Original article

Histomorphometry of the vesicourethral junction of rats at different ages

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Abstract

To evaluate the elastic system fibers in the vesicourethral junction of wistar rats at different ages, histological and histomorphometric studies were conducted. The histology study of the elastic system fibers for selective staining methods showed the presence of three types of elastic fibers. In all age groups studied, histology study satisfactorily showed the structurals differences between the mature elastic fibers, elaunin and oxytalan fibers, which were located among the intercellular spaces of the muscular layer as well as between collagen fibers. Histomorphometric studies have revealed that with the advanced age, there is a decrease in the linear density of the mature elastic and oxytalan fibers, while the linear density of elaunin fibers has increased. Thus, it could be inferred that in the vesicourethral junction of old animals, there is a fall in the elasticity, elastic recoil and anchorage properties due a loss of elastin and microfibrils, but they are compensated by the elaunin fibers, since they show intermediate characteristics between mature elastic fibers and oxytalan fibers. It could be concluded that the aging process of the elastic fibers in the vesicourethral junction does not contribute alone and directly in the urinary incontinence state, but it compensates and gives muscular support, mainly due to the increase of the elaunin fibers, what makes the elastic system in the vesicourethral junction responsible for the maintenance of the urinary continence.

Keywords: vesicourethral junction, elastic fiber, aging.

1 Introduction

The vesicourethral junction controls the urine storage and emptying, and at the time this occurs, the superficial muscular layer of the trigone muscles is already in contraction to prevent the vesicoureteral reflux, acting with the colon structures to collaborate with its funneling and opening, facilitated by the relaxation of the pelvic and urogenital diaphragms (TANAGHO, MEYERS, SMITH, 1968; TANAGHO and SMITH, 1968; MENESES, 2000). The urinary continence is maintained through a complex relationship that involves structures of the lower urinary tract, which is divided into bladder and urethra associated with the bladder neck.

The elastic system fibers are present in various structures of our body (JOHNSON, CHETTY and MOORE, 1982) and their arrangement contributes for the tissue architecture, as it promotes an action on the tissue when it is exposed to mechanical stress, so that it could return to its original position. Along with collagen fibers, the elastic fibers are important structures for the maintenance of normal tissue resistance (BARROS, RODRIGUES and RODRIGUES, 2002), thus, the fibroelastic tissue, present in the vesicourethral junction (VUJ) is considered the main factor in generating closure pressure of the urethra and the VUJ at rest (AWAD and DOWNIE, 1976). The elastic fibers arranged circularly in the VUJ may be partly responsible for elasticity and plasticity of the region, allowing its expansion and fast backward, with the urine flow. Therefore, the elastic fibers present in this region work together with the smooth musculature and nervous control, forming an important group for the maintenance of the urinary continence (WOODBURNE, 1961).

The elastic system was studied by light microscopy (GAWLIK, 1965; COTTA-PEREIRA, RODRIGO and DAVID-FERREIRA, 1977; PEREIRA, LIMA, CONEGERO et al., 2010) and transmission electron microscopy associated with the tannic acid technique (KAJIKAWA, YAMAGUCHI, KATSUDA et al., 1975; COTTA-PEREIRA, RODRIGO and DAVID-FERREIRA, 1976; SACCHETTI, 1996), which enables evidencing and classifying three types of elastic fibers that make up this system.

However, there are some issues not clarified regarding the elastic system and its participation in the urinary continence/incontinence states; thus, this paper aims to study the elastic system of the VUJ, with qualification and classification through light microscopy and quantification by means of histomorphometric studies in relation to advancing age.

2 Material and methods

This study used 15 male Wistar rats (*Rattus norvegicus*) obtained from the Central Animal Facility of the Institute of Biomedical Sciences, University of São Paulo, which were kept under controlled temperature (21 °C) and lighting (12 hours of light/12 hours of darkness), with balanced diet and water offered *ad libitum*, without nutritional constraints, which criteria followed the Ethics Research Committee and the laws in force (Law 1.153/95). The animals were divided into three groups distinguished by different age groups, with five animals per group: Neonate Group (21 days), Adult Group (200 days), and Old Group (450 days).

The study material was obtained by removing the vesicourethral junction containing the bladder neck, urethral orifices and proximal urethra.

Light microscopy: To obtain the study material for light microscopy, five animals from each group were used and anesthetized by intraperitoneal injection with the use of anesthetic cocktail (40% distilled water + 25% ketamine + 25% xylazine + 10% acepromazine), whose heart rate was maintained until perfusion. The structures were dissected through laparotomy to visualization of the urinary bladder, ureters and proximal urethra, which were removed with the aid of a stereoscopic microscope (magnifying glass), thus obtaining the VUJ. The blocks were processed according to histological tests with sections of 5 μm stained through the following techniques: iron hematoxylin (Verhoeff, demonstration of elastic fibers), Resorcinol-fuchsin (Weigert, demonstration of elastic and elauninic fibers), Resorcinol-fuchsin after oxidation with 1% oxone aqueous solution (Weigert-Oxone, demonstration of elastic, elauninic and oxytalan fibers).

Histomorphometry: The histomorphometric study of the elastic system fibers was performed by calculating the linear density (LD), which is measured in square micrometers. The estimated length of the elastic system fibers was performed using the following formula: $L = 2Q \times EV$, where L is the length of fibers per volume unit, Q = number of intersections of elastic fibers in a flat section, and EV is the volume unit. The sections were examined by light microscope with 100 × objective in immersion, and compensation ocular Kf 10 × 18, with integration graticule (×400), displaying 20 parallel lines. The distance (l) between the points in this system is 5 μm and the distance (d) between lines is 4.94 μm. To estimate the error of 5%, five fields (n = 15) were analyzed using the 400-point testing system.

Statistics: The statistical analysis of the LD values were obtained by analysis of variance (ANOVA) followed by multiple comparisons using the Tukey method. The assumption of homogeneity of variances was checked and the significance level adopted was $p < 0.05$. The linear density values were also expressed by a quadratic regression model using the following equation $LD = \alpha + \beta \times age + c \times age^2$, thereby obtaining a correlation coefficient (r), which must be between 1 and -1 relating LD as a function of age.

3 Results

The analysis of our results for the histological study showed the presence of three types of fibers in all age groups, which are distributed throughout the vesicourethral junction along the muscle layer that is located just below the epithelium lamina propria. The elastic fibers appear in both the surface layer and in the deep layers of the muscle and also in regions surrounding the ureteral ostium and urethral ostium. The direction of elastic fibers in the VUJ is longitudinal and oblique and they are not arranged in blades or bundles, appearing scattered in the muscular layer of the VUJ. Among the stains used, all satisfactory and specifically evidenced the fibers of the elastic system, i.e., as for the specificity, Verhoeff stain (iron hematoxylin) evidenced the mature elastic fibers (Figure 1a), Weigert (resorcin-fuchsin), the mature and elauninic elastic fibers (Figure 1b) and Weigert-Oxone (resorcin-fuchsin with previous oxidation) evidenced the mature elastic fibers, elauninic, and oxytalan fibers (Figure 1c).

This study used mathematical relationships between linear density and ages of animals in two respects, firstly in relation to the staining methods employed, and subsequently for each type of fiber present in the studied region, resulting in comparisons between groups. Figure 2 show the linear density of the elastic system fibers in relation to age (neonate, adult and old animals) in the three staining methods used (Verhoeff, Weigert and Weigert-oxone). According to methods of Verhoeff, which evidences the mature elastic fibers, Weigert, which evidences mature elastic fibers and elauninic fibers and Weigert-Oxone, which evidences elastic fibers mature, elauninic and oxytalan fibers, it could be inferred that the LD value of elastic fibers stained by these methods, respectively, increases from the neonate group (21 days) to the adult group (200 days) and on the other hand, decreases with aging, i.e., from the adult group (200 days) to the old group (450 days) ($p < 0.05$), and there was no significant relationship between the LD values between neonate (21 days) and old groups (450 days).

Figure 3 shows the analysis the LD values of each type of fiber that makes up the elastic system (mature elastic fibers, elauninic and oxytalan fibers) according to age through the following relations: LD Verhoeff = LD mature elastic fibers, LD Weigert - LD Verhoeff = LD elauninic fibers; LD Weigert-Oxone - LD elauninic fibers - LD Verhoeff = LD oxytalan fibers, it seems that the mature elastic fibers increase from the neonate group (21 days) to those of the adult group (200 days) ($p < 0.05$) and decreases the LD value from the adult group (200 days) to the old group (450 days), with no significance between neonate (21 days) and old groups (450 days). The LD value of elaunin fibers showed no significant differences between neonate, adult and old groups. In turn, the LD values of oxytalan fibers decreased with increasing age from adult animals (200 days) to old animals (450 days), with no significant values between newborn (21 days), adult (200 days) and old groups (450 days).

The quadratic regression analyses showed that the LD values of elastic fiber stained through Verhoeff, Weigert and Weigert-Oxone increase from the neonate (21 days) to the adult group (200 days), and from this group to the old group (450 days), and this decrease in the LD values is associated with the aging of the animal (Figure 4). When the elastic system fibers are observed separately, it could be observed that the LD values of mature elastic fibers and oxytalan fibers, the latter stained by the Verhoeff method and the former by the Weigert method, increase from the neonate group (21 days) to the adult group (200 days) and decrease from the adult group to the old group (450 days); however, the LD value of elaunin fibers (stained with Weigert) increases with increasing age from 21 days to 450 days, i.e., in old animals, these fibers have increased linear density values (Figure 5).

4 Discussion

The elastic fiber system has been demonstrated in various tissues such as aorta (GAWLIK, 1965; GOLDFISCHER, SCHWARTZ and BLUMENFELD, 1983; PRATT and CURCI, 2010), skin (COTTA-PEREIRA, RODRIGO and BITTENCOURT-SAMPAIO, 1975), periodontal tissues (FULLMER and LILIE, 1958; FULLMER, 1960; SCULEAN, KARRING and THEILADE, 1997; SAWADA, SUGAWARA and ASAI, 2006; NAKATOMI, TSURUGA, NAKASHIMA et al., 2011), vas deferens (PANIAGUA, REGADERA and NISTAL, 1983),

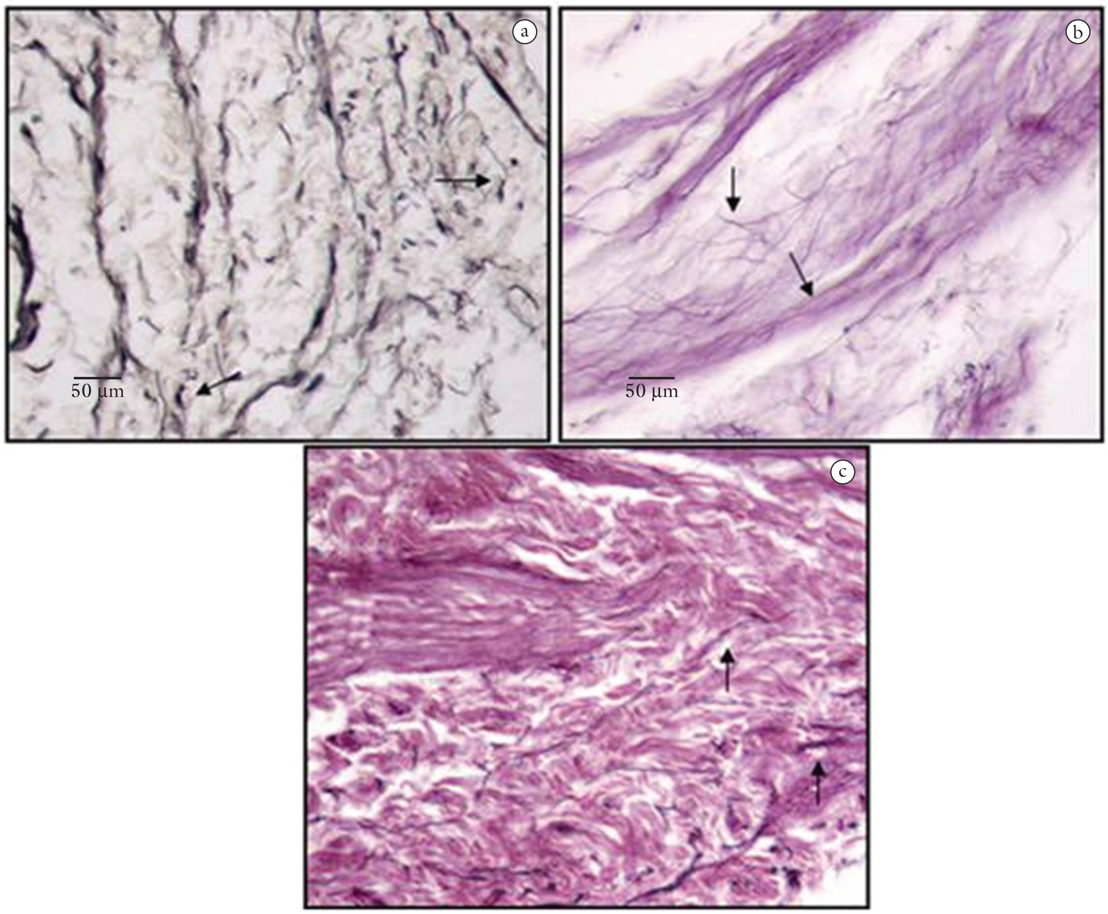

Figure 1. Histological section of 5 mm showing the elastic fibers of the vesicourethral junction of Wistar rats. a) Old group, Verhoeff method, 40×. b) Old group, Weigert method, 20×. c) Old Group, Weigert-oxone method, 40×. Bar: 50 μm.

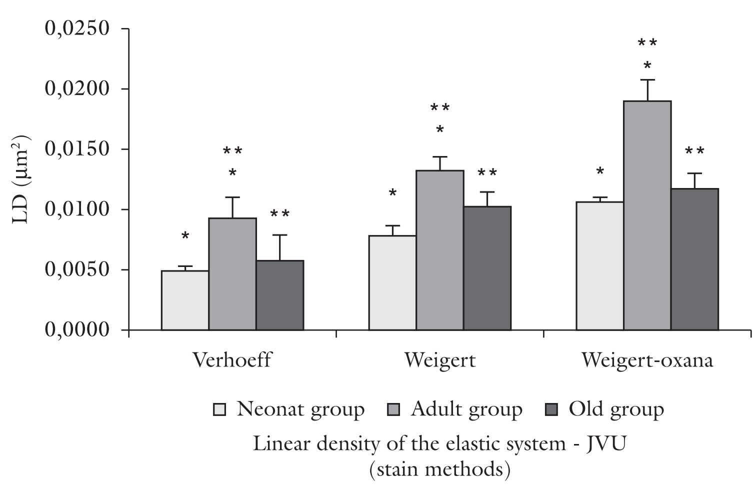

Figure 2. Linear density analysis of the elastic system, in different stains, in relation age. Increase of the LD of elastic fibers of neonat group in relationship between adult group* in staining Verhoeff, Weigert e Weigert-Oxona ($p < 0,05$); Decrease of the LD of elastic fibers in relationship of adult group to with old group** in staining Verhoeff, Weigert e Weigert-Oxona ($p < 0,05$).

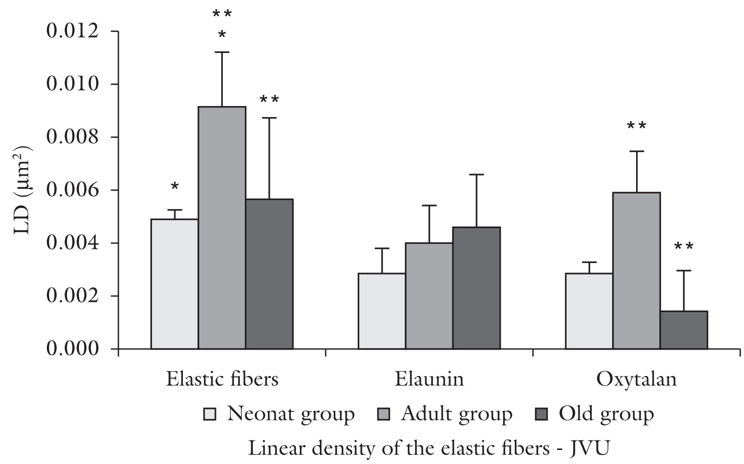

Figure 3. Linear Density analysis in the different types of elastic fibers, in relation age. Increase of the linear density of elastic fibers in adult group in relationship to neonat group* ($p < 0,05$). Decrease of the linear density of elastic fibers and oxytalan in adult group in relationship to old group** ($p < 0,05$).

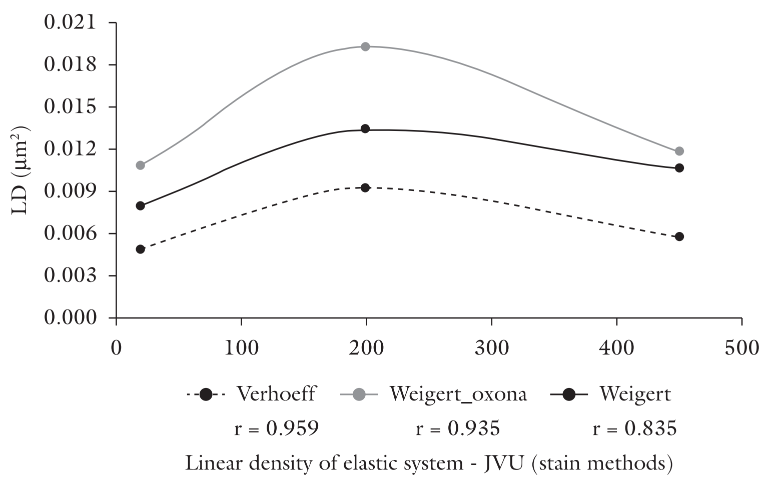

Figure 4. Regression for quadratic curve of values of linear density of the fibers elastic system, staining by Verhoeff, Weigert-Oxona na Weigert methods, in relation age. Correlation coefficient (r) are mentioned for each different stain.

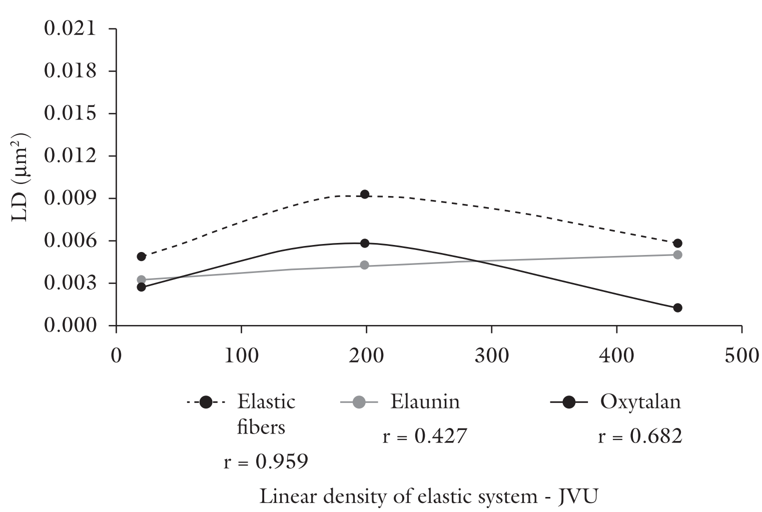

Figure 5. Regression for quadratic curve of values of linear density, in the different types of elastic fibers, in relation age. Correlation coefficient (r) is mentioned for each different type of elastic fibers.

splenic capsule (SACCHETTI, 1996), smooth muscles (CARVALHO and LINE, 1997), vesicourethral junction (AWAD and DOWNIE, 1976; DASS, McMURRAY and BRADING, 1999), urethra (BUMP, FANTL and HURT, 1988), prostate (KARLINSKY, SNIDER and FRANZBLAU, 1976), transverse inguinal fascia (RODRIGUES JUNIOR, TOLOSA and CARVALHO, 1990), lung (NIEWOEHNER and KLEINERMAN, 1977), bladder neck (ROTHER, LÖFFLER, DORSCHENER et al., 1996) and meninges (PEREIRA, LIMA, CONEGERO et al., 2010). This study also confirmed the distribution of elastic system fibers in the vesicourethral junction, corroborating finding of Awad and Downie (1976) and Dass, McMurray and Brading (1999) concerning the same structure.

Previous work such as those conducted by Beertesen, Everts and Van de Hoof (1974), Cotta-Pereira, Rodrigo, David-Ferreira (1977), Everts, Niehof, Jansen et al. (1998), Fullmer and Lilie (1958), Gawlik (1965), Sakay, Keene and Engvall (1986) and Pereira, Lima, Conegero et al. (2010) reported that the fibers that make up the elastic system are evidenced in light microscopy through the following staining methods: iron hematoxylin (Verhoeff), resorcin-fuchsin (Weigert) and resorcin-fuchsin with previous oxidation (Weigert-oxone), staining mature elastic fibers, mature elastic fibers and elauninic fibers, mature elastic fibers, elauninic and oxytalan fibers, respectively. In our study, it was possible to identify and classify the elastic system by light microscopy with the use of these specific staining methods. It was also possible to demonstrate the mature elastic fibers, elauninic and oxytalan fibers in the VUJ, and to observe that these fibers are located throughout the muscle layer of the VUJ, which are not arranged into bundles, chains or blades like those found in skin (KIELTY, SHERRATT and SHUTTLEWORTH, 2002) and vas deferens (PANIAGUA, REGADERA and NISTAL, 1983).

Several studies have reported some changes of the elastic system in relation to aging tissues, and with respect to the elastic system in the VUJ of Wistar rats and age group of these animals, this study allowed observing that the linear density of elastic fibers is different between neonate, adult and old animals, agreeing with the findings of Carvalho and Line (1997), who reported that during the aging process, changes occur in the elastic system fibers.

As for the linear density of each type of fiber of the elastic system, it could be observed that the linear density of mature elastic fibers increased from the neonate to the adult group, but decreased from the adult to old group, which is in line with findings of Sephel and Davidson (1986) and Uitto, Fazio and Olsen (1989), who reported that with advancing age, there is a decline in the elastin biosynthesis rates, stating that in the VUJ, the reverse process occurs when compared to the vas deferens. Paniagua, Regadera and Nistal (1983) found that there is an increase of elastin in relation to advancing age, except for oxytalan fibers, that this structure is less numerous, as in the VUJ, where the linear density of fibers also decreases in older animals. The same was found by Rodrigues Junior, Tolosa and Carvalho (1990), who studied the transverse fascia of the inguinal region, and found that with increasing age, there is a decrease of oxytalans and an increase of mature elastic fibers, agreeing with Barros, Rodrigues and Rodrigues (2002) that with aging, there is an induction in the disappearance of oxytalan fibers. The elaunin fiber in our study was the only type of fiber of the VUJ that showed increasing linear density both from the neonate to the adult animal and from the adult to the old animal, in other words, with aging. Therefore, if there is a difference in linear density of elastic fibers, then there will also be a change in the VUJ functions with aging, and these functions are related only with the elastic system, thus, if the linear density of mature elastic fibers decreases with aging, it could be inferred that this event leads to a proportional decrease in the amount of elastin in the VUJ.

According to Cotta-Pereira, Rodrigo and David-Ferreira (1977), the elastic properties and the elastic recoil property is determined by the presence of elastin; therefore, if there is a reduction of mature elastic fibers, the elastic recoil ability of the region studied decreases with the aging process. Regarding the oxytalan fibers that are present in tissues submitted to stress (FULLMER and LILLIE, 1958) and with the anchorage and maintenance functions of elasticity, these functions in VUJ will be decreased in old animals, whereas in our study, the linear density of oxytalan fibers is decreased in these animals. Regarding the elaunin fibers, our results showed an increase in linear density of these fibers, which are formed by microtubules and elastin (COTTA-PEREIRA, RODRIGO, DAVID-FERREIRA, 1977). Since it is also related to the anchorage, during aging, as these fibers have characteristics intermediate between mature elastic fibers and oxytalan fibers, they will keep the tensile strength and plasticity of the VUJ in the old animal, as well as some elasticity and elastic recoil property, whose functions will probably be reduced, but not absent.

This study came to the conclusion that the elastic system changes that occur in older animals are associated with the natural aging process, and certainly the decrease in muscle tonicity leads the physiological sphincter to become more permeable, promoting gradual processes of urinary incontinence, and that alone, the elastic fibers are not responsible for this pathological condition, because studies have shown that urinary incontinence occurs due to urodynamic problems such as deficiency or absence of detrusor activity, urethral pressure on active or incompetent, leading to undesirable intra-vesical pressure changes (FENELEY, THOMAS, BLANNIN, 1982), systemic and psychological diseases and use of certain drugs (RESNICK and YALLA, 1985).

We believe that in the VUJ, the distribution of elastic fibers is homogeneous and there is a myoelastic system composed of the elastic system along with the muscle system and collagen fibers. Thus, if we transfer the findings in animals to humans, it could be hypothetically assumed that the elastic system in the VUJ serves as a compensatory support of the trigone muscle layer by presenting more functional changes in the aging process and also for being a propitious target for diseases and surgical interventions. With increasing elaunin fibers, there is a remodeling of the elastic system during the natural aging of the vesicourethral junction, and the entire lower urinary tract. We attribute to the elastic system in the VUJ, and indirect responsibility for the maintenance of the urinary continence regardless of the age of the animal.

5 Conclusion

From the methodology used and results obtained, it could be inferred that:

- In the three age groups studied, all types of fibers of the elastic system are present;
- The mature elastic fibers and oxitalan fibers showed their length decreased with advancing age, whereas elaunin fibers had theirs length increased with aging;
- The elastic system of the VUJ is not a component that determines the onset of urinary incontinence in the physiological aging process;
- The elastic system is indirectly responsible for the urinary continence in all age groups and in old animals, it acts as a support for the smooth muscle that probably has the most relevant changes with advancing age than the elastic system itself.

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