

## Effect of castration on male rabbit lower urinary tract tissue enzymes

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**Abstract** *Objectives* The influence of testosterone on the prostate and corpus cavernosum have been studied extensively. However, the influence of testosterone on the lower urinary tract (bladder and urethra) have not been investigated to any great extent. The aim of this study was to determine whether androgen deprivation alters lower urinary tract metabolism. *Methods* A total of 16 rabbits were divided into four groups of four rabbits each. Each rabbit in groups 1–3 underwent surgical bilateral castration for duration of 1, 2, and 4 weeks, and group 4 underwent sham operations. Sections of bladder body and base wall and mucosa, urethra and corpora were isolated, frozen, and stored under liquid nitrogen. The activ-

ities of citrate synthase-thapsigargin sensitive  $\text{Ca}^{2+}$  ATPase (Sarco/Endoplasmic Reticulum  $\text{Ca}^{2+}$  ATPase [SERCA]), and choline acetyl-transferase were examined as markers for mitochondrial function, sarcoplasmic reticular calcium storage and release, and cholinergic nerve function, respectively. *Results* The activity of SR function indicator,  $\text{Ca}^{2+}$  ATPase was significantly higher in the control corpora than in the control bladder or urethra. Castration resulted in decreased activity in the mitochondria specific enzyme, citrate synthase, the activity of which was greatest in the urethra and lowest in the corpora. Cholinergic nerve density indicator, choline acetyl-transferase activity was greatest in the bladder body and lowest in the urethra. *Conclusions* Our data indicate that (1) significant differences exist in the activities of all three enzymes in the various organs associated with the lower urinary tract; and (2) that castration results in significant alterations in the activities of all three enzymes in the bladder body, base, urethra, and corpora.

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### Introduction

Bladder dysfunction secondary to benign prostatic hyperplasia (BPH) is a common consequence in the aging male. The frequent urinary symptoms include the storage symptoms of urgency, frequency and nocturia and the voiding symptoms of incomplete emptying, intermittency, weak stream, and voiding with straining. It is well-known that prostate enlargement is androgen

dependent, and the effect of prostate enlargement induced partial bladder outlet obstruction is extensively studied in many animal models [1–3]. Clinically, the  $\alpha$ -blocker and anti-androgen medications are widely used as treatments for BPH and lower urinary tract syndromes (LUTS) in the aging males. However, the impact of androgen on lower urinary tract function and biochemistry is not well-known. In clinical practice, the size of the prostate does not always correlate with the severity of urinary symptoms. Some men with greatly enlarged glands may have little obstruction and few symptoms while others with prostate glands less enlarged have more outlet blockage and severe problems. By immunohistochemical methods, it has been proved that estrogen receptor and androgen receptor are widely distributed in the bladder, proximal urethra and autonomic ganglia of the prostatic plexus, suggesting the importance of sex hormone in regulating lower urinary tract function [4].

In the rabbit bladder outlet obstruction study, bladder dysfunction is mainly mediated by three cellular processes, including (1) progressive denervation, (2) cellular mitochondria malfunction, and (3) dysregulation of intracellular calcium storage and release from the sarcoplasmic reticulum (SR) [3]. Biomarkers for these three functions are calcium ATPase for calcium regulation; citrate synthase for mitochondrial function, and choline acetyl-transferase for cholinergic innervation. The aim of this study is to determine whether androgen deprivation alters the activities of these biomarkers in the various organs of the lower urinary tract, namely, bladder body, bladder base muscle and mucosa, urethra and corpora.

## Materials and methods

These studies were approved by the Institutional Animal Care and Use Committee of the Stratton Affairs Medical Center, Albany, NY.

### Animal preparation

A total of 16 adult male rabbits were divided into four groups of 4 rabbits each. Each rabbit was anesthetized with isoflurane (1–3%). The rabbits in groups 1–3 were bilaterally castrated through two small incisions, which were then closed with 3–0 silk. The rabbits in group 4 were sham operated.

The rabbits in groups 1–3 were anesthetized at 1, 2, and 4 weeks following castration. The following tissues were surgically excised, frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until analyzed: bladder body muscle

and mucosa; bladder base muscle and mucosa, urethra, and corpus cavernosum. The mucosa of the urethra could not be separated from the muscle. The rabbits were euthanized immediately following surgery. The rabbits in group 4 were anesthetized one rabbit with group 1, two rabbits with group 2, and one rabbit with group 3. Tissues were removed as described above and the rabbits euthanized. The sham rabbits showed no significant differences and were combined into one group.

### Mitochondrial function

#### *Citrate synthase (CS) assay* [5]

Frozen tissue samples are homogenized in ice-cold Tris buffer (50 mM, pH 7.6) at 50 mg/ml and centrifuged at 2500g for 10 min to remove the cell membranes and nuclei. A sample aliquot (100  $\mu\text{l}$ ) of supernate is added to a 0.5 cm cuvette, along with 1.0 ml 0.05 M Tris buffer (pH 7.6), 50  $\mu\text{l}$  0.2–10 mM oxaloacetate (substrate), 30  $\mu\text{l}$  12.3 mM acetyl-coenzyme A, 100  $\mu\text{l}$  1 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and 100  $\mu\text{l}$  10% Triton X-100. The free coenzyme-A generated by CS activity reacts with DTNB to form a colored compound that is quantified at 412 nm. Absorbance is recorded every 30 s for 6 min (reaching steady state), using a Hitachi spectrophotometer. Protein concentration is determined using the Lowry method. Citrate synthase activity is given as nmoles Coenzyme-A generated per min. per mg protein.

### Sarcoplasmic reticulum (SR) function

#### *Ca<sup>2+</sup> ATPase Activity* [6]

SR function is evaluated by measuring thapsigargin-sensitive calcium ATPase activity (SERCA) activity. Frozen tissue samples are homogenized at 10 mg/ml in ice-cold Tris buffer (50 mM, pH 7.4) and centrifuged at 2,500g for 10 min to remove the cell membranes and nuclei. Aliquots of particulate preparations are incubated at  $37^{\circ}\text{C}$  in TRIS buffer with 4 mM ATP (substrate) and  $\text{CaCl}_2$  (1 mM). The reaction system for a typical assay designed to measure ATPase activity is as follows: 75 mM TRIS, 0.25–4 mM ATP, 4 mM  $\text{MgCl}_2$ , 100  $\mu\text{l}$  tissue extract (pH 7.4; total volume 2.0 ml). At the end of the incubation (e.g. 30 min) the reaction is stopped by adding 1 ml 12.5% TCA. Reaction tubes are centrifuged at 1,000g for 20 min to remove protein precipitates, and inorganic phosphate is determined. Inorganic phosphate present in the ATP reagent and in

the tissue extracts is measured initially, and then subtracted from total inorganic phosphate. Results are expressed as  $\mu\text{mol phosphate/mg protein/min}$ . Thapsigargin inhibition is assessed by incubating the reaction system in the absence and presence of  $10 \mu\text{M}$  thapsigargin; % inhibition of activity is calculated.

### Cholinergic nerve density

#### *Choline acetyl-transferase (ChAT) activity [7]*

Frozen tissue samples are homogenized in ice-cold phosphate buffer (50 mM, pH 7.4) containing 10 mM EDTA and centrifuged at 20,000g for 30 min to remove the cell membranes, mitochondria, and nuclei. Aliquots are incubated at  $37^\circ\text{C}$  for 10 and 20 min with 200  $\mu\text{l}$  of reaction mixture consisting of 0.2–10 mM acetyl-coenzyme A (acetyl-CoA) (substrate), 0.2 mM  $^3\text{H-acetyl-CoA}$  (200 mCi/mmol), 8 mM choline, 50 mM sodium phosphate, 0.3 M NaCl, 20 mM EDTA, and 96 nM physostigmine. After incubation, each solution is diluted with 5 ml of 0.01 M sodium phosphate and the reaction stopped with 2 ml of acetonitrile containing 5 mg/ml tetraphenylboron. Then, the contents of each reaction tube are transferred to a 20 ml scintillation vial and 10 ml of Betamax scintillation fluid are added slowly to each vial. The vials are shaken lightly. Samples stand for 1 h while the phases separate, extracting acetylcholine ( $^3\text{H-Ach}$ ) into the Betamax phase, while  $^3\text{H-acetyl-CoA}$  stays in the aqueous phase. The aqueous phase is removed and  $^3\text{H-ACh}$  is measured using scintillation spectroscopy. ChAT activity is reported as fmoles Acetyl-Co-A generated per min per mg protein/30 min.

### Data analysis

All values are presented as mean  $\pm$  SEM. Analysis was performed by Analyses of Variance and Bonferonni

test for individual differences. A  $P < 0.05$  was required for statistical significance.

## Results

**Bladder weight:** Bladder weight decreased progressively, after 2 weeks of castration reached statistical significance compared to the control (sham) group. The weights (in grams) for the groups were:  $3.0 \pm 1.0$  (control)  $2.5 \pm 0.4$  (1 Wk castrated);  $2.1 \pm 0.36^*$  (2 wk castrated); and  $2.2 \pm 0.18^*$  (4 wk castrated). \* = significantly different from control,  $P < 0.05$ .

The basal activities of the three enzymes are presented in Table 1. The calcium ATPase activity of the corpora was significantly greater than all other tissues; whereas the corpora had the lowest citrate synthase activity. The citrate synthase activity of the mucosa of the bladder body and base were significantly greater than the activities of the bladder body and base muscle. The ChAT activity of the urethra was significantly lower than the activities of the bladder body and base. Interestingly, the corpora had a surprisingly high activity of ChAT.

### Effect of castration

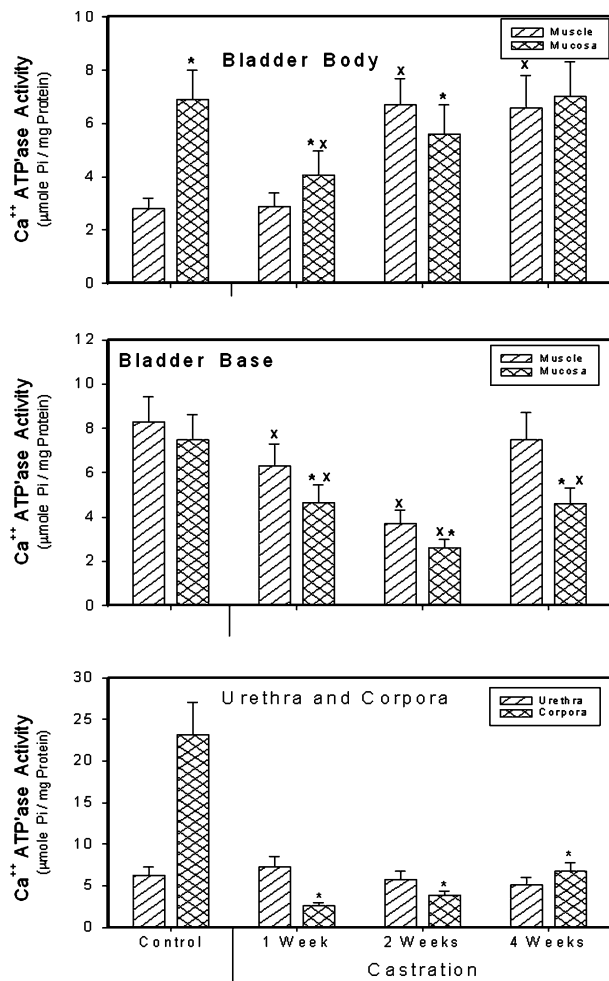
#### *Calcium ATPase activity*

Figure 1 shows the effect of castration on  $\text{Ca}^{2+}$  ATPase activity. The activity of the bladder body mucosa was significantly greater than the activity than bladder body muscle layer. The activities of body muscle increased by 2 weeks following castration and remained elevated at 4 weeks. The body mucosa significantly decreased at 1 week following castration and increased to control activity by 4 weeks. For the bladder base, the activity decreased progressively at 1 and 2 weeks following castration. By 4 weeks, the activity of the base muscle increased to control values, whereas the activity of the

**Table 1** Basal activities of calcium ATPase, citrate synthase, and choline acetyltransferase in lower urinary tract tissues obtained from control male rabbits

	$\text{Ca}^{2+}$ ATPase $\mu\text{molPi/mg protein}$	Citrate Synthase nmol Co-A/min/mg protein	ChAT fmol Acetyl Co-A / 30 min
Body muscle	$2.8 \pm 0.5$	$129 \pm 19$	$743 \pm 110$
Body mucosa	$6.9 \pm 1.0^*$	$261 \pm 39^*$	
Base muscle	$8.3 \pm 1.0^+$	$175 \pm 26^+$	$550 \pm 100$
Base mucosa	$7.5 \pm 1.2$	$263 \pm 39^*$	
Urethra	$6.2 \pm 0.9$	$342 \pm 51^x$	$388 \pm 80^a$
Corpora	$23.0 \pm 4.0^x$	$44 \pm 6^x$	$469 \pm 48$

\* = significantly different from muscle; + = bladder base significantly different from body; <sup>a</sup> = significantly different from bladder body and base; x = significantly different from all other tissues,  $P < 0.05$ . ChAT was not performed on mucosa

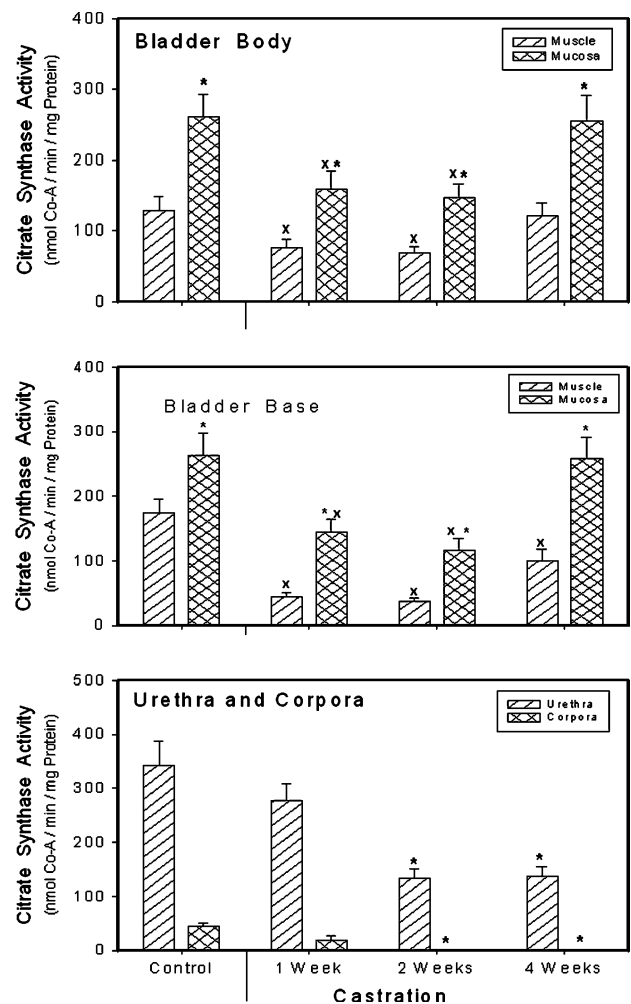


**Fig. 1** Effect of castration on calcium ATPase (SERCA) Each bar is the mean  $\pm$  SEM of four individual animals. \* = significantly different from muscle; x = significantly different from control,  $P < 0.05$

base mucosa was still significantly lower than that of the muscle. Castration had no effect on the calcium ATPase activity of the urethra, whereas the activity of the corpora decreased significantly by 1 week following castration to 20% of control activity and remained low throughout the 4-week study.

#### Citrate synthase activity

Figure 2 presents the effect of castration on citrate synthase activity. The citrate synthase activity of the bladder body and base, muscle and mucosa decreased over the first week following castration, remained low at 2 weeks and then returned toward control values by 4 weeks following castration. The citrate synthase activities of the urethra and corpora progressively decreased over the course of the 4 weeks study such that at 4 weeks following castration the activity of the urethra was approximately 1/3 of the control values



**Fig. 2** Effect of castration on citrate synthase. Each bar is the mean  $\pm$  SEM of four individual animals. \* = significantly different from muscle; x = significantly different from control,  $P < 0.05$

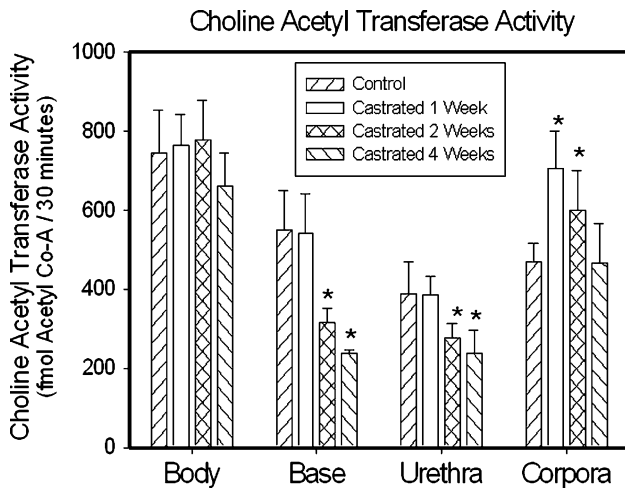
and the activity of the corpora fell below detectable levels.

#### Choline acetyl-transferase activity

The effect of castration on ChAT activity is presented in Fig. 3. Castration had no significant effect on the ChAT activity of the bladder body, whereas the activities of the base and urethra were significantly reduced at both 2 and 4 weeks following castration. Surprisingly, the activity of the corpora increased at 1 and 2 weeks following castration and returned to control values by 4 weeks after castration.

#### Discussion

The basic function of bladder is to store urine in a low intravesical pressure and efficiently expel the urine



**Fig. 3** Effect of castration on choline acetyl transferase. Each bar is the mean  $\pm$  SEM of four individual animals. \* = significantly different from control,  $P < 0.05$

after the bladder is full. Lower urinary tract symptoms (LUTS), including frequency, urgency, and nocturia are major problems for the aging male population. It has been estimated that approximately 30% of men aged over 50 have mild to severe micturition problems. Bladder dysfunction, including smooth muscle hyperplasia and contractile dysfunction in aging male was considered as a consequence of BPH. However, in aging men, the testosterone levels also declines as a result of both decrease in Leydig cell mass and alteration of the hypothalamic-pituitary-gonadal axis [8]. Secretion of the testosterone is regulated by a precise positive and negative biofeedback interaction in the hypothalamic-pituitary-gonadal axis. In addition, the testosterone can be converted to other hormones by several enzymes, to dihydrotestosterone (DHT) by 5 $\alpha$ -reductase and to estrogen by aromatase. The action of the testosterone is mediated by the androgen receptor located in the cellular nucleus [9]. Comparing with the female menopause, the decline in testosterone in aging male occurs gradually and had few acute clinical manifestations. However, long-term androgen deficiency (as may occur during the treatment of androgen-sensitive prostate cancer) are characterized by decreased muscle mass and strength, increase fat mass, impaired cognitive function, and osteoporosis [10, 11].

Despite androgens' effect on central and peripheral nervous system which are well-known, the local effect of androgen on bladder and urethra has not been extensively studied [12–15]. Morita et al. reported that  $\alpha$ 1-adrenergic and muscarinic cholinergic receptor densities decreased significantly after castration and were not affected by estrogen administration [16].

$\beta$ -Adrenergic receptors were not affected either by castration or estrogen administration [16].

The indicator of cholinergic innervation, choline acetyl-transferase activity, significantly decreased in bladder base and urethra after 2 weeks of castration. This finding can concur with the previous observation that a decrease in extracts of hypogastric ganglion taken from castrated rats [12] and indicating the importance of testosterone in maintaining cholinergic nerve function. A change in the cholinergic to adrenergic nerve ratio in the bladder base and urethra would be expected to enhance bladder emptying during micturition, in that during cholinergic stimulation which is what generates tension in the bladder during micturition would have a relatively lower contractile effect on the bladder base and urethra, thus reducing urethral resistance during micturition.

The choline acetyl-transferase activity in corpora increased after 1 week of castration and progressively returned to control level after 4 weeks. Testosterone enhances the erectile response of cavernous nerve stimulation. The sites of action for testosterone or its metabolites are situated on neurons rather than on penile erectile tissue. Postganglionic parasympathetic neurons seem to be the exact target for androgen steroids [17]. However, the exact cause of this phenomenon is still unclear and may need further experiment to investigate this observation.

Bladder contraction can be divided into phasic and tonic period, the phasic response depends on the adenosine triphosphate (ATP) concentration in the bladder wall, whereas the tonic phase requires mitochondrial oxidative activity to generate energy [18]. Mitochondria also have a critical role in Ca<sup>2+</sup> buffering in bladder smooth muscle [19]. There are some evidences suggesting that low testosterone level may be associated with insulin resistance and decreased expression of genes involved in oxidative metabolism in mitochondria [20]. Mitochondrial function was evaluated by the mitochondrial marker enzyme citrate synthase. We observed that citrate synthase activity decreased significantly after castration in all of the lower urinary tract, including bladder body, base, urethra, and especially in corpora. However, interestingly, the citrate synthase activity of the bladder body and base returned toward control activity by 4 weeks following castration whereas in the urethra and corpora, the citrate synthase activity continued to decrease. This would indicate that in regards to micturition, castration would significantly decrease high-energy phosphate generation for the urethra and corpora, which would decrease the ability of the urethra to sustain tension during bladder filling and might be significantly in-

volved in the increased incidence of incontinence in aging men; especially following TURP or other prostatic surgery.

Consistent with previous studies, the bladder mucosa has a significantly higher activity than the muscle in both bladder body and bladder base. This goes along with the demonstrations that the mucosa has a significantly higher metabolic rate and high-energy phosphate turnover than the muscle.

In rat animal studies, DHT treatment will increase micturition frequency and increase the incidence of unstable detrusor contractions [21, 22]. Smooth muscle contractile activity is regulated by intracellular  $\text{Ca}^{2+}$  concentration. The sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA), plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA), and plasmalemmal  $\text{Na}^+/\text{Ca}^{2+}$  exchanger are responsible for lower intracellular  $\text{Ca}^{2+}$ , which lead to smooth muscle relaxation [23]. By thapsigargin-sensitive  $\text{Ca}^{2+}$ -ATPase enzyme activity assays, we determined the SERCA activity in urinary bladder, urethra, and corpus cavernosum after castration.

Interestingly, the mucosa of the bladder body has a significantly higher SERCA activity than the muscle compartment, whereas in the bladder base, the activities of the mucosa and muscle are approximately equal, and equal to the mucosa activity of the body. The SERCA activity of the urethral smooth muscle is approximately equal to the activity of the muscle of the bladder base. This may be related to the fact that during bladder filling (representing over 90 percent of the micturition cycle), both the urethra and bladder base are under tonic alpha adrenergic stimulation and must maintain active tension, whereas during bladder filling the bladder body is under beta adrenergic stimulation and maintains a relaxed state to allow for filling at low intravesical pressures. The activity of the corpora was surprisingly high. This may be related to the fact that during erection, the corpora smooth muscle needs to maintain a relaxed state and thus needs to have a well-defined calcium storage capacity, which would be directly related to the high SERCA activity.

The SERCA activity in bladder body wall is significantly increased after 2 weeks of castration, indicating that bladder will increase relaxation after castration. This phenomenon also demonstrates that the effect of testosterone in inhibiting smooth muscle contraction is calcium ion dependent. The SERCA activity significantly decreased after castration in corpus cavernosum, pointing out the importance of testosterone in corporal myocyte relaxation and related to erectile dysfunction after testosterone deficiency.

It is interesting to note that the four tissues studied responded very differently to castration. For the

bladder body, no change was seen in regard to choline acetyl transferase activity. For both citrate synthase and SERCA, whereas the activity fell immediately following castration, the activities returned toward normal by 4 weeks following castration for both muscle and mucosa. In regard to the bladder base, choline acetyl-transferase decreased throughout the castration period, whereas for both citrate synthase and SERCA the activities decreased immediately then recovered toward control by 4 weeks following castration. In regard to the urethra, the SERCA activity did not change following castration whereas both citrate synthase and choline acetyl-transferase decreased throughout the castration period. For the corpora tissue, the choline acetyl-transferase increased then returned to control activity by 4 weeks; whereas for both SERCA and citrate synthase the activities continually decreased throughout the castration period. One last point, even though the net activities of the bladder body and base muscle and mucosa had different activities, castration had very similar effects on both tissues.

## Conclusions

Our data clearly demonstrate that castration (androgen deprivation) has significant short-term effects on three important enzymes involved with lower urinary tract muscle and mucosal function and are consistent with the known effects of testosterone and androgen deprivation on bladder, urethral, and corporal function. Future studies will be directed at looking at the long-term effects of androgen deprivation.

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