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Heinz R. Augsburger · Luis M. Cruz-Orive

Influence of ovariectomy on the canine striated external urethral sphincter (*M. urethralis*): a stereological analysis of slow and fast twitch fibres

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Abstract Slow and fast twitch fibres were stereologically analysed in the morphologically defined and most strongly developed ventral and ventrolateral region of the external urethral sphincter (*M. urethralis*) using five sexually intact anestrous and five ovariectomized nulliparous beagles. The estimated mean total cross-sectional area of the investigated part of the muscle consisted of 4.2 mm^2 (15.1%) type I fibre, 10.0 mm^2 (32.1%) type II fibre, and 16.3 mm^2 (52.8%) connective tissue in the control group. The corresponding absolute mean value of type I fibres (3.7 mm^2/13.5%) was statistically lower in ovariectomized animals. No significant difference between groups was observed in the relative number of transverse profiles of type I and II fibres; type I fibres comprised 23.8% and type II 76.2% of all muscle fibres in the sexually intact group, but 21.8% and 78.2% in the ovariectomy group, respectively. The ovariectomized dogs exhibited a statistically significant lower type I and II fibre number and a concomitant slightly larger mean single profile area (diameter) of fibre type II compared with the control animals. The significantly reduced number and decreased total cross-sectional area of the fatigue-resistant type I fibres in ovariectomized dogs suggest a predominant weakening of the fibre type I portion of the *M. urethralis* as consequence of ovariectomy. The effect could be mediated by sex hormonal factors and may contribute to the development of postspaying urinary incontinence in female dogs.

Key words Urethra · External sphincter · Urinary incontinence · Fibre type · Female dog · Stereology

H. R. Augsburger (✉)
Institute of Veterinary Anatomy,
University of Zürich, Winterthurerstrasse 260,
CH-8057 Zürich, Switzerland

L. M. Cruz-Orive
Faculty of Sciences, University of Cantabria,
E-39005 Santander, Spain

Introduction

The *M. urethralis* is the striated musculature of the female canine urethra. It is macroscopically confined to the distal third of the urethra and most strongly developed in the most distally located urethral quarter [4, 6, 7, 17], where the fibres of the *M. urethralis* surround the urethra transversely at its ventral and lateral aspects. From the middle of the distal urethral quarter caudally, these fibres pass dorsally and progressively onto the vagina. They are separated into groups of various sizes by thick connective tissue septa [5].

In sexually intact female beagles, the fibres of the ventral and ventrolateral region of the *M. urethralis* are composed (in number) of 24% type I (slow twitch) and 76% type II (fast twitch) fibres. In this paper, the term "fibre" is generally used as a synonym of "fibre profile" normal to the fibre axis. Thus, "fibre number" really refers to the number of fibre profiles in a section normal to the fibres. Two main subtype II fibres can be distinguished enzyme-histochemically as classical type IIA and a dog-specific (IIS) fibre with a similar oxidative potential [5]. As in other skeletal muscles of the dog, the classical type IIB fibres are absent [25, 34, 35, 50].

Among other urethral tissue components such as smooth musculature, vascular plexus and collagenic and elastic fibres, the striated musculature of the urethra is involved in the complex urethral closure mechanism that provides urinary continence [5, 8, 9, 12, 19, 20, 33, 40, 46, 52]. The predominance of fast twitch II fibres in the female canine *M. urethralis* suggests that this muscle is not primarily designed for sustained contracture but rather to prevent leakage of urine in case of sudden increase of abdominal and intravesical pressures [5]. Based on its slow contracting, fatigue-resistant type I fibres and the relatively high oxidative capacity of all type II fibres, the *M. urethralis* may also play a role in the urethral closure mechanism during rest.

A possible consequence of ovariectomy or ovariohysterectomy in dogs is urinary incontinence, observed

as early as immediately following surgery or up to 10 years later [10, 30, 38, 44, 45, 54]. This urological dysfunction shows some similarities to stress incontinence in women. In both conditions substantially lower urethral pressures are measured compared with continent individuals and hormonal factors are likely to be involved [3, 11, 28, 29, 41, 43, 51, 53]. The precise pathophysiological mechanism of these types of urinary incontinence has not been elucidated. One recent stereological analysis of the female urethra in sexually intact and spayed dogs suggests that ovariectomy leads to a general weakening of the urethral wall due to decreased smooth muscle mass and lower connective tissue content [7].

The present study was aimed at investigating the influence of ovariectomy on the female canine *M. urethralis*, the effect on its fibre-type composition, total cross-sectional area of fibre types I and II, as well as their mean diameters and absolute numbers. The functional implications of the results are discussed.

Materials and methods

Animals, muscle sampling and preparation

A homogenous group of 10 sexually intact nulliparous beagles in anoestrus, 1.5–3 years of age and weighing between 9 and 11.5 kg were used for this investigation. Five randomly chosen animals of this group were ovariectomized under general anaesthesia. The dogs were kept for 1 year and then killed with an overdose of pentobarbital. The urethra, including the urinary bladder, vestibulum vaginae and the distal vagina was removed from the pelvic cavity by a ventral approach through the pelvic floor. The tissue was sampled according to our definition of the target space. Ideally, the target structure would be the entire *M. urethralis*. To make the estimation of fibre number and size possible, however, we chose a relevant subset of this muscle which excluded nearly all fibres that do not run normal to the urethral axis. The subset was located at the most distal urethral quarter, and it was encompassed between two parallel cutting planes a quarter of the urethral length apart and normal to the urethral axis. The corresponding tissue cylinder was cut open; the target structure was centered at the ventral part (i.e. the lower part of the urethra) and it was comprised between two parallel planes 6 mm apart, normal to the urethral wall and parallel to the urethral axis. The stereological estimates were obtained from three systematic sections about 1 mm apart, parallel to the preceding two planes. The target structure therefore resembled a ''bricks'' one urethral quarter in length, 6 mm wide, and as thick as the whole muscle. Naturally, the sections were approximately normal to all muscle fibres, a necessary condition that would fail to be fulfilled if the target structure extended beyond the chosen limits. The tissue ''bricks'' were laid on a piece of plexiglass with their largest face dissected from the urethra down, and immediately frozen in melting 2-methylbutane previously cooled in liquid nitrogen. The samples were stored at −70°C until further processing.

Histochemical demonstration of mATPase activity

For demonstration of myofibrillar actomyosin ATPase (mATPase), serial cross-sections (10 μm) were cut with a Reichert-Jung Frigocut E cryostat microtome at −18 to −20°C. The sections were mounted on glass cover-slips, air-dried for 60 min and histochemically analysed. To differentiate type I and II fibres, a procedure modified after Brooke and Kaiser [13] was employed [5]. Following preincubation for 15 min at 21°C in the incubation medium adjusted to pH 10.35 with NaOH and 5 min washing in distilled water, the sections were incubated in 75 mM sodium barbital and 70 mM sodium acetate at pH 9.4 containing 0.1 M $CaCl_2$ and 2.7 mM ATP (Fluka, Buchs, Switzerland) for 30 min at 21°C. Sections were then rinsed twice with 0.2 M $CaCl_2$ for 5 min and subsequently immersed in 2% (w/v) $CaCl_2$ for another 5 min. After three successive 30-s washings with distilled water, they were treated with 1% (v/v) $(NH_4)_2S$. Prior to embedding in Eukitt (Kindler, Freiburg, Germany), the sections were washed five times for 1 min, dehydrated in ethanol and cleared in xylene.

Stereological analysis

Type I and type II fibres were stereologically analysed using the three longitudinal (i.e. parallel to the urethral axis, hence approximately normal to the fibres), 1 mm apart systematic 10-μm sections described above. The following target parameters were estimated (all obviously defined for the adopted reference subset of muscle):

1 Mean cross-sectional area of the investigated muscle region (including connective tissue) referred to as reference area A (ref), in mm^2
2 Volume fraction V_V (structure/ref) of all type I and type II fibres as well as connective tissue (CT) per reference volume (area), in per cent
3 Relative number of type I and type II fibre profiles N_N(I,II/I + II), in per cent
4 Average fibre profile area $\bar{a}$(I,II), in μm^2
5 Conventional average fibre diameter $\bar{D}$(I,II), in μm expressed as the diameter of the circular area $\bar{a}$(I,II)
6 Mean absolute analysed cross-sectional area A(structure) of total type I and II fibres as well as connective tissue, in mm^2 expressed as:

$$A\ (\text{structure}) = V_V(\text{structure/ref}) \cdot A(\text{ref}) \tag{1}$$

7 Number of fibre profiles I and II per area (mm^2) N_A (I,II/ref)
8 Total number of type I and type II fibre profiles N (I,II) expressed as:

$$N(\text{I,II}) = N_A(\text{I,II}) \cdot A(\text{ref}) \tag{2}$$

The mean absolute analysed cross-sectional muscle area was estimated by point counting (27, 56) in the three systematically obtained cross-sections. The microscopical section images were projected onto grid of regularly spaced test points $d = 1$ cm apart at a final magnification of $M = 14$, using a Wild Leitz Aristoplan microscope equipped with a mirror. The estimate of the total mean cross-sectional area was computed as:

$$\text{est}\,A(\text{ref}) = \frac{1}{3} \cdot \frac{d^2}{M^2} \cdot \sum_{i=1}^{3} P_i \tag{3}$$

where $\Sigma\ P_i$ denotes the total number of test points counted in the three sections.

For the estimation of relevant volume fractions (V_V) and the relative number of fibre profiles (N_N) the same sections were used at a final magnification of $M = 375$. Each section was subsampled by an approximately rectangular array pattern of 19–33 systematic microscopic fields, identical for all animals. Each field was projected onto test system composed of 36 regularly spaced ($d = 2.4$ cm) points and nine counting frames [26] within a 14.4-cm square frame. The 36 points were used to quantity each relevant structure. The 9 test points enclosed by the counting frames were employed for the reference space.

Fibre type and connective tissue volume fractions were estimated as follows:

$$\text{est}\,V_V(\text{structure/ref}) = \frac{1}{4} \cdot \frac{\sum P_i(\text{structure})}{\sum P_i(\text{ref})} \tag{4}$$

The multiplication factor of 1/4 corresponds to the test point ratio 9/36, ΣP_i represents the total number of points counted in the four compartments (fibres types I and II, connective tissue, reference

space) in the three sections. The absolute cross-sectional areas of the single structures, A(structure), were obtained using equation (1). The relative number of fibres I and II were estimated by the following equation:

$$\text{est } N_N(\text{I,II/I+II}) = \frac{\sum Q_i(\text{I,II})}{\sum Q_i(\text{I+II})} \quad (5)$$

where ΣQ_i denotes the total unbiased number of fibre profile captured by the counting frames in the three sections.

The average profile area ($\bar{a}$) of the two fibre types and their conventional mean diameter $\bar{D}$ were calculated according to the following equations:

$$\text{est } \bar{a}(\text{I,II}) = \frac{1}{4} \cdot \frac{d^2}{M^2} \cdot \frac{\sum P_i(\text{I,II})}{\sum Q_i(\text{I,II})} \rightarrow \bar{D} = \sqrt{\frac{4\bar{a}}{\pi}} \quad (6)$$

The number of fibre profiles per area was obtained as follows:

$$\text{est } N_A(\text{I,II/ref}) = \frac{M^2}{d^2} \cdot \frac{\sum Q_i(\text{I,II})}{\sum P_i(\text{ref})} \quad (7)$$

where ΣQ_i is as in equation (5), d^2 denotes the area of one of the nine counting frames used to count the fibres, and ΣP_i is the total number of test points in the centre of each of the nine frames hitting the reference space.

Statistical analysis

The estimated parameters of sexually intact and ovariectomized dogs were compared and statistically evaluated using the non-parametric Mann-Whitney U-test. Statistical tests with $P < 0.05$ were considered significant. Data are presented as means with the corresponding coefficient of variation (CV%) among animals.

Results

Following the preincubation at pH 10.35, the mATPase reaction allowed a clear differentiation between the light staining slow-twitch type I and the dark fast-twitch type II fibres (Fig. 1). The pertinent means of the compiled estimated parameters from the various components of the *M. urethralis* are presented in Table 1 and 2. The estimated mean total cross-sectional area (volume) of the striated musculature in the ventrolateral part of the most distally located urethral quarter in the control group amounted to 30.2 mm^2 and was composed of 16.3 mm^2 (52.8%) connective tissue, 4.6 mm^2 (15.1%) fibre type I and 10.0 mm^2 (32.1%) fibre type II profile area (Figs. 2, 3). Comparing these values with the measurements of the ovariectomized animals, it was noteworthy that the corresponding absolute means of this spayed group were lower without exception. However, only the difference in the total mean profile area of type I fibres proved to be statistically significant, $P < 0.05$ (Table 1).

The average number of fibres obtained per analysed muscle subset was 2039 in the sexually intact and 1448 in the spayed dogs. Type I fibres constituted 23.8% and type II 76.2% of all muscle fibres in the control group, whereas in the ovariectomized animals the mean relative numbers were 21.8% and 78.2%, respectively. The mean number of fibres per mm^2 in the sexually intact group amounted to 158.6 for type I and 509.2 for type II; the corresponding means in the spayed animals were lower

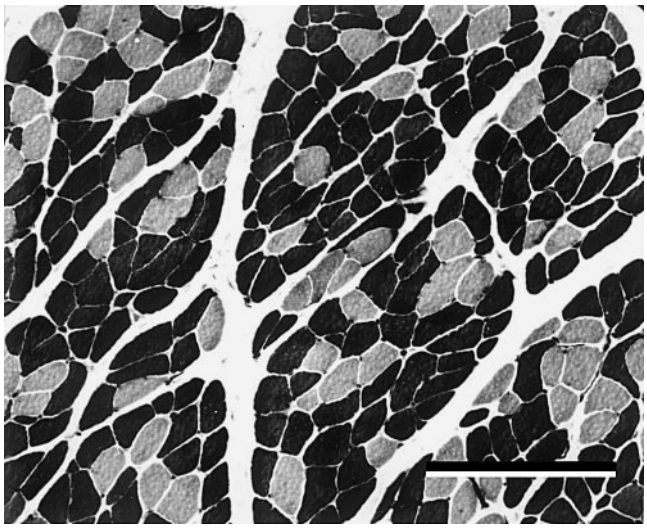

Fig. 1 Cross-section from the *M. urethralis* of a 2-year-old beagle stained for mATPase activity following alkaline preincubation at pH 10.35 with light-staining type I and dark-staining type II fibres. Bar 200 μm

(113.1 and 405.0, respectively) but only in the case of type I fibres of statistical relevance. The spayed dogs exhibited 35% lower total average type I fibre and a 27% lower type II fibre number, compared with the sexually intact group; both differences were significant ($P < 0.01$ and $P < 0.05$, respectively). In contrast, ovariectomy seemed to lead to a slight relative increase of the mean single profile area in both fibre types, whereby only the change of type II fibres was statistically relevant ($P < 0.05$). Accordingly, the spayed animals showed an increased mean diameter of 39.0 μm in type I and 31.8 μm in type II fibres, compared with 34.9 μm and 28.5 μm in the control dogs, respectively.

Discussion

The stereological and histochemical analysis of the fibre type composition of a striated muscle allows conclusions regarding its activity and function [1, 2, 14, 15, 16, 36, 39]. The female canine *M. urethralis* being composed of one quarter of type I and three quarters of type II fibres in the analysed part, seems not designed to maintain a high tonus over longer periods of time. Nevertheless, due to its slow contracting, fatigue-resistant type I fibres and the relatively high oxidative capacity of all type II fibres, the striated urethral musculature may also contribute to urinary continence at rest [5]. Contradictory data have been reported concerning the fibre type composition of the external urethral sphincter in women. Schroder and Reske-Nielsen [48] demonstrated a large proportion of type I and also some type II fibres. They suggest that type I fibres play a role in the maintenance of continence at rest, while type II fibres would be fully activated during stress conditions. In contrast, Gosling et al. [23] found exclusively fibres of type I,

Table 1 Mean values of estimated volume fractions (V_V) for type I and type II fibres and connective tissue (CT); absolute cross-sectional areas (A) of all type I and II fibres as well as CT; and absolute cross-sectional areas (A ref) of the M. urethralis in the ventral most distal urethral quarter of sexually intact and ovariectomized (spayed) female beagles ($n = 5$)

Groups ($n = 5$)	V_V(%)			A (mm^2)			A ref (mm^2)
	I	II	CT	I	II	CT	
Intact $\bar{x}$ (CV%)	15.1 (11.6)	32.1 (9.6)	52.8 (3.4)	4.6 (13.0)*	10.0 (21.2)	16.3 (12.6)	30.9 (14.2)
Spayed $\bar{x}$ (CV%)	13.5 (18.6)	32.1 (6.7)	54.4 (8.0)	3.7 (13.5)*	8.9 (8.1)	15.2 (17.3)	27.9 (11.5)

* Significant difference between corresponding means ($P < 0.05$)
Values in parentheses are coefficients of variation among all animals, given as a percentage (CV%)

Table 2 Mean values of estimated total number (N), relative number (N_N), average cross-sectional area ($\bar{a}$) and average diameter ($\bar{D}$) for type I and II fibres in the M. urethralis of sexually intact and ovariectomized (spayed) female beagles ($n = 5$)

Groups ($n = 5$)	N		N_N (%)		$\bar{a}$ (μm^2)		$\bar{D}$ (μm)	
	I	II	I	II	I	II	I	II
Intact $\bar{x}$ (CV%)	4904 (18.4)**	15744 (21.8)*	23.8 (17.7)	76.2 (5.6)	963.5 (16.4)	642.4 (20.0)*	34.9 (8.3)	28.5 (9.6)*
Spayed $\bar{x}$ (CV%)	1315 (13.2)**	11282 (14.1)*	21.8 (10.1)	78.2 (2.8)	1203.1 (22.3)	799.1 (15.3)*	39.0 (10.6)	31.8 (7.7)*

* Significant differences between corresponding means ($P < 0.05$) **($P < 0.01$)

which they thought were functionally capable of maintaining tone over a prolonged time without fatigue. Furthermore, Gosling [24] states that the striated urethral sphincter is the most important factor achieving urethral closure at rest, as urodynamic studies consistently demonstrate that maximum closure pressure normally occurs in the middle portion of the urethra, which corresponds to the location of maximum thickness of this muscle in women.

This study demonstrated a significantly lower estimated total fibre number of both types and a concomitant slight increase in fibre II size in the *M. urethralis* of ovariectomized, as compared with sexually intact beagles. The number of fatigue-resistant type I fibres was found to be decreased to a larger extent than that of type II fibres, with a significantly reduced total cross-sectional area. Furthermore, the slightly larger average diameter of type II fibres in spayed animals could cause a small increase of contraction power that may compensate to some extent for the reduction of fibre II numbers. This implies a predominant weakening of the type I fibre portion of the muscle, which is supposed to contribute to urinary continence at rest. This, in turn, may affect the closure mechanism of the urethra between micturitions. Kobori and Yamamuro [32] who studied the influence of ovariectomy on rat skeletal muscle found a significant increase in the size of all types of fibres in the soleus and caudofemoralis muscles of ovariectomized compared with sexually intact animals. In accordance with the present study the

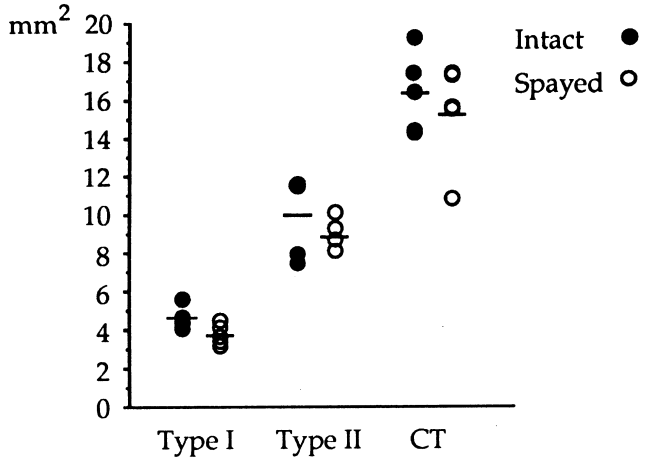

Fig. 2 Individual absolute total cross-sectional areas of type I and type II fibres and connective tissue (CT) of the *M. urethralis* in five sexually intact and five ovariectomized beagles. Bars represent corresponding means

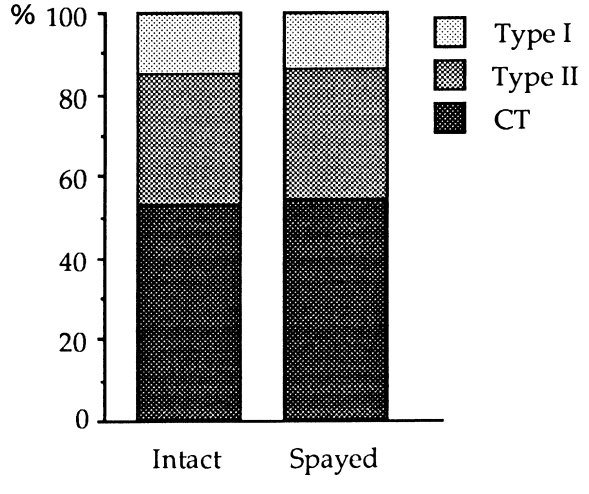

Fig. 3 Mean cross-sectional areas (volumes per unit fibre length) of type I and type II fibres and connective tissue (CT) of the *M. urethralis* in five sexually intact and five ovariectomized beagles (percentage of total)

change was most pronounced in type IIA fibres. Oestrogen administration resulted in a decrease in the size of all types of fibres in both male and female gonadectomized rats. The total number and composition of the different fibre types, however, was not affected by ovariectomy. In contrast, Knudsen et al. [31] reported a reduction in cross-sectional area of striated anal sphincter muscle tissue as a result of oophorectomy in rats. This was due to a reduced mean cross-sectional area of muscle fibres, whereas the total number of fibres was unchanged. The discrepancy between the results of these two reports may be due to the age of the animals at the time of ovariectomy and the duration of the experiment. Kobori and Yamamuro [32] ovariectomized their animals at the immature age of 3 weeks and killed them at 20 weeks. Whereas Knudsen et al. (1991) operated on mature rats at 6 months of age and killed them at 24 months. The contradictory results could also depend on the individual muscles concerned. The influence of ovariectomy on the size of striated muscle fibres may be mediated through hormone-binding receptors, which have been demonstrated in the levator ani muscles of rats [18] and women [49].

The involvement of hormonal factors is probably due to the fact that ovariectomy affects hormonal homeostasis. Nickel et al. [37] detected significantly decreased 17-β-oestradiol and progesterone blood levels following ovariectomy of anoestrous beagles. Richter and Ling [41], however, found no essential difference between oestrogen serum concentrations of anoestrous, sexually intact and spayed female dogs. In either case, ovariectomized animals lack periodical elevated blood oestrogen levels because of eased cycling.

Canine post-spaying incontinence as well as postmenopausal urinary stress incontinence in women are in some way related to hormonal factors, because estrogens have been effective in alleviating these urological disorders [10, 11, 21, 22, 47]. It is not known how ovariectomy effects a decline of muscle fibre numbers. Direct nerve damage during surgery can be excluded on anatomical grounds. This surgical intervention has been observed to change gonadotropin-releasing hormone neuronal morphology in the rat and rhesus monkey [42, 57]. Oestrogen, for example, induces axonal outgrowth in the nucleus retroambiguous-lumbosacral motoneuronal pathway in the adult female cat [55] showing that female sex steroids can influence motoneurons. It is imaginable that oestrogen depletion (ovariectomy) not only affects skeletal muscle directly [31, 32] but also, via the innervation, is what may influence the number of muscle fibres.

The significantly reduced number and the decreased total cross-sectional area of type I and the lower number of type II fibres in the *M. urethralis* of ovariectomized beagles compared with sexually intact animals suggest a weakening of the *M. urethralis* due to sex hormonal influences (e.g. oestrogen depletion). This will predominantly affect the fibre type I portion of the muscle because there is a smaller reduction of fibre type II numbers, which in addition would be compensated for to some extent by their slight increase in diameter. This, in turn, may affect the urethral closure mechanism at rest and contribute to the development of post-spaying urinary incontinence in dogs.

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