washed in PBS for 1 h. Post-fixation was done in 1% osmium tetroxide in PBS for 3 h, after which the samples were washed in PBS for 1 h and dehydrated in an ascending graded series of ethanol. Samples were then "critical point" dried with liquid carbon dioxide, mounted on aluminum stubs, and coated with gold using a sputter coater. The samples were examined in a SEM with an acceleration voltage of 15 kV. This SEM technique was also used to monitor the efficiency and extent of the cellular solubilization process. All descriptive data are representative of the 21 FT samples used in this study.

Results

Qualitative analysis

The reported findings are exclusively from the *ampulla* region of the assessed human FT. The hematoxylin-eosin stains showed that all samples were free of disease. The normal FT description has already been vastly described in the literature. However, it was seen to have a slight degree of reduction of vascular elements and a more pale mucosa. The histologic aspects of the FT (hematoxylin-eosin) stain can be seen in **Figure 1**.

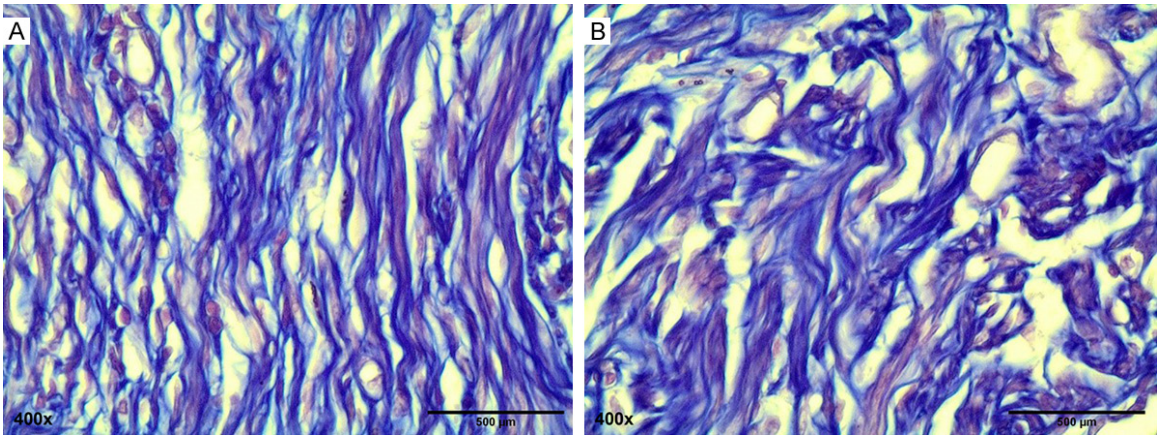

Figure 3. Ampulla from the G1 (A) and G2 (B). The smooth muscle expression is decreased, while the collagen expression is increased in the G2 samples in comparison to the G1 samples. Masson's trichrome, 400×.

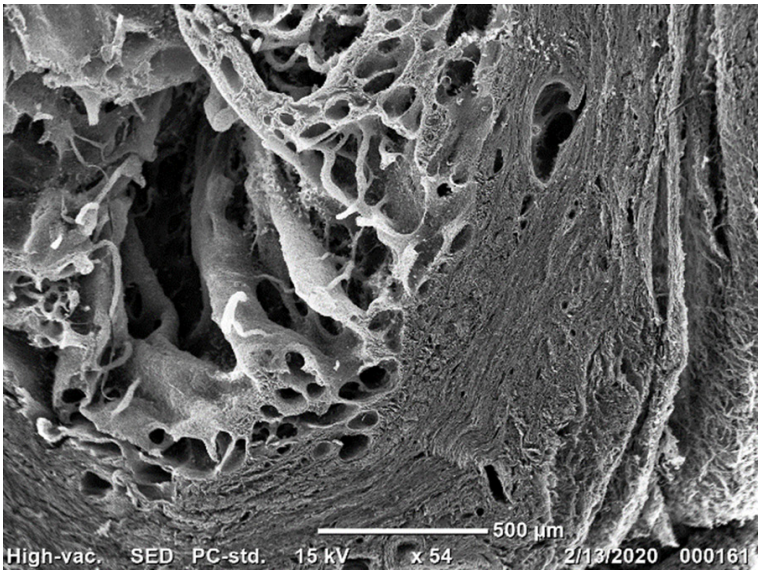

Figure 4. FT specimen from a woman in fertile age (G1). Mucosa and submucosa surrounding stromal wall in a decellularized preparation of the ampulla as seen under high-vacuum SEM.

In the G1, according to Weigert's and Masson's stains, it could be observed beneath the mucosa that the submucosa contained a regular amount of connective tissue (collagen e elastic fibers) interspersed with longitudinal bundles of smooth muscle (**Figures 2A** and **3A**). These two parallel layers constituted the major component of the FT wall.

In the climacteric group (G2), a greater amount of fibrotic tissue was seen and it also showed a more condensed pattern while the collagen and smooth muscles were seen in less quantities and with a more disoriented distribution. The mucosa showed a more atrophic appearance in comparison to the G1 (**Figures 2B** and **3B**).

Scanning electron microscopy analysis

To investigate in greater detail the three-dimensional organization of this framework, and especially of its constituent connective tissue, FT samples from the two groups were treated with a 2M NaOH solution, which solubilizes cells, and were then examined under high-vacuum SEM. These acellular preparations showed that a relatively narrow fibrous septum formed a dense and supportive scaffold for irregular mucosa (**Figures 4** and **5**) in the G1. These fibrous components of the ECM were mostly distributed in concentric layers around the luminal surface of the FT ampulla and were in close apposition to the basal epithelial cells (**Figures 6** and **7**). Also, several mucosal projections were seen in the lumen of the FT arranged randomly.

Quantitative analysis

525 images were analyzed in the study to obtain the quantitative data of collagen, smooth muscle, and elastic fibers. An increase of collagen in the G2 and a decrease of smooth muscle

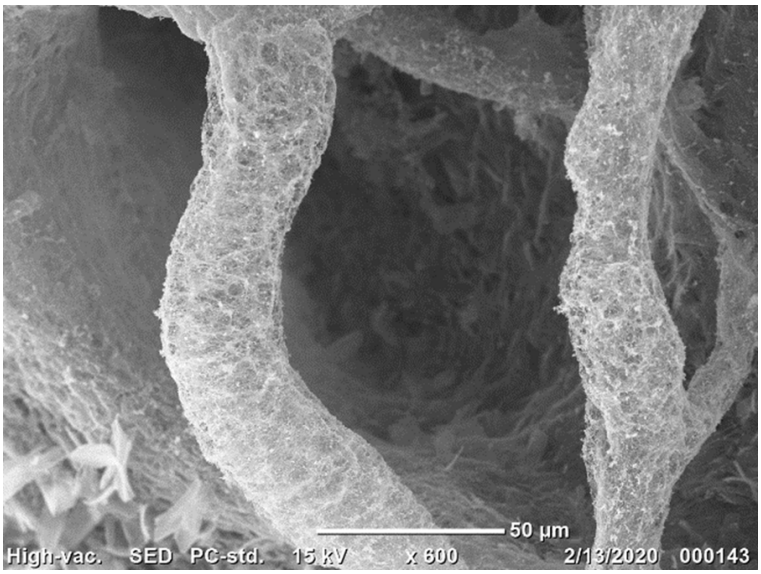

Figure 5. FT specimen from a woman in fertile age (G1). Higher magnification of **Figure 3** showing the lumen of the UT. The organization results from the presence of a delicate fibrous network and empty cellular spaces.

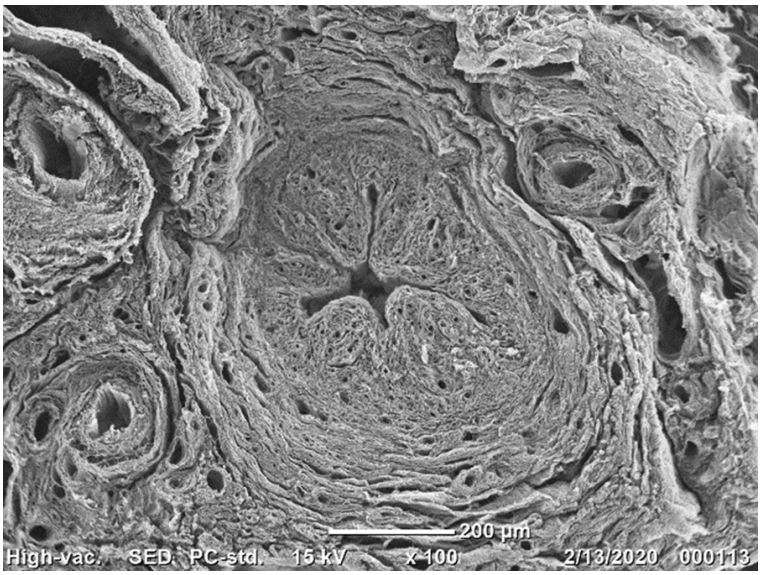

Figure 6. FT specimen from a woman in the climacteric period (G2) showing the wall FT atrophied and lumen occluded in a decellularized preparation of the ampulla as seen under high-vacuum SEM.

fibers and elastic fibers were noted in comparison to the samples of the G1, as shown in **Figures 2** and **3**.

An increase in the Vv% of collagen (27.38%) was observed in the G2 in comparison to the G1. Furthermore, the Vv% of elastic fibers (10.49%) and smooth muscle (12.86%) were decreased in the G2 in comparison to the G1 samples. All the results of the stereologic analysis presented statistically significant differences. These results are summarized in **Table 1**.

Discussion

Stereological evaluations are design-based, in contrast to assumption-based techniques, such as histomorphometry. Thus, it is a more unbiased and more accurate way of quantifying the characteristics of a certain tissue by using geometrical and statistical principles. Due to these benefits, stereology is an excellent method to evaluate changes in various types of tissues [18]. Stereological methods have been used in previous quantification studies specifically to determine the number or proportion of fibrous components of the ECM from the urogenital system [19-22].

We opted to use a manual point-counting method, which has proven to be very efficient. It avoids the bias that frequently occurs with computerized image analysis, which may overestimate or underestimate the proportion of the analyzed structures. Furthermore, manual point-counting has lower intra and inter-observer variability [23].

The ECM is especially important in the development of tissues and organs, as they rebuild and repair damage while also maintaining tissue normality. Therefore, the location and arrangement of the ECM can be associated with the functional features of each kind of tissue, pointing out their particular biomechanical proprieties. Changes in the elements of the ECM of an organ also change these proprieties [24, 25].

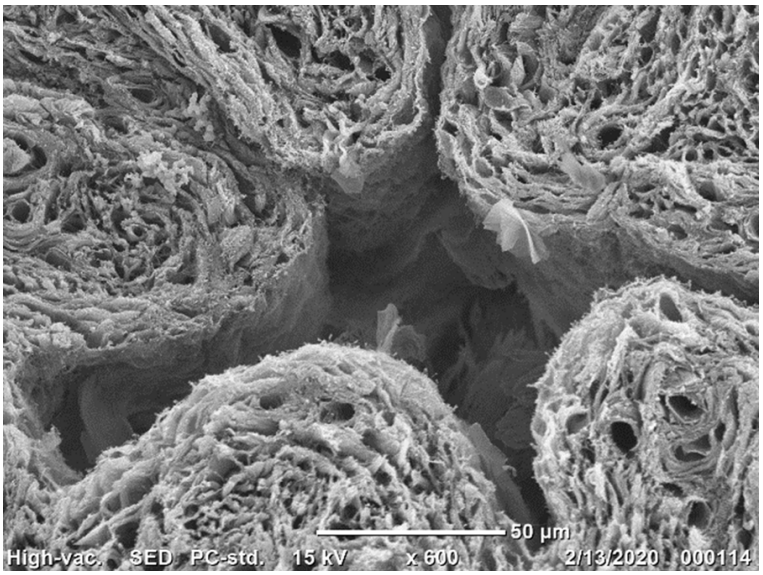

Figure 7. FT specimen from a woman in the climacteric period (G2). Higher magnification of **Figure 5**. The organization results from the absence of regular mucosa.

Table 1. Volumetric density (Vv%) of the fibrous elements and smooth muscle of the extracellular matrix in both groups (Data presented as mean percentage ± standard deviation)

Element:	Group 1	Group 2	*p* value
Collagen	25.43±2.83	52.81±12.97	< 0.01*
ESF	43.01±5.05	32.52±10.92	< 0.01*
SM	40.23±7.36	27.37±7.52	< 0.01*

ESF = elastic system fibers; SMC = smooth muscle cells.
*Statistically significant, Wilcoxon-Mann-Whitney test.

The collagen fiber architecture of ECM plays a critical role in determining its biomechanical behavior. The alignment and organization of collagen fibers are dependent on the function of the source tissue from which the ECM is derived [24, 25].

Our study showed a significant increase of collagen and smooth muscle in the samples belonging to the climacteric period group (G2). This increase of collagen was also identified by Mao et al. [26] in the anterior wall of Sprague-Dawley rat vaginas. Therefore, it is likely that this age-related increase of collagen is not restricted to a single structure from the female reproductive system.

Our findings were in accordance with the results presented by Schultka et al. [27], which observed through electron microscopy that the connective tissue framework of the uterine tube changed its architecture due to the aging process, as the glycoprotein expression changed. The authors also observed that the basal membrane of older women was found to be thicker than in the young ones, thus highlighting greater deposits of collagen in older women.

The intrinsic musculature of the FT has a noted function in motility and, consequently, in fertility [28]. A great difference was found between the G1 and G2 in the quantitative analysis of smooth muscle, such that, while the collagen in the tissues increased, there was a decrease in the expression of muscular tissue and elastic fibers. The morphologic alterations due to menopause can present interesting information about the functioning of the tubes. We can hypothesize that the stiffening found iznd, as a consequence, the decrease of the tubal motility can influence the motility of the mucosa fimbriae, thus, becoming another factor for infertility [7].

A study performed by Godoy-Guzmán et. (2018) [7] showed that during the menstrual cycles, the arrangement and deposition of collagen and collagen-related proteoglycans did not alter during menstrual cycles, although the authors used only samples of fertile patients between 25 and 45 years old. The authors also showed that the arrangement was different according to the studied region, whereas the mucosa displayed a specific distribution of versican and fibromodulin, and the lamina propria displayed decorin and fibromodulin, which suggests a different biomechanical function needed for the oviductal transport and subsequent embryogenesis.

The phenotypic aspect of these morphologic alterations produces changes in the function and activity of the FT. When performing hysteroscopy in women in pre and postmenopausal periods, Siftar et al. [29] evidenced that in the postmenopausal group there was a liquid leak

to the perineal cavity when compared to the premenopausal group, which could be partly due to structural changes found in the present work: a reduction of smooth muscle and elastic fibers and an increase of collagen expression, all caused by aging.

Conclusion

Our results show that in the ampulla of the human FT-employing stereology and SEM methods-there was a significant increase of collagen and a significant decrease of smooth muscle and elastic fibers of women over the age of 60 in comparison to younger women.

Disclosure of conflict of interest

None.

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