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ORIGINAL ARTICLE

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Effects of ovariectomy and hormone replacement on collagen and blood vessels of the urethral submucosa of rats

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Abstract Collagen and blood vessels of the urethral submucosa of ovariectomized rats were studied following 28 daily subcutaneous injections of 17-ß estradiol ($n=6$, group 1), medroxy-progesterone acetate ($n=6$, group 2), both drugs ($n=6$, group 3) or vehicle (n = *6*, control) and after sham surgery without castration or injection ($n=6$). Investigations included the immunohistochemistry of estrogen and progesterone receptors and collagen fibres, Western blot analysis of collagen types I and III and counting periurethral vessels by light microscopy. Our results showed positive immunostaining with estrogen, progesterone and collagen types I and III in all samples. Collagen type I and III levels were lower in the controls than in the sham group. The other groups showed increases (2 > 3 > 1) over the controls with a relatively higher increase in type III. The type I/III collagen ratio progressively decreased (control > 1 > 2 > 3) below sham levels. The mean vessel count was significantly lower in control than in sham animals ($P<0.00001$). However, only estrogen treatment significantly increased the vessel number compared to controls ($P=0.04$). Our results indicate that estrogen and progesterone, alone or in combination, have an effect on collagen types I and III, and that estrogen has an effect on blood vessels of the urethral submucosa in female rats.

Keywords Collagen · Estrogen · Progesterone · Rat · Urethra · Urinary incontinence

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Introduction

Urinary incontinence in women can result from a number of factors including failure of the urethral sphincter mechanism (genuine stress incontinence-GSI) in about 60% of cases [4, 19]. GSI is believed to result from functional or anatomical changes in the urethra that cause negative urethral closure pressure during stress [19]. The factors thought to be important in maintaining the closure pressure are the mucosa, connective tissue, vascular tissue and the urethral musculature. Each of these components has some dependence on estrogen and possibly progesterone [3, 11, 12, 26, 27, 28]. Several studies have also shown the presence of estrogen and possibly progesterone receptors in the proximal urethra of animals and humans using different techniques [12, 24]. Suggested mechanisms underlying the failure of urethral closure pressure include hormone-induced changes in the biochemical compliance of soft tissues or submucosal blood flow [4, 7, 9, 11, 12, 15, 22, 28].

Of the 19 types of collagen currently identified, only types I and III occur in the connective tissues of the female lower urinary tract. These are located particularly in the urethral submucosa where they significantly influence biological functions [5, 9, 14, 15, 17, 19, 25]. Advances in immunohistochemical and cytogenetic

techniques now allow the identification of qualitative and quantitative changes, respectively, in collagen with a high degree of consistency and reproducibility [1, 17, 19]. The aim of our study, therefore, was to: (1) ascertain whether the administration of estrogen and/or progesterone influences the amount or distribution of collagen fibres types I and III and the number of vascular plexus of the urethral submucosa in ovariectomized rats, (2) compare such effects with those of a placebo. This work may provide evidence for a possible role of connective tissue in mediating estrogen and/or progesterone effects on urinary control in women, and has obvious therapeutic potential.

Materials and methods

In vitro experiments were performed on the urethra of Wistar female rats weighing 200–250 g and aged 5–6 months, in the approved animal care facility of the Faculty of Medicine and Health Sciences, United Arab Emirates University. The study protocol was approved by the Animal Research Ethics Committee of the Faculty and conformed to the Code of Practice for the use and care of laboratory animals for research. The series consisted of 18 rats in the study group and six in the control group ($n = 24$). All underwent bilateral ovariectomy under brief general anesthesia with diethyl ether using a standard dorsal surgical approach. Six more animals underwent sham surgery without castration or further postoperative intervention. The animals were housed two per cage with free access to water and food and maintained without medication for 30 days. Complete surgical castration was determined by the absence of mature epithelial cells on vaginal cytological smears.

The study group was further assigned to three subgroups of six rats each. Group 1 received 17-ß estradiol (Sigma, St. Louis, Missouri, USA) 10 μg/kg/day, group 2 received medroxyprogesterone acetate (Upjohn, Crawley, UK) 0.2 μg/kg/day and group 3 was treated with both drugs in the same doses used for groups 1 and 2. The control group received 0.1 ml of a diluted vehicle medium, made up of 95% corn oil and 5% benzyl acid. All drugs were administered subcutaneously. After 28 days of continuous treatment, the animals were killed, the abdomen opened and the lower urinary tracts containing the urinary bladder and urethra removed for evaluation. The urethra was transected at its junction with the bladder, opened by a vertical incision and divided longitudinally into two halves. One half was rapidly frozen in liquid nitrogen for the analysis of collagen types and the other half fixed in formalin for qualitative assay of estrogen and progesterone receptors and collagen fibres and for measuring the periurethral blood vessels. The investigators performing the laboratory studies did not know from which group of animals the tissue samples were taken.

The immunohistochemistry of estrogen and progesterone receptors and collagen types I and III was performed on 5 μm thick transverse sections obtained from formalin-fixed, paraffin-embedded tissue. The sections were heated in a 750 W household Panasonic microwave oven twice for 5 min to retrieve the antigens and immunostained, as previously described [1, 17, 19, 24]. In brief, the specimens were incubated with the primary monoclonal antibodies to types I and III collagen (Sigma) diluted 1:100 and 1:400, respectively, with 0.15 M NaCl in 0.05 M Tris-HCL buffer (pH 7.4) and pre-diluted primary monoclonal antibodies to estrogen and progesterone receptors (Dako, Copenhagen, Denmark) overnight at 4°C. The sections were allowed to warm to room temperature and then incubated with the link antibody comprising biotinylated anti-mouse IgG (Dako, Copenhagen, Denmark) in cold phosphate-buffered saline (PBS) for 30 min and then peroxidase labelled-streptavidin tertiary antibody (Dako) for 1 h. One 10 mg tablet of diaminobenzidene hydrochloride (Sigma) was dissolved in 15 ml of 0.05 M tris-bovine serum albumin buffer and filtered, then 12 ml of 30% hydrogen peroxide was added to the filtrate. The light brown solution was placed on the sections for 3-5 min. The sections were then counterstained within hematoxylin and examined on a Zeiss axiophot photomicroscope. Tissue sections of cartilage known to be collagen I and III positive and sections of breast carcinoma known to be estrogen and progesterone receptor positive were used as positive controls. The primary antibodies were replaced by non-immune serum for negative controls. Fibres or cells with distinct staining or nuclear staining, respectively, were considered immunopositive. Any immunopositive fibre or cell was included in the analysis irrespective of the number or intensity of staining.

The periurethral vessel count was performed using light microscopy on formalin-fixed and paraffin-embedded tissue blocks perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) and stained with standard hematoxylin and eosin using a 40× lens, as previously described [6]. The results were expressed as the mean number of vessels per high power field (400×) in each group.

Analysis of collagen types I and III was performed by Western blot [17]. Freshly isolated tissue was homogenized at 4°C in homogenization buffer (1 M Tris-HCl pH 7.4, 5 M NaCl, 10% NP-40, 100 mM NaF, 100 mM $NaVO_4$, 500 mM DTT and 100 mM fresh PMSF containing protease inhibitors; 10 mg/ml leupeptin, 10 mg/ml aprotinine, 10 mg/ml trypsin and 1 mg/ml pepstatin A). The homogenate was centrifuged at 15,000 rpm for 15 min at 4°C. An aliquot of the supernatant was kept for protein determination and a SDS sample buffer containing beta-mercaptoethanol was added to the rest. The samples were boiled for 3 min, and after cooling the equivalent of 30 ug of protein/lane were loaded on a 15% polyacrylamide gel. The proteins were subjected to electrophoresis under constant voltage and transferred onto nitrocellulose membranes blocked with 5% non-fat milk. The blots were incubated with the monoclonal antibodies to collagen types I and III (Sigma) at a dilution of 1:500 at room temperature for 2 h, washed and incubated with the secondary antibody conjugated to horseradish peroxidase. Anti-collagen antibody binding was visualized by enhanced chemiluminescence. Signal intensity was analyzed using an image analyzer (Vilber Lourmat, France and Bio-Rad Image System, Bio-Rad Laboratories, Calif., USA) and normalized against known collagen standards in each study group.

The results in the drug and each of the control and sham groups were compared by the Student's *t*-test (two-tailed). A probability of < 0.05 was considered to be statistically significant.

Results

There was positive immunostaining with estrogen and progesterone receptors in the submucosa of all urethral samples studied. Furthermore, all sections showed positive immunostaining with collagen types I and III in the submucosa (Fig. 1). Levels of collagen type I were decreased in all study groups compared to the sham group (Figs. 1A, 2). The greatest decrease was seen in the control group followed by groups 3, 2 and 1 (Fig. 2). However, the levels of collagen type I were higher in groups 1and 2 than in the control group. Levels of collagen type III were also decreased in the control group but increased in other groups compared to the sham group (Figs. 1B, 2). The greatest increase was in group 2 followed by groups 3 and 1 (Fig. 2). The total collagen level (collagen type I + collagen type III) decreased in the control group and increased in other study groups compared to the sham group (Fig. 2). The greatest increase was in group 2 followed by groups 3 and 1. The type I/III collagen ratio, however, progressively decreased in the control group followed by groups 1, 2 and 3 compared to the sham group (Fig. 2).

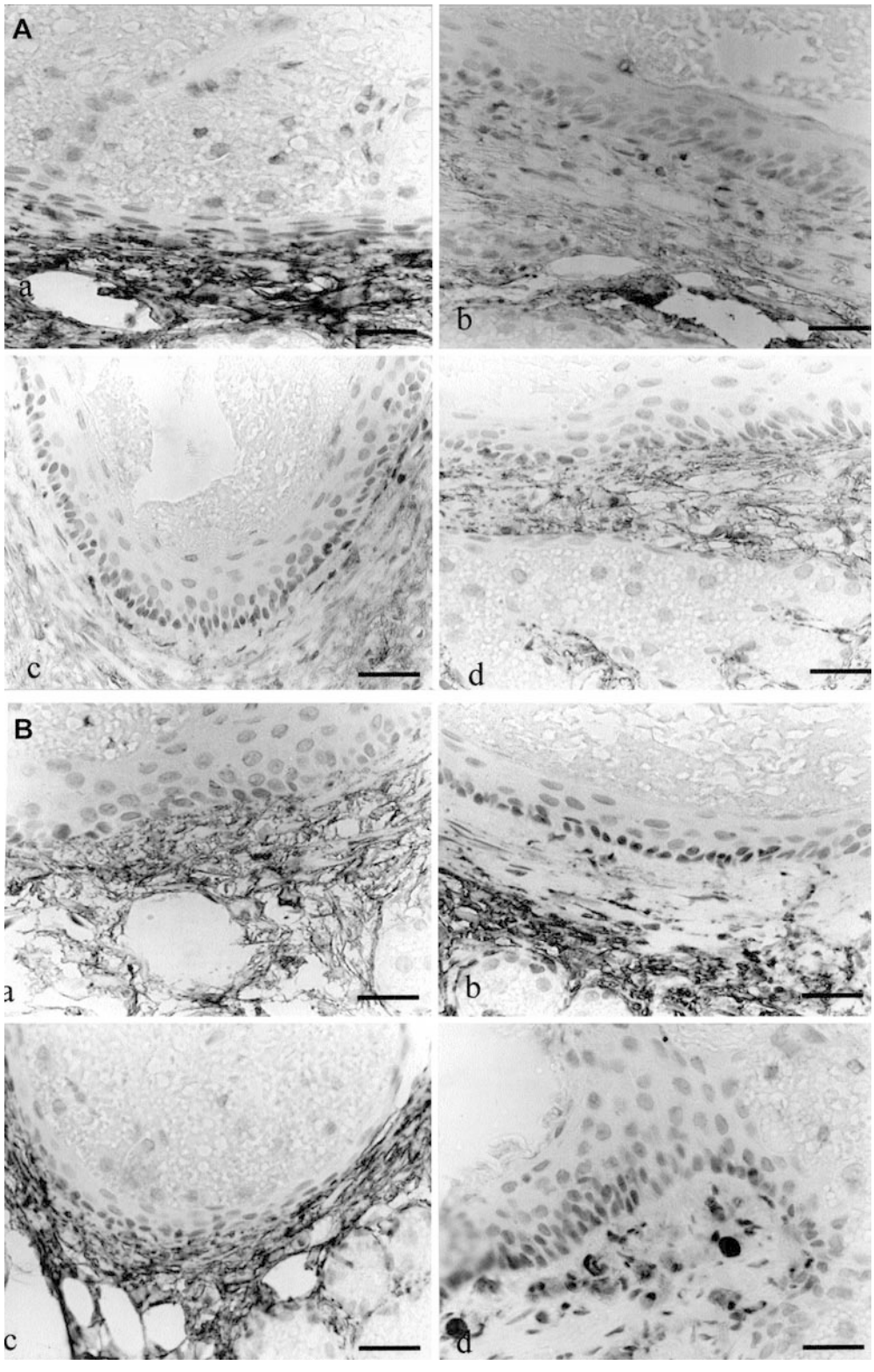

Fig. 1 Immunostaining with collagen types I (**A**) and III (**B**) in the urethral submucosa (*bar* = 6 μm) of sham *a*, groups 1 *b* and 2 *c* and controls *d*. The immunostaining of collagen type I is less in all groups compared to the sham group (**A**). Immunostaining of collagen type III is less in the control group but more in other groups compared to the sham group (**B**)

The mean (± SD) periurethral blood vessel count of all study groups was lower than that of the sham group (27.5 ± 2.5) as shown in Fig. 3. The difference was significant in the control group (6.5 ± 2.5, $P < 0.00001$). The mean vessel count in group 1 (9.5 ± 2.4) was significantly greater than that of controls ($P = 0.04$). The differences between the vessel counts in the control group and each of groups 2 (5 ± 2.1) and 3 (6.2 ± 4.4) were not significant (Fig. 3).

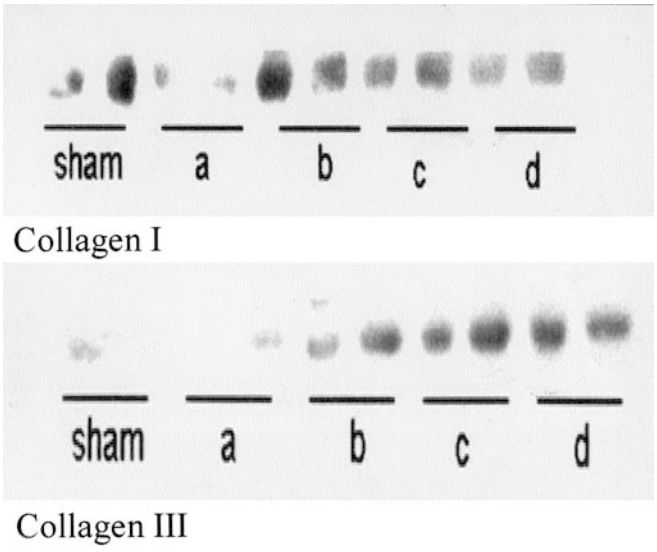

Fig. 2 Western blot analysis of collagen types I and III in the urethral submucosa of sham rats, controls *a* and groups 1 *b*, 2 *c* and 3 *d*. Signal intensity of collagen type I is decreased in all groups compared to the sham group. The greatest decrease is seen in the control group followed by groups 3, 2 and 1. The signal intensity of collagen type III is decreased in the control group but increased in other groups compared to the sham group. The greatest increase is seen in group 2 followed by groups 3 and 1

Discussion

The presence of high-affinity estrogen and progesterone receptors in female periurethral connective tissue and the ability of these hormones to induce structural or functional changes in individual components such as collagen types I and III and periurethral blood vessels would provide evidence for the involvement of estrogen and/or progesterone in urinary control. This study attempts to investigate such an hypothesis in adult female rats. Our results showed the presence of estrogen and progesterone receptors in the urethral submucosa of female rats. They also showed that collagen levels in the urethral submucosa decrease after castration with a relative decrease in the collagen I component. Estrogen and progesterone, alone or in combination, increase collagen above castration levels, mainly because collagen type III is increased. The periurethral blood vessel count also decreases after castration but partially increases following the administration of estrogen.

It is widely believed that an underlying abnormality of connective tissue in the urethra is a cause of GSI since collagen provides the strength and flexibility of this organ [4, 5, 8, 9, 15, 16, 19, 20, 21, 22]. There is also presumptive clinical, histological, biochemical and experimental evidence that urethral collagen fibres are affected by variations in estrogen and progesterone levels [7, 15, 21, 22, 25, 27]. In our study, estrogen and progesterone, alone or in combination, increased collagen III above castration levels but had less effect on collagen I levels, thus shifting the balance in favor of collagen III. Changes in the type I/III collagen ratio have been shown to correlate with changes in the mechanical properties of the urethra because type I is more rigid and supportive while type III contributes more to its elastic properties [14, 22, 23]. Increased urethral type I collagen or decreased type III collagen were, respectively, clinically associated with the loss of urethral compliance or decreased urethral pressure in most [1, 2, 19, 22, 23] but not all [8, 16] studies. Hence, in the present study, the decreased collagen type I/III ratio after estrogen or progesterone administration, whether alone or in combination, supports the positive effects of both hormones on periurethral collagen and consequently on urethral compliance and pressure.

The periurethral vessel 'cushion' is an important component in the intrinsic urethral function [10, 27]. Sex hormone influences on the development and involution of these vascular plexus have been described in the proximal portion of the female urethra in anatomical, experimental and clinical studies [6, 7, 13, 26, 28]. In castrated female rats, the periurethral blood vessel count was increased following estrogen replacement but not following the combined administration of both hormones [7]. Our findings are in line with the latter study [7] and support the suggestion [6, 7, 26, 28] that part of

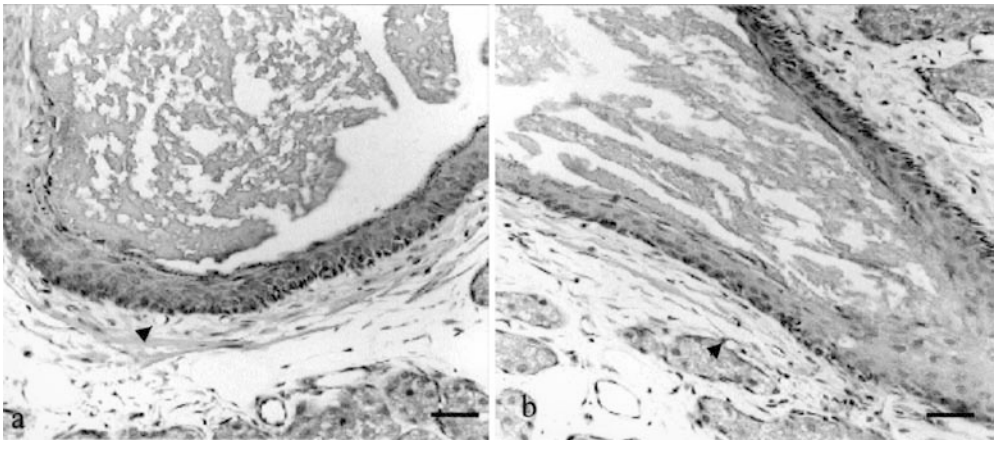

Fig. 3 Periurethral blood vessel count (hematoxylin and eosin, *bar* = 8 μm) of **a** controls and **b** group 1 . The count in group1 is greater than that in controls

the beneficial action of estrogen on female urinary control is possibly mediated via the increased number or improved function of periurethral blood vessels, with a corresponding increase in urethral pressure. Progesterone probably has an anti-estrogenic action on these vessels since progesterone replacement, either alone or combined with estrogen, did not increase the vessel count in our experiments or in previous animal studies [7]. Furthermore, urethral blood flow in the luteal phase of the menstrual cycle was reduced compared to the follicular phase in continent premenopausal women [26].

The use of rats as animal models to study normal, lower urinary tract structure and function as well as experimentally induced dysfunctions is well established [18]. However, a study examining the relationship between connective tissue factors, reproductive hormones and urethral closure pressure in women should ideally involve the measurement of collagen and examination of the periurethral blood vessels in a specimen obtained through a direct biopsy from the human female urethra. It was not possible to perform this procedure in a population of normal individuals in our institution for ethical reasons. Moreover, biopsy samples are usually obtained from accessible urethral tissue far from the key supportive elements of interest that are often difficult to approach surgically [5, 15, 21].

In conclusion, our study suggests that the influence of estrogen and progesterone on urethral function in adult female rats may be mediated through changes in differential collagen levels and the number of periurethral vessels in the urethral submucosa. The significance of our experimental data, however, needs to be further investigated before its clinical impact can be determined.

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