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ORIGINAL ARTICLE

Immunohistochemical analysis of collagen types I, III, IV and α-actin in the urethra of sexually intact and ovariectomized beagles

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Abstract Urinary incontinence is a widespread problem in both postmenopausal women and ovariectomized dogs. The objective of this study was to investigate the influence of ovariectomy on the immunoreactivity and the distribution pattern of collagens I, III, IV and α-actin in the canine urethra. The immunohistochemical results were evaluated in five sexually intact and five ovariectomized beagles. The immunostaining of both collagens I and III delineated urethral connective tissue fibres and co-localized within in the fibres of both groups. The basement membranes of smooth muscle cells and sinusoids showed marked type IV collagen expression, whereas only faint immunoreactivity was present at the urothelial–stromal interface. No differences could be detected in the expression or distribution of the assessed collagen types and actin between ovariectomized and control animals. In conclusion, ovariectomy does not appear to have an effect on urethral collagens I, III, IV and smooth muscle actin in the dog, as ascertained by immunohistochemistry.

Keywords Collagen · Connective tissue · Immunohistochemistry · Ovariectomy · Urethra · Urinary incontinence

Introduction

Urinary incontinence is a widespread problem in both women and female dogs. In women, stress urinary incontinence is prevalent during reproductive senescence, whereas in dogs, urinary incontinence is the most common side effect of ovariectomy [1–4]. Menopause in women and ovariectomy in dogs are accompanied by decreased ovarian steroid hormone plasma concentrations [5, 6]. In both postmenopausal women and spayed dogs, urinary incontinence is associated with reduced urethral closure pressures and impaired closure function of the urethra [7–9]. The underlying pathophysiological mechanism has not been clearly elucidated.

The complex closure mechanism of the urethra depends on several urethral tissue components, such as smooth and striated musculature, the vascular plexus, connective tissue, as well as periurethral structures [10–14]. Connective tissue, including elastic fibres, is by far the predominant constituent and comprises about 77% of the total volume of the female canine urethra [15]. Collagen types I and III are the most abundant fibrillar collagen subtypes in fibrous connective tissue and are important for the function of the extracellular matrix. Collagen type I provides supportive rigidity and confers tensile strength, whereas collagen type III lends flexibility and distensibility [10, 16, 17]. In contrast, type IV collagen is a non-fibrillar collagen subtype forming sheetlike networks comprising the basal laminae framework [17]. Collagen has been ascribed a significant role in the maintenance of urinary continence [18]. Several publications have described differences in collagen content and metabolism of urogenital and pelvic floor tissues between pre- and postmenopausal women [19, 20], as well as between continent and incontinent pre- and postmenopausal subjects [21–23]. The results of these studies, however, are highly controversial. In terms of change in the ratio of type I and type III collagen, one investigation showed a decreased ratio in periurethral biopsies from stress incontinent premenopausal patients compared to

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continent control women [21]. Furthermore, urethral biopsy specimens from stress incontinent women have been found to exhibit altered collagen morphologic characteristics [10].

The objective of the present investigation was to establish the immunohistochemical expression pattern of collagen types I, III and VI as well as smooth muscle actin in the female canine urethra and to determine if ovariectomized beagles exhibited differences in the expression and distribution of collagen types and actin when compared with sexually intact controls.

Materials and methods

Animals and tissue processing

A homogenous group of ten sexually intact nulliparous beagles (2–3 years of age) in anestrus weighing between 9 and 11.5 kg were used for this study. Five randomly chosen dogs were subjected to ovariectomy under general anaesthesia, whereas the remaining five served as controls. All animals were continent, kept in identical environmental conditions for 12 months, and were then killed by intravenous injection of pentobarbital. The dogs were housed and cared for according to the principles of laboratory animal care prescribed by Swiss national law for the protection of animals.

Each urethra was then removed from the pelvic cavity through the symphysis of the osseous pelvic floor and split into four parts of equal length. The specimens were fixed with 3.7% paraformaldehyde in 0.1 M calcium acetate (1.45 osM, pH 7.3) for 24 h at 5°C. The fixed urethral specimens were routinely embedded in paraffin and cut into 5 μm serial cross-sections. For the immunohistochemical analysis, serial cross-sections were selected from the middle of the proximal and distal halves of the urethra and mounted on SuperFrost Plus glass slides (Milian, Basel, Switzerland).

Immunohistochemistry for collagens and actin

In a pilot study, primary antibodies were evaluated for distinct reactivity with canine antigens. Optimal dilutions and digestion conditions were determined by titration experiments. The immunohistochemical analyses were carried out using the Streptavidin–biotin-complex–peroxidase technique (StreptABComplex–HRP Duet; DakoCytomation, Glostrup, Denmark). The sections were deparaffinized and rehydrated. For retrieval of collagen I, III and IV epitopes, sections were treated with 0.25% Pepsin (Sigma-Aldrich, Buchs, Switzerland) in 10 mM HCl for 15, 60 and 120 min, respectively, at 37°C. After blocking endogenous peroxidase activity with 0.5% H_2O_2 in methanol for 20 min and washing in Tris-buffered saline (TBS) for 5 min, the sections were transferred to a humidity chamber and immersed in 10% normal goat serum (NGtS; Kirkegaards & Perry Laboratories, Gaithersburg, USA) to eliminate non-specific protein binding. After tapping the slides to remove excess serum, primary antibodies diluted in TBS containing 1% NGtS were applied and allowed to incubate for 60 min at 25°C. Polyclonal rabbit anti-human collagen I (1:200; Rockland, Philadelphia, USA), IV (1:50; Quartett, Berlin, Germany), monoclonal mouse anti-human collagen III (1:50; Quartett), and smooth muscle actin (1:100; DakoCytomation) were employed as primary antibodies. The sections were washed in TBS for 3 min, incubated with the corresponding biotinylated anti-mouse and anti-rabbit secondary antibodies (DakoCytomation) for 30 min at 25°C, washed again in TBS and treated with a peroxidase conjugated streptavidin label (DakoCytomation) for 30 min, followed by a final TBS wash. For the peroxidase reaction, diaminobenzidine (DakoCytomation) served as the chromogen. The sections were then rinsed with distilled water (2×5 min), counterstained with haematoxylin (DakoCytomation), dehydrated and embedded in Histoclear (Raymond A Lamb, Eastbourne, UK).

Tissue sections of human ureter and skin were used as positive controls, and the primary antibody was replaced by non-immune serum (rabbit and mouse isotype control; DakoCytomation) for negative controls.

For the evaluation of the immunohistochemical expression and distribution of investigated antigens, three randomly chosen immunostained sections from the proximal and distal urethral halves of each animal were used. The immunoreactivity and the distribution pattern of the different collagens were assessed in every urethral connective tissue compartment (subepithelial, sinusoid, smooth and striated muscle layers) and compared between groups.

Results

Distinct immunohistochemical expression of collagens I, II and IV and α-actin was detected in the urethral cross sections of both ovariectomized and control animals.

The positive immunostaining of collagen types I and III labelled the collagen fibres of all urethral connective tissue compartments. Both collagen types seemed to be co-localized resulting in a similar staining distribution pattern. The labelling intensity of collagen type III, however, was generally stronger than collagen type I. This was particularly evident in the fine fibre network immediately underlying the urothelium (Fig. 1a–d). The intense staining signal of collagen type III was mainly found in the strongly positive collagen fibre contours (Fig. 2b), thus giving the connective tissue a more "fibrous" appearance in the

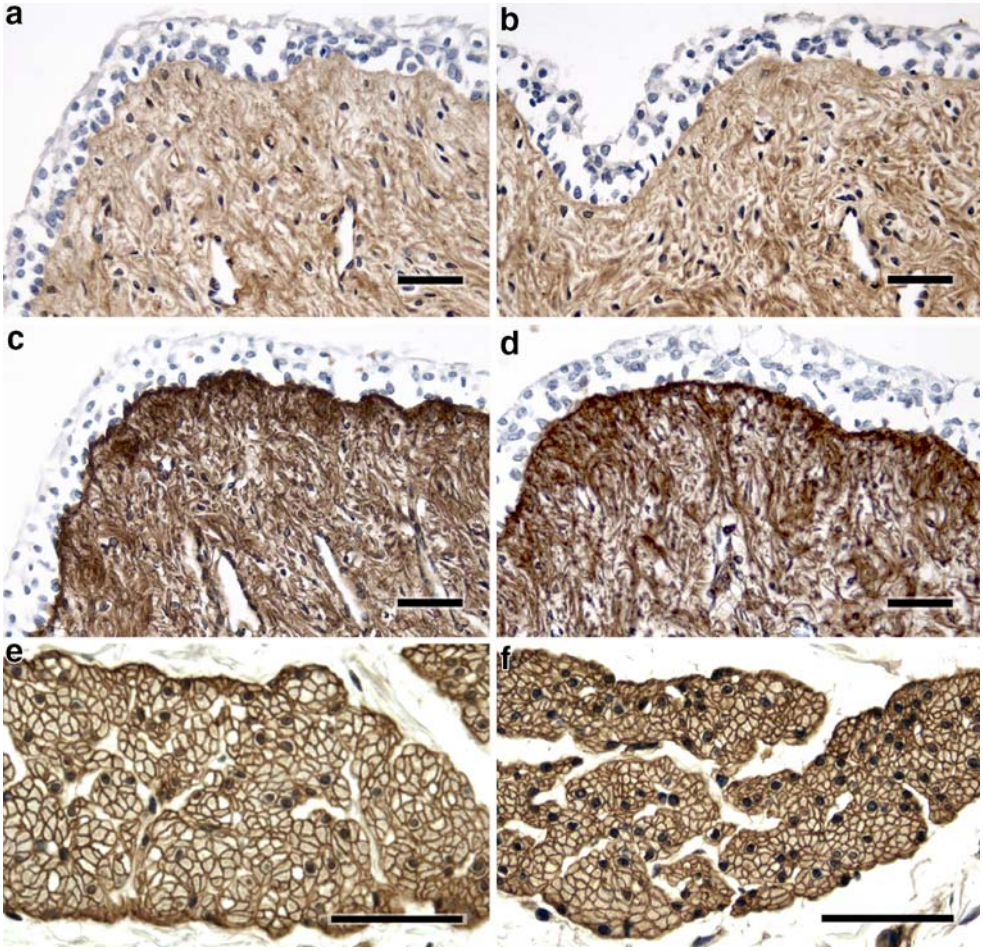

Fig. 1 Immunohistochemical reactivity of collagens I, III and IV in the urethra of sexually intact (CO) and ovariectomized (OX) beagles; bars, 50 μm. **a** Collagen I (CO); **b** collagen I (OX); **c** collagen III (CO); **d** collagen III (OX) depicting the fine subepithelial collagen fiber network with a stronger labelling intensity of collagen III compared to collagen I; **e** collagen IV (CO); **f** collagen IV (OX), demarcating the basement membranes of smooth muscle cells

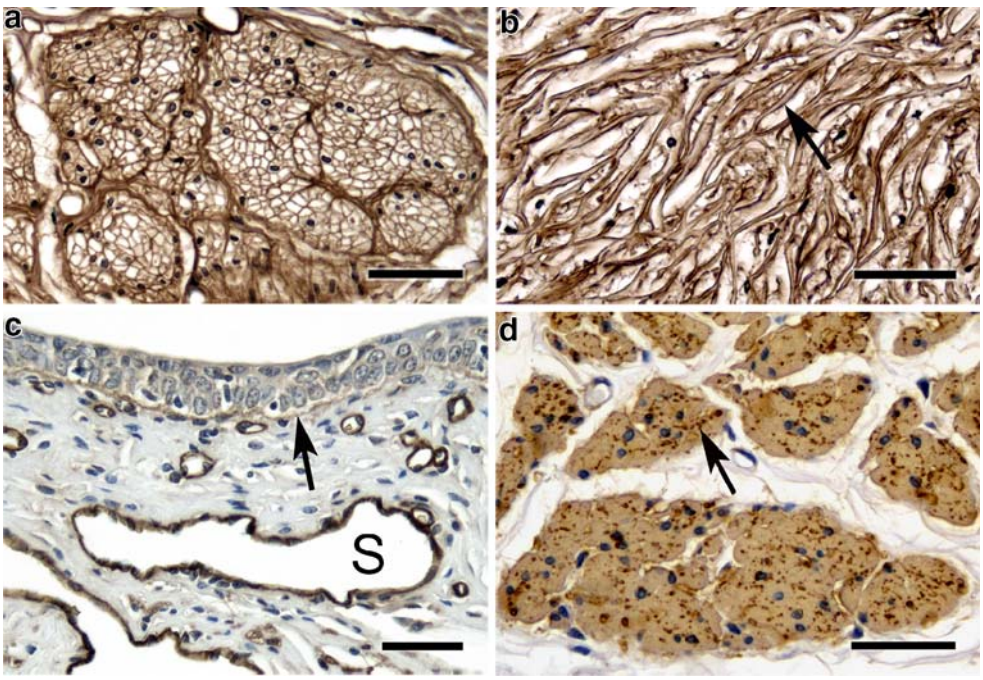

Fig. 2 Immunohistochemical reactivity of collagens I, III and IV and α-actin in the urethra of sexually intact (CO) and ovariectomized (OX) beagles; bars, 50 μm. **a** Collagen I (CO), demarcating the endo- and perimysium of smooth muscle cell fascicles; **b** collagen III (OX), showing intense staining of collagen fiber contours (*arrow*); **c** collagen IV (CO), delineating the weakly stained epithelial (*arrow*) and the strongly stained vascular basement membranes (*S*, sinusoid); **d** α-actin (OX), showing the granular reaction product in smooth muscle cells (*arrow*)

overview. The collagen fibres exhibited variable diameters and formed a dense network underlying the epithelium and surrounding the sinusoids of the vascular plexus. A loose meshwork of fine and coarse fibres was observed in the smooth muscle layer connecting bundles of smooth muscle cells. The endo- and perimysium of these muscle cell fascicles were markedly stained with both anti-type collagen I and III antibodies (Fig. 2a). This was also true for the striated muscle fibre bundles, which were localized solely in the distal urethral half. The collagen fibre network between the striated muscle fibre bundles, however, was of higher density when compared with that of the smooth musculature. The adventitia consisted of a loose meshwork of various-sized undulating collagen fibres. Differences in the expression or the distribution of collagen types I and III immunoreactivity could not be detected between ovariectomized and control animals (Fig. 1a–f).

The presence of collagen IV was revealed in the staining of basement membranes underlying the urethral epithelium and the vascular endothelia as well as encircling smooth and striated muscle cells or fibres. The basement membranes of vascular and non-vascular smooth muscle cells and sinusoids displayed marked type IV collagen expression, whereas only faint immunoreactivity was discernable at the urothelial–stromal interface (Figs. 1e,f and 2c) in both the ovariectomized and control animals.

Immunolocalization of smooth muscle actin was confined to vascular and non-vascular smooth muscle cells resulting in a granular reaction product (Fig. 2d). No difference was observed between either group.

Discussion

To our knowledge, this is the first report on the immunohistochemical expression and distribution pattern of different collagen types and smooth muscle actin in the urethra of sexually intact and ovariectomized bitches. Immunostaining of both collagen type I and III demarcated the collagen fibres of all urethral connective tissue compartments resulting in a similar distribution pattern. Concomitant localization of collagen types I and III has been identified in the stromal connective tissue of various organ systems, indicating a mixed composition of collagen fibres [20, 24, 25]. Fleischmajer et al. [25] demonstrated the coexistence of type I and type III collagen within individual fibrils comprising collagen fibres of differing diameters. Likewise, heterotypic fibres consisting of collagens I, III and V have been identified in the arcus tendineous fasciae pelvis of pre- and postmenopausal women [20]. Furthermore, collagen fibres can be coated with type III collagen. This could account for the increased collagen type III immunoreactivity of collagen fibre contours observed in the urethral connective tissue. The stronger labelling intensity of collagen type III, compared to type I, implies a relatively high percentage of collagen type III in the composition of urethral collagen fibres. This, apart from tensile strength, would lend flexibility and distensibility to the tissue [16, 18, 20, 21]. Immunolabelling of collagen type III revealed an intensely stained thin layer of a fine fibre mesh just beneath the urothelium, suggesting the existence of a lamina fibroreticularis. This reticular structure is usually found under multi-layered epithelia as part of the epithelial basement membrane zone and aids attachment of the epithelium to the extracellular matrix [25–27].

Type IV collagen is one of the major meshwork components of mature basement membranes. It is encoded by six genetically distinct α-chains, which constitute different collagen type IV isoforms. The isoform composition of basement membranes varies from tissue to tissue accordingly [17, 26, 28]. This could explain the discrepancy between the very faint staining urothelial basement membrane and the intensely immunolabelled vascular and muscular membranes.

Both menopause in women and ovariectomy in dogs are accompanied by decreased ovarian steroid hormone plasma levels and increased prevalence of urinary incontinence [1–6]. Female steroid hormones are known to influence the complex metabolism of collagen and this, in turn, can affect the connective tissue, contributing to urinary incontinence [19, 20, 23, 29]. Our results, however, revealed no differences in the expression and distribution of immunoreactivity of the assessed collagen types between ovariectomized and control dogs. This indicates that ovariectomy and concomitant decreased ovarian steroid hormone plasma levels have no obvious structural impact on the female canine urethral connective tissue. These findings seem to be supported by a study of Dündar et al. [30] who investigated the influence of estrogen treatment on urethral connective tissue in ovariectomized rats. The authors conclude that early and late estrogen replacements do not seem to have an ascertainable effect on the urethral connective tissue. In contrast, Clark et al. [29] investigated the impact of estrogen treatment on the pelvic floor connective tissue of ovariectomized rhesus macaques and found that estrogen increased collagen gene transcription and stimulated collagen synthesis. The influence of menopause on collagen of urogenital connective tissues has also been controversially discussed [19, 20, 23]. The discordant results of studies dealing with the relationship between collagen metabolism, collagen expression, menopause and urinary incontinence appear to be mainly due to differences arising from variation within study populations and the use of small tissue samples obtained from differing locations.

In conclusion, immunostaining of both collagen type I and III delineated the collagen fibres of all urethral

connective tissue compartments suggesting co-localization of these collagens in the stromal connective tissue of the female canine urethra. No difference in the immunoreactivity and staining pattern of collagens I, III and IV as well as of α-actin could be detected between ovariectomized and control dogs. These findings suggest that ovariectomy does not seem to have an immunohistochemically ascertainable effect on the assessed urethral collagens and smooth muscle actin in the dog.

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