Quantitative evaluation of collagen and muscle fibers in the lower urinary tract of castrated and under-hormone replacement female rats

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Summary

Objective: To evaluate the number of collagen and muscle fibers in the muscle layer of the urethra and in the bladder wall of castrated and under-hormone replacement female rats.

Method: We studied 37 castrated female rats assigned to the following groups: Group C (n=9): received no medication; Group P (n=8) was given 0.1 ml of placebo, subcutaneous (SC) route; Group E (n=10): 17ß-estradiol, 10 µg/kg/day, SC route; Group PR (n=9): medroxyprogesterone acetate, 0.2 mg/kg/day, SC route; Group E+PR (n=9): association of 17ß-estradiol and medroxyprogesterone acetate. Sections were taken from the bladder wall and from the middle third of the urethra, and the specimens were stained with picrosirius for collagen and muscle fiber identification.

Results: Groups C and P showed a similar amount of collagen in the bladder and in the urethra, however greater than the other groups. Group E showed the smallest number of collagen fibers in the urethra. Groups E and E+PR presented a larger number of muscle fibers in the bladder. Group PR presented a larger number of muscle fibers than groups C and P, however smaller than groups E and E+PR. In the muscle layer of the urethra, the number of collagen fibers was smaller in Group E than in all the other groups, which were similar among one another. In regard to the urethral muscles, Group E was found to present the largest number of muscle fibers as compared to the other groups analyzed, while Group PR showed a significant decrease in the muscle layer, even in relation to the groups that were given no hormone medication.

Conclusion: Estrogens significantly decrease the amount of collagen fibers, increase the amount of muscle fibers and determine a significantly decreased collagen/muscle ratio in both the detrusor muscle and in the urethral muscle layer. It is also noticed that isolated progestogen decreases the amount of collagen fibers and increases the number of muscle fibers in the detrusor muscle, but with less intensity than replacement with estrogens alone. It neither alters the number of collagen fibers nor decreases the muscle fibers in the muscle layer of the urethra, with increased collagen/muscle ratio in that structure. Finally, the estrogen-progestogen combination determines significantly decreased collagen fibers and increased muscle fibers in the detrusor muscle, causing no alteration to the collagen or muscle fibers in the muscle layer of the urethra.

Key words: Collagen; Urinary tract; Hormone replacement.

Introduction

Several urinary alterations either start or worsen at climacterium, such as urge incontinence, pollakiuria, dysuria, nocturia, increased micturition frequency and stress or urge incontinence of urine [1]. The common embryological development of both the lower urinary tract and the genital tract causes steroid receptors, of estrogens in particular, to be found in both [2].

The elements responsible for the suspension and support of the pelvic organs, such as ligaments and fasciae, also present receptors for estrogens and progesterone. Thus they are influenced by hypoestrogenism, becoming thinner and weaker and causing displacement of the urethrovesical junction of the bladder and also of the uterus [3]. Several urinary disorders, such as urgence, difficulty emptying the bladder and stress urinary incontinence, can arise from slackness of the vaginal wall or of its supporting ligaments. Therefore, such symptoms would be different effects of the same micturition reflex, prematurely activated [4].

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The results of estrogen therapy in postmenopausal urogenital alterations are controversial [5]. A large portion of the studies show improvement of urinary symptoms and an increased urethral pressure profile with estrogen replacement in postmenopause [6]. Progestogens determine decreased urethral pressure in female dogs [7], an event not demonstrated in humans [8].

Thus, several authors have tried to analyze the alterations that occur following the use of steroid hormones in the urethral mucosae, in the periurethral vessels, in the muscles and in the periurethral connective tissue [9, 10].

As for the mucosa, estrogen replacement in castrated female rats – whether or not combined with progestogens – promoted metaplasia, hyperplasia and thicker lower urinary tract epithelium [11].

Through digital color doppler velocimetry, a smaller number of periurethral vessels and increased resistance to blood flow in postmenopausal incontinent women are found [12]. Estrogen replacement, however, increases the blood supply to the urethra, the arterial pulsation and the number of periurethral vessels [13, 14]. Progesterone reduces the effects observed with estrogens [15, 16].

The connective tissue, an important element in the continence mechanism, has collagen as its major component, accounting for 30% of the body's total protein [17]. Over 18 types of collagen have been reported; those mostly studied are types I, II and III, which are also affected by estrogens [18].

Estrogen and androgen receptors have also been shown in skin fibroblasts, which suggests the sensitivity of such collagen-producing cells to those hormones [19]. And, as in the dermis collagen represents its major constituent, thus skin can be studied as a model for further protein sites in the body [20]. On the other hand, a significant correlation is shown between the amount of collagen in the skin and urethral sphincter function, and estrogen therapy in postmenopause is claimed to improve urethral function as a result of increased amounts of collagen in the urogenital tissues [21].

Hormonal influence on connective tissue may differ in other organs. While estrogens increase collagen in the skin and bones [20], *in vivo*, they reduce the production of such protein by the kidney's mesangial cells [21], as well as its synthesis and accumulation on the vascular wall of female rats and rabbits [22]. Even fewer studies on the lower urinary tract can be found.

The deposit of collagen fibers on the detrusor muscle may alter bladder contractility and thus determine several urinary alterations such as urge, incontinence, vesicoureteral reflux, and hydronephrosis, among others [23].

A larger amount of collagen is found in the bladder of women over 50 years of age as compared to men of the same age [23]. In experimental studies, in the absence of estrogens, the bladder is shown to be smaller and to contain a larger amount of collagen, which is reversed with hormone replacement [24, 25], suggesting that hypoestrogenism may imply deposits of collagen on the bladder wall. Collagen quantitative or qualitative alterations on the pelvic tissues could foster urogenital dystopia [26].

In relation to urinary continence, incontinent women are found to have a smaller amount of collagen in the skin, in the ligaments and pelvic fascia [27]. This could be only a decline of type III collagen since the total amount of protein does not seems to be altered [28].

Hormone replacement at climacterium would increase the production of collagen, particularly of type III [29], as well as promote increased organization of the fibers, with increased metabolism and, as a result, more elastic fibers [30].

This study sought to assess the effects of replacement with estrogens, progestogen or of the combination of both, on the number of both collagenous and muscle fibers in the bladder and in the urethra of castrated female rats.

Materials and Methods

Forty-six adult female OUTB EPM-1 Wistar Br Escola Paulista de Medicina (2c) virgin rats (Rattus norvergicus albinus, Rodentia, Mammalia), approximately three months old, weighing between 170 and 330 grams, with an average weight of 218 grams were used. They were castrated through a dorsal incision.

Thirty days after castration the animals were assigned to the following groups:

– Group C: consisted of 10 rats that were given no medication;

– Group P: included 8 rats which were given 0.1 ml of placebo solution, made up of 95% maize oil and 5% benzyl acid by subcutaneous (SC) route;

– Group E: was composed of 10 rats that were given 17ß-estradiol, 10 µg/kg/day by SC route [31];

– Group PR: consisted of 9 rats which were given medroxyprogesterone acetate, 0.2 mg/kg/day, dissolved in an aqueous solution by SC route [32];

– Group E+PR: was composed of 9 rats that were given the combination of 17ß-estradiol and medroxyprogesterone acetate, in the same doses and administration route as groups E and PR.

The rats received medication for 28 consecutive days, after which they were put to death and their lower urinary tract was withdrawn. Sections were taken from the bladder and in the middle third of the urethra, and stained with hematoxylin-eosin and picrosirius [33].

For the quantification of collagen and muscle fibers, a Zeiss I Kpl-W 10x integration eyepiece, with a geometrically distributed 25-point reticule was used. The reticule cast on the sections enabled the points that fell on the collagen and muscle fibers to be counted. The integration eyepiece was coupled to a Zeiss 100 x objective lens, resulting in an end magnification of 1,000 diameters. Forty fields were counted, totaling 1,000 points, by region studied and by animal.

Thus, one was able to count the collagen and muscle fibers present in the detrusor muscle of the bladder, as well as in the periurethral muscles.

Statistical Method

The groups were homogeneous in regard to animal age and weight, which was verified through the variance homogeneity test.

Analysis of variance was used to verify uniformity in the collagen fibers and muscle fibers by counting means among the different groups studied. The Tukey multiple comparison test was used for the analysis of collagen and muscle fibers in the bladder and the urethra, of the studied groups. Accordingly, one was able to identify the groups presenting significant differences.

In all tests, a level of 0.05 or 5% (alpha $\leq$ 0.05) was fixed for rejection of null hypotheses; significant values are indicated with an asterisk.

Results

Table 1 shows the results of the collagen and muscle fiber count in the bladder and urethra muscle layer in the five groups studied.

The castrated ras (C) and placebo given (P) groups presented the same amount of collagen which was higher than the other groups. The groups medicated with progestogen (PR and E+PR) showed a similar number of collagen fibers, however smaller than that found in group E (Figure 1).

In relation to the number of muscle fibers found in the bladder, groups C and P, as well as groups E and E+PR, were found to have similar values when compared. The groups that received estrogens alone (E) or in combination with medroxyprogesterone (E+PR) showed a higher number of muscle fibers than the other animals. A higher

Table 1. — *Number of collagen fibers (COL) and muscle fibers (MUSC) in the detrusor muscle and in the middle third muscle layer of the rat urethra, according to the studied groups: C (castrated); P (placebo); E (estrogens); PR (medroxyprogesterone); E+PR (estrogen-medroxyprogesterone combination).*

		GROUPS				
		C	P	E	PR	E+PR
COL	Mean	417.00	435.00	308.00	278.89	257.80
Bladder	SD	32.30	16.90	23.48	14.53	30.70
MUSC	Mean	523.00	532.50	648.00	582.22	648.90
Bladder	SD	53.80	27.65	39.40	19.86	88.20
COL	Mean	331.11	322.50	245.44	327.80	323.33
Urethra	SD	18.33	18.32	28.93	33.50	24.49
MUSC	Mean	339.44	337.50	548.90	298.89	315.60
Urethra	SD	21.57	25.50	30.60	20.28	31.30

Variance analysis: p<0.001*; Tukey multiple comparison test.

COL (bladder):		
C=P (p=0.170)	P>E (p<0.001)*	E>PR (p=0.005)*
C>E (p<0.001)*	P>PR (p<0.001)*	E>E+PR (p=0.001)*
C>PR (p<0.001)*	P>E+PR (p<0.001)*	PR=E+PR (p=0.080)
C>E+PR (p<0.001)*		
MUSC (bladder):		
C=P (p=0.660)	P<E (p<0.001)*	E>PR (p<0.001)*
C<E (p<0.001)*	P<PR (p<0.001)*	E=E+PR (p=0.980)
C<PR (p=0.006)*	P<E+PR (p=0.003)*	PR<E+PR (p=0.040)*
C<E+PR (p=0.001)*		
COL (urethra):		
C=P (p=0.350)	P>E (p<0.001)*	E<PR (p<0.001)*
C>E (p<0.001)*	P=PR (p=0.700)	E<E+PR (p<0.001)*
C=PR (p=0.800)	P=E+PR (p=0.940)	PR=E+PR (p=0.750)
C=E+PR (p=0.460)		
MUSC (urethra):		
C=P (p=0.870)	P<E (p<0.001)*	E>PR (p<0.001)*
C<E (p<0.001)*	P>PR (p=0.003)*	E>E=PR (p<0.001)*
C>PR (p<0.001)*	P=E+PR (p<0.140)	PR=E+PR (p=0.200)
C=E+PR (p<0.077)		

number of muscle fibers was found in the groups that received progestogen alone (PR) than in the groups of castrated rats (C) or the group receiving placebo (P). However, the number was smaller than in the groups treated with estrogens alone or combined with medroxyprogesterone (E and E+PR) (Figure 2).

In the muscle layer of the urethra the amount of collagen was found to be significantly smaller in group E than in the other groups. Both rats without hormone therapy (C and P) and those that received progestogen (P and E+PR), showed similar results (Figure 3).

As for the urethral muscles, the group that received estrogens (E), presented a higher number of muscle fibers than all the other groups analyzed, while the PR (medroxyprogesterone) group had significantly reduced muscle layers, even in relation to the groups that were not treated with hormones (Figure 4).

Discussion

In this study, we assessed the number of collagen and muscle fibers, both in the bladder and in the urethra of

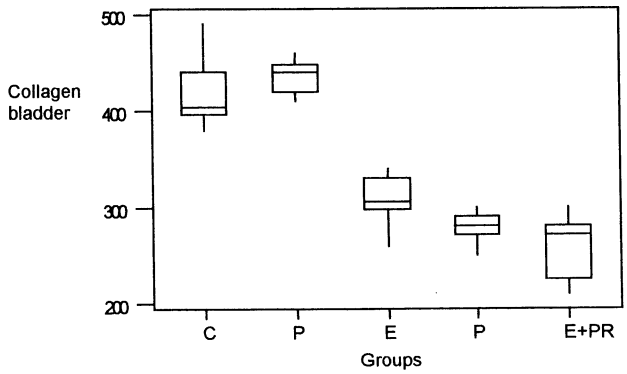

Figure 1. — Boxplot of collagen count in the muscle layer of the bladder in the studied groups.

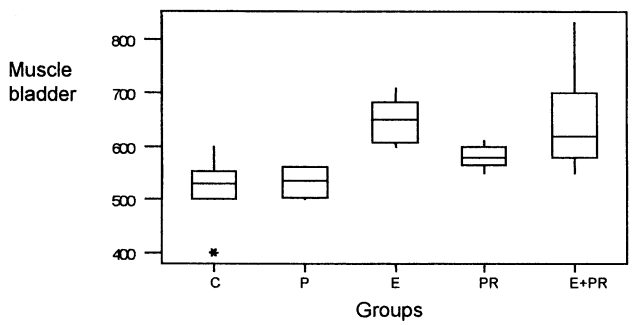

Figure 2. — Boxplot of muscle fiber count in the muscle layer of the bladder in the studied groups.

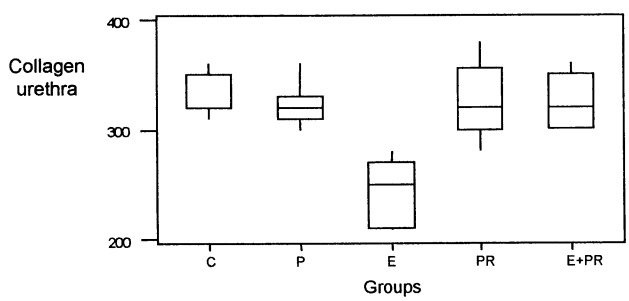

Figure 3. — Boxplot of collagen count in the muscle layer of the urethra in the studied groups.

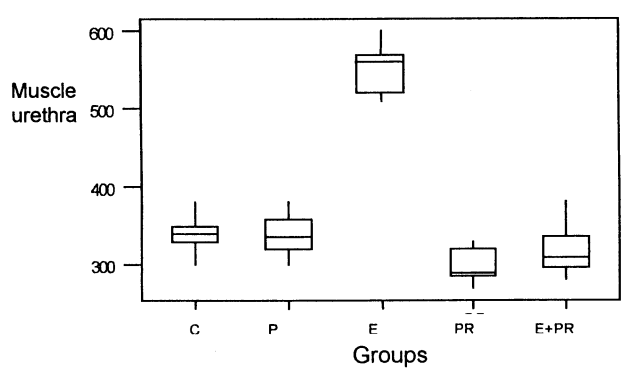

Figure 4. — Boxplot of the muscle fiber count in the muscle layer of the urethra in the studied groups.

castrated adult rats, before and during hormone replacement. It should be mentioned that quantified collagen refers to several types that form the fibers, even if of a small size, such as types I and III, since the method used shows collagen as a whole.

The use of estrogens alone was found to promote an important decline in the amount of collagen among the muscle fasciculi of the detrusor muscle and in the muscle layer of the urethra. On the other hand, replacement of estrogen alone contributed to a significant increase in the number of muscle fibers, both in the bladder and in the urethra. Such findings were also corroborated by Susset *et al.* [23], who identified a higher amount of collagen in women with hypoestrogenism and also by Phillips and Davies [24], who found that the older the age, the smaller the amount of smooth muscle in the urethra. Estrogen replacement in castrated rats also reverted the increase in collagen among the muscle fibers in the urinary tract, according to Eika *et al.* [25]. However, our results differ from the ones achieved by Persson *et al.* [34], who found similar amounts of collagen, both in the urethra and in the bladder of rabbits, whether castrated or not.

In regard to progestogen, an effect similar to that of estrogen was found in the bladder, although less intensely in relation to the muscle fibers.

Nevertheless, in the bladder the estrogen-progestogen combination promoted a marked reduction in the amount of collagen and significantly increased the number of muscle fibers – as much as the replacement of estrogen alone.

According to Hassager *et al.* [29], the use of such hormones would increase the production of collagen, although type III, which is more flexible.

Therefore, our results show the replacement of estrogen alone or the estrogen-progestogen combination to be effective in reducing collagen and increasing the muscles in the bladder; progestogen also causes such an effect, albeit to a lesser degree as far as muscle fibers are concerned.

In this respect, Falconer *et al.* [30] emphasized that hormone replacement therapy would increase only the metabolism of collagen by taking the less rigid fibers without necessarily altering the number of fibers.

Our results enable one to infer that hormone replacement in postmenopausal women could contribute to improved muscle contractility, reducing the amount of collagen among the muscle fibers of the detrusor muscle. Thus, improvement in urinary disorders, such as increased bladder capacity and improved emptying of the bladder would occur, as well as a smaller post-voiding residual volume, as found in a previous study [6]. Whereas progestogen replacement, despite showing opposed action to estrogens in a large portion of tissues [7], would apparently have a similar effect in the bladder, although to a lesser degree.

In regard to the urethra, we found that a reduction in collagen was provided only by estrogen replacement alone. As for its muscles, estrogen accounted for a significant increase in muscle fibers and progestogen determined the contrary. Thus, the administration of progestogen does not appear to be ideal in improving muscle trophism of the urethra in the presence of hypoestrogenism.

By transferring such results to women, we would say that if hormone replacement improves the activity of the urethra, the ideal would be to employ estrogens alone which would promote a further increase in the intrinsic muscles of the urethra. Progestogen could reduce such effects, particularly in cases with stress urinary incontinence.

Thus, the hormones studied are found to have different effects according to the organ analyzed, suggesting there are receptors that react differently to hormonal stimulation.

Increased investigation in this area may help in the prevention of urinary disorders, identifying women prone to urinary dysfunction arising from alterations in collagen metabolism, either by preventing injuries to the pelvic floor, or indicating hormone replacement at the onset of climacterium.

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