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Neutering affects urinary bladder function by different mechanisms in male and female dogs

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Abstract

Acquired urinary incontinence is a significant, incurable problem, prevalent in neutered bitches but rarely seen in entire bitches or males. Decreased urethral closure pressure has been proposed as a causative factor for altered detrusor contractility in the neutered bitch. In post menopausal women, acquired urinary incontinence is associated with acquired urinary incontinence and changes in collagen deposition within the bladder wall. The aim of this study was to determine effects of neutering on smooth muscle in the canine urinary bladder. Tissue bath studies were used to assess contractile function and morphometric analysis to determine percentage of collagen in the bladder wall from male and female, neutered and entire canines. Maximal response to both carbachol and neurogenic stimulation was significantly lower in bladder strips from neutered animals of both sexes. Sensitivity to carbachol was also significantly reduced by neutering in both sexes. The percentage of collagen was significantly higher in the bladder wall from neutered vs. entire females, which were similar to that of both neutered and entire males. Neutering a canine decreases urinary bladder responsiveness to muscarinic stimulation *in vitro*, in both sexes, but only increases the percentage of collagen in the bladder wall in females. While increased percentage collagen may predispose bitches to acquired urinary incontinence, the sex difference in this parameter indicates that more than one mechanism underlies the changes in bladder responsiveness seen following neutering. This alternative effect of neutering may be in the muscarinic receptor effector pathway and act as a therapeutic target for acquired urinary incontinence treatment.
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1. Introduction

Acquired urinary incontinence is a debilitating and so far incurable condition that causes significant welfare problems in affected individuals. Two populations have been identified that are particularly susceptible to acquired urinary incontinence, post menopausal women and spayed female canines. Information relating to the exact pathophysiology of the condition in these two groups is not well enough understood to allow determination of commonality of cause; however, both groups of affected individuals are subject to a reduction in gonadal steroids and associated hormonal changes. In the bitch, acquired urinary incontinence can occur anytime from one week after neutering and is associated with severe management problems which often lead to euthanasia of the animal. A better understanding of this condition and how it can be treated would thus have significant benefits with regard to animal welfare.

Urinary incontinence is defined as the involuntary leakage of urine (Abrams et al., 2002) and in the bitch, is an increasingly recognised clinical problem. Interestingly, acquired urinary incontinence is reported to affect approximately 20% of neutered bitches (Arnold et al., 1989) but is seen in less than 1% of intact bitches (Holt and Thrusfield, 1993) and is rarely reported in male canines regardless of gonadal status. A direct relationship between neutering and acquired urinary incontinence has been reported (Thrusfield, 1985) which is proposed

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to occur as a consequence of neurological (Thrusfield et al., 1998), vascular or hormonal changes, rather than mechanical damage of the lower urinary tract sustained during surgery (Gregory, 1994). Incontinence in the neutered bitch can be associated with a decrease in maximal urethral closure pressure (Holt, 1988; Rosin and Barsanti, 1981), however, as this is not a defining characteristic of acquired urinary incontinence, multiple causative factors are likely to be involved, not all of which will be a consequence of gonadectomy. Thus it has been hypothesized that factors in addition to those that decrease urethral closure pressure, lead to a bitch developing urinary incontinence after neutering. In post menopausal women, acquired urinary incontinence has been reported to be caused by impaired contractility of the bladder, either alone or in conjunction with acquired urinary incontinence (Elbadawi et al., 1993a,b) and similar results have been published for rats (Fleischmann et al., 2002; Zhu et al., 2001). These aspects of bladder function have not been reported in the canine. In addition, studies in humans suffering from idiopathic detrusor instability have also shown changes in collagen deposition within the bladder (Charlton et al., 1999; Chen et al., 2002), and it is hypothesized that this may also play a role in the development of urinary incontinence in these patients. This has been looked at in other species but again has not been studied in the canine.

In this study, to try to understand the factors that result in urinary incontinence in neutered canines, we investigated the effects of neutering on three aspects of the bladder wall that could affect its functionality. We studied responsiveness to the muscarinic receptor agonist carbachol and responsiveness to electrical field stimulation, both of which affect the muscarinic pathway, which is the primary pathway responsible for bladder emptying in the normal animal. We also examined the percentage of collagen within the bladder wall, due to its effects on bladder contractility and elasticity and thus the bladder's ability to relax and expand to store urine.

2. Materials and methods

2.1. Tissue harvesting

The study was approved by The University of Glasgow Veterinary School's ethical review committee. A total of 52 canines were included in the study, with a mean age of 6 years (range 1–14 years) and a mean weight of 24.4 kg (range 8–45 kg). The majority of dogs were cross bred, with no pedigree breeds appearing more than once.

In all cases, tissue was collected from canines euthanased (intravenous overdose of pentobarbatone), with full informed owner consent, for reasons other than scientific investigation. The majority of animals were destroyed for severe behavioural problems, the remainder for a number of different complaints, none of which involved the urinary system. In all cases a detailed history of each animal was taken and within 2 h of death, a gross postmortem study of the entire urinary system performed and urinary bladders harvested and stored at 4 °C in Krebs solution. Any animals with a history or signs of gross pathological urinary tract disease (e.g. tumours, cystitis) were excluded from the study. The animals were split into four groups depending on sex and gonadal status, entire males, entire females, neutered males and neutered females. Based on visual inspection, neutered animals were all assessed as being more than 6 months post gonadectomy. Two females within the neutered female group had been identified as suffering from acquired urinary incontinence based on clinical history.

2.2. Tissue preparation for tissue bath studies

Tissue was used within 48 h of collection as validation studies indicated that responses were maintained for 48 h (maximal response to carbachol 24 h 1.06±0.29 g/mg $n=5$, 48 h 1.02±0.34 g/mg $n=5$, 72 h 0.37±0.11 g/mg, $n=5$). Following removal of the lining urothelium, strips of smooth muscle (2 mm×10 mm) were dissected from the dome of the bladder. The strips were mounted under 4 g resting tension (the optimum tension demonstrated in previous length-tension studies) on a fixed hook. For neurogenic electrical field stimulation tissues were passed through an Ag–AgCl ring electrode. Tissues were maintained in a 15 ml organ bath bathed at 37 °C in Krebs solution (NaCl 118 mM, KCl 4.8 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 24 mM and glucose 11 mM) gassed with 95% O_2/5% CO_2. Tension was measured with a BIOPAC TSD125C isometric force transducer and displayed using the AcqKnowledge 3.8.1. Biopac data acquisition system (Linton Instruments). Tissues were allowed to equilibrate for 60 min before experiments were begun, during which time the resting tension was re-adjusted to 4 g if required. For all studies, a minimum of 2 muscle strips per animal were included.

2.3. Assessment of carbachol-induced contraction

To determine the response to the non-specific muscarinic agonist carbachol, cumulative concentration response curves to carbachol (1 nm–30 μM) were generated in strips of bladder smooth muscle.

2.4. Assessment of neurogenic contraction

Electrical field stimulation (0.5–100 Hz, 100 pulses) was delivered from a Digitimer Ltd MultiStim System-D330 stimulator at a pulse width of 0.5 ms and at supramaximal voltage. Stimulations were applied at five minute intervals to allow the establishment of normal resting tone between each stimulation. Frequency dependant contractions were observed. Confirmation that the responses were neurogenic was obtained by treatment of tissue strips with Tetrodotoxin (1 μM) which abolished the contractile response at all frequencies studied.

2.5. Morphometric analysis

Full thickness sections of bladder wall (2 cm×4 cm) were dissected from the lateral dome of the bladders of studied animals. Sections were fixed in 10% phosphate buffered formalin before dehydration and embedding in paraffin. Transverse

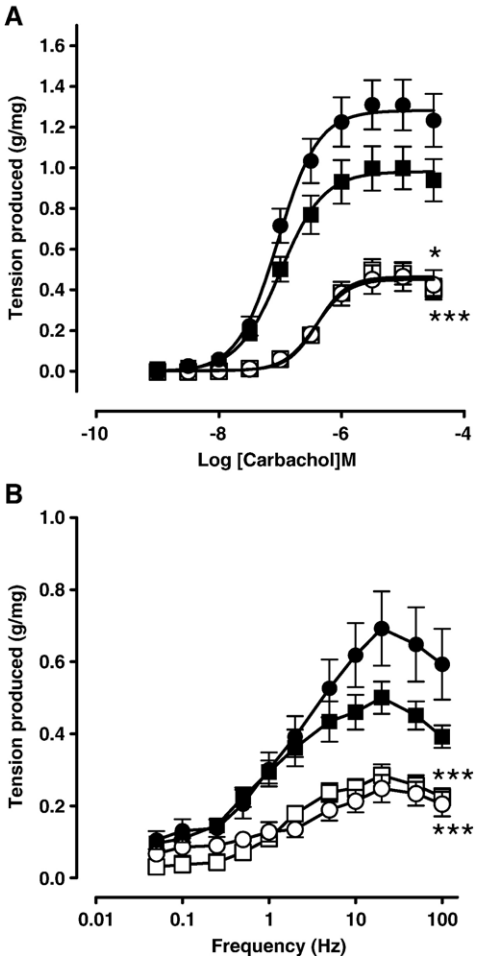

Fig. 1. *In vitro* detrusor muscle strip responses to muscarinic and electrical field stimulation. A. Cumulative concentration response curves to carbachol. B. Response to electrical field stimulation. ● Female entire, ○ F emale neuter, ■ Male entire, □ Male neuter. Data are mean±S.E.M., $n \geq 7$. Two way ANOVA was used to evaluate full curve data. *$P<0.01$, ***$P<0.001$ compared to entire animal of same gender.

tissue sections (2 μm) were cut using a Finesse Microtome, for Masson Trichrome Orange G staining. Morphometric evaluations were performed by light microscopic stereologic analysis, at a magnification of 40×, as reported previously (Bartsch et al., 1979; Hashimoto et al., 1999; Shapiro et al., 1991). At least five fields were evaluated for each bladder over 2 tissue sections; images were captured and analyzed using image analyses software (Leica, UK) to determine percentage of collagen.

2.6. Statistics

Results from all tissue bath studies were normalised relative to tissue weight and results presented as g/mg of wet tissue. For morphometric analysis mean percentage collagen was calculated for each animal. Data from all studies are expressed as mean±S.E.M. (n=number of dogs). Comparisons between groups were made using 2 way analysis of variance (ANOVA) with Bonferroni post-test. A probability (P) less than or equal to 0.05 was considered significant.

3. Results

3.1. Response to muscarinic stimulation

In all groups, concentration dependant contractions were observed in response to carbachol (Fig. 1A). Neutering was associated with a significant decrease in the maximum contractile response of isolated strips of bladder smooth muscle to carbachol in neutered compared to entire animals ($P<0.01$). This decrease in maximal response was accompanied by a significant decrease in sensitivity to carbachol, as measured by the $LogEC_{50}$ values ($P<0.001$) (Table 1). The maximum contractile responses for the females identified as suffering from acquired urinary incontinence were the lowest in the neutered female group (Canine A max response 0.23 g/mg: Canine B max response 0.18 g/mg) and were below the 95% CI (0.3395–0.6385) for the group. Multivariant analysis of the results indicated that there was no effect of gender, age or weight of the animal on responses to carbachol, and furthermore the effects of gonadal status were independent of gender.

3.2. Response to neurogenic field stimulation

In all groups, frequency dependant contractions were observed in isolated strips of bladder smooth muscle in response to electrical field stimulation, with maximum tension observed at 20 Hz. The effects of neutering on the response to neurogenic electrical field stimulation were similar to those of carbachol, with a significant decrease in the maximal contractile response in neutered, compared to entire animals, regardless of gender ($P<0.001$) (Fig. 1B). As with carbachol, there was no effect of age, weight or gender and no interaction between gender and gonadal status on the effects of neutering on the response to neurogenic field stimulation. The response of the two neutered females known to suffering from acquired urinary incontinence fell within the range of the remaining females in this group (Canine A max response 0.18 g/mg, Canine B max response 0.36 g/mg).

3.3. Percentage of collagen

Staining with Masons Trichrome Orange G allowed visualisation of collagen and smooth muscle in sections of bladder wall (Fig. 2A). The percentage of collagen in the bladder wall of entire male and female canines was similar. No difference in percentage collagen was observed in neutered

Table 1
Maximum and $LogEC_{50}$ values for carbachol

	ME (n=14)	MN (n=11)	FE (n=17)	FN (n=10)
Emax	1.00±0.11	0.50±0.053[a]	1.31±0.12	0.49±0.07[b]
Log EC_{50}	−7.00±0.01	−6.34±0.05[b]	−6.99±0.05	−6.42±0.06[b]

Maximum (Emax) and Log EC_{50} values for Carbachol in isolated strips of detrusor muscle from entire and neutered male and female dogs (ME, MN, FE and FN respectively). Data are expressed as g/mg of wet tissue with results given as mean±S.E.M. where n=number of animals. [a]$P<0.01$, [b]$P<0.001$ indicates different from entire of same gender.

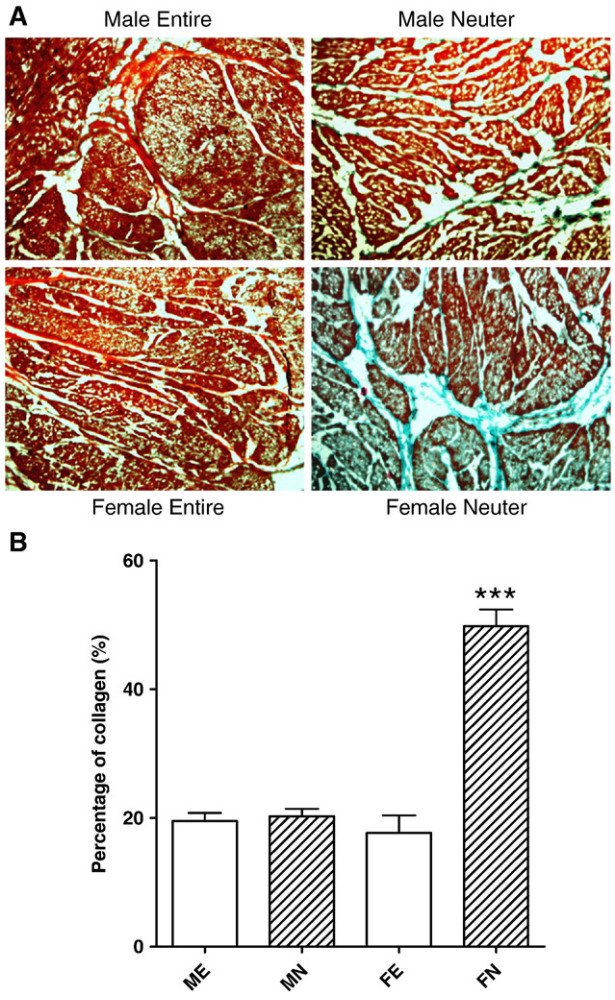

Fig. 2. The percentage of collagen in the wall of the urinary bladder. A. Representative sections of bladder wall from each group stained with Masson Trichrome Orange G to allow visualization of collagen (green) and smooth muscle (red). B. Summary data from entire and neutered male and female dogs. Data are mean ± S.E.M., $n \geq 8$. ***$P < 0.001$ compared to all other groups.

compared to entire males. In stark contrast, the percentage of collagen in neutered females was significantly increased ($P < 0.001$) relative to both the entire female and neutered male canines (Fig. 2B). The greater percentage of collagen in the bladder wall of the neutered female animals was due to greater collagen deposition both between and within muscle bundles in the bladder wall. The two acquired urinary incontinence neutered females had the highest collagen percentages within the group (57% and 59%), these values being above the 95% CI values (43.49–55.91) for the group.

4. Discussion

Our results demonstrate that following neutering, regardless of gender, there is a marked decreased in the magnitude of response of the bladder wall to both carbachol and electrical field stimulation. Furthermore, a decrease in sensitivity to carbachol was also observed in bladder tissue from neutered compared to entire animals. These changes could lead to a decrease in bladder muscle strength and contractility and thus could contribute to susceptibility to acquired urinary incontinence. Carbachol is a non-specific muscarinic agonist that causes contraction of the bladder via activation of the muscarinic pathways which are thought to be the primary pathways responsible for bladder emptying (Chess-Williams, 2002) and the pathway stimulated by electric field stimulation in our experiments (Creed et al., 1983; D'Agostino et al., 1989). The differences in function within these pathways in entire and neutered dogs observed in the current study could dramatically alter the bladder's ability to contract fully as a single functional unit and thus affect normal bladder function. This possibility is supported by our limited data from neutered female animals known to be suffering from acquired urinary incontinence whose data fell below the 95% CI for the group. This combined with other factors, could possibly explain why these particular animals were suffering from urinary incontinence. While the mechanisms underlying the observed changes in bladder responsiveness were not investigated in this study, a decrease in response to carbachol/electrical field stimulation could be due to a change(s) in the receptor effector pathway, such as the number and/or subtype of muscarinic receptors within the bladder or changes in intracellular signalling pathways. Further factors that may interact with the receptor effector pathway are the changes in sex hormone concentrations and receptor levels that occur post-neutering (Reichler and Welle, 2005; Welle et al., 2006).

The decrease in responsiveness of the detrusor muscle observed in bladder tissue from neutered canines in this study, is similar to that described in human patients suffering both from impaired contractility of the detrusor muscle (Resnick and Yalla, 1987) and idiopathic detrusor instability (Mills et al., 2000), conditions which are recognised as predisposing factors for urinary incontinence in post menopausal women. In these conditions there are changes in the functional properties of the urinary bladder characterised by either slow, weak contractions of the detrusor that lead to only partial emptying of the bladder and retention of urine (impaired contractility), or unstable bladder tone with partial contraction of the bladder during the storage phase of micturition which is out with patient control (idiopathic detrusor instability). We hypothesize that the decrease in response of the detrusor muscle in the canine shown in our study may cause similar functional conditions to those described above and therefore may be a causative factor for acquired urinary incontinence in the spayed bitch.

Our study demonstrated that changes in responsiveness occur in bladder smooth muscle in both neutered male and female canines. While the functional changes observed in this study could support the development of acquired urinary incontinence in the neutered bitch it is worthy of note that similar changes occurred in the bladder of neutered male dogs, which do not typically become incontinent. This sex difference in the propensity to develop acquired urinary incontinence could reflect an interaction between post-neutering effects on smooth muscle function and anatomical differences in the urethra of a male dog, that make the development of acquired urinary incontinence less likely. Namely, the increased urethral length and passage of the urethra through the penile structures in the male may provide a greater urethral closure pressure that counters

effects of neutering on the detrusor muscle and explain why urinary incontinence is less prevalent in neutered male dogs.

Gonadectomy, or more specifically ovariectomy, has been documented to result in structural changes in the bladder wall (Fleischmann et al., 2002; Hashimoto et al., 1999; Zhu et al., 2001) which could affect bladder function and thus contribute towards acquired urinary incontinence. These reported changes, however, are not consistent and include a decrease in relative smooth muscle content, and an increase in percentage of collagen, in rodents (Fleischmann et al., 2002; Zhu et al., 2001) and a decrease in the relative amount of collagen in the bladder in the rabbit (Hashimoto et al., 1999). While these differences in the effects of gonadectomy on bladder wall structure could be species specific, differences in the experimental designs mean that they could also reflect acute (Hashimoto et al., 1999) and chronic (Fleischmann et al., 2002; Zhu et al., 2001) effects of ovariectomy on smooth muscle structure. Our results are in general agreement with those obtained in the latter studies whereby neutered bitches have a greatly increased percentage of collagen within the bladder wall, and a concomitant decrease in the proportion of smooth muscle relative to entire bitches. The observed increase in collagen occurred both within and between the muscle bundles of the bladders of the neutered bitches and this has the potential to affect the bladder by decreasing conduction of action potentials throughout the muscle fascicle. This could lead to decreased contraction and/or compliance of the bladder (Fleischmann et al., 2002), and could alter the sensory threshold for cholinergic stimulation as seen in women suffering from acquired urinary incontinence (Charlton et al., 1999). The possibility that these changes in collagen may result in functional changes that predispose animals to acquired urinary incontinence is supported by our data for the neutered females suffering from acquired urinary incontinence which had the highest percentage collagen of all the animals studied, but the lowest responses to muscarinic stimulation.

The changes in muscle structure and percentage collagen described above have been proposed to be brought about by the changes in female reproductive hormones that occur after the menopause or ovariectomy (Thom and Brown, 1998). This conclusion is supported by data from the rat and rabbit where muscle structure was demonstrated to be affected by ovariectomy and supplemental oestrogen therapy (Fleischmann et al., 2002; Hashimoto et al., 1999; Zhu et al., 2001). These studies highlighted the role of oestrogen in the maintenance of the smooth muscle to collagen ratio within the female bladder, thus we could hypothesize that it is the removal of oestrogen that leads to the observed changes in percentage collagen demonstrated in the neutered bitch in this study. A complication relative to the proposal that the removal of oestrogen is key to the development of urinary incontinence, however,demonstrated is the clinical observation that oestrogen levels in an entire bitch are low for the majority of the year (Jeffcoate, 1993) but the entire bitch remains continent. Thus, while it is of interest that percentage collagen increases in the bitch bladder following neutering and this change may indeed contribute to acquired urinary incontinence, our functional results in both sexes indicate that some other mechanistic change must occur following gonadectomy. Specifically, whilst the post-neutering changes in percentage collagen are seen in females but not in males, the significant attenuation of contractility post-neutering is observed to a similar extent in bladder strips from both male and female canines.

In conclusion we have shown that neutering a canine, regardless of gender, is associated with a reduction in the response of the detrusor muscle to muscarinic and electrical field stimulation *in vitro*. We also demonstrate that only in bitches does a change in gonadal status result in a change in the percentage of collagen in the urinary bladder wall. This result would suggest that although loss of oestrogen may be responsible for the increased collagen content of the bladder of neutered bitches, it cannot be solely responsible for the changes in contractile function as had previously been postulated by studies that only looked at the effect of neutering on female animals. It is hypothesized that the changes to muscarinic and electrical field stimulation seen in neutered animals are due to an as yet unidentified mechanism involving the muscarinic receptor effector pathway, which could result in a condition similar to acquired urinary incontinence in human. The results from the neutered animals suffering from acquired urinary incontinence provide evidence that this may be an important predisposing factor in the development of acquired urinary incontinence in the bitch. The muscarinic pathway may, therefore, present a potential therapeutic target for treatment of this debilitating condition in the bitch, and an understanding of this pathway may help further our understanding of certain forms of the condition in the human model.

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