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Effect of aging on the response of biochemical markers in the rabbit subjected to short-term partial bladder obstruction

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Abstract *Purpose* Partial bladder outlet obstruction (PBOO) results in marked biochemical alterations in the bladder. In this study, we focused on comparison of thapsigargin sensitive sarco/endoplasmic reticulum Ca^{2+} ATPase activity (SERCA) and Citrate Synthase after short term PBOO in young versus old rabbits. *Materials and methods* A total of 20 young and 20 mature male rabbits were divided into 4 sub-groups of 5 rabbits each (4 obstructed and 1 sham-control rabbit). The rabbits in the groups were evaluated after 1, 3, 7, and 14 days of obstruction, respectively. The activities of SERCA and citrate synthase were examined as markers for sarcoplasmic reticular calcium storage and release and mitochondrial function, respectively. *Results* The SERCA activity of bladder body smooth muscle in the young animals increased at 7 and 14 days. For the old rabbits, the SERCA activity decreased significantly by 1 day and remained this level throughout the course of obstruction, and was significantly lower than young at all time periods. The citrate synthase activity in the young animals decreased over the 1–7 days, and then returned toward control level by 14 days following obstruction. In the old animals, citrate synthase activity of bladder body smooth muscle progressively decreased over the course of the study, and was significantly lower in the old than the young animals after 14 days obstructed. *Conclusion* The urinary bladders of the young rabbits have a considerable greater ability to adapt to PBOO than do those of the old rabbits. The deterioration of mitochondrial and SR function may be important mechanisms underlying geriatric voiding dysfunction.

Keywords Bladder · Citrate synthase · SERCA · Obstruction · Mitochondria · Sarcoplasmic reticulum

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Introduction

The urinary bladder is a smooth muscle organ whose function is to collect and store urine at low intravesical pressure and then expel urine via a highly coordinated, sustain contraction [1, 2]. Detrusor contraction depends on an increase in cytosolic Ca^{2+} concentration, which can result from release of intracellular Ca^{2+} stored in sarcoplasmic reticulum (SR) through channels in the SR membrane known as inositol trisphosphate receptors and ryanodine receptors and/or influx of extracellular Ca^{2+} through sarcolemmal calcium channels. Increased Ca^{2+} concentration activates myosin light chain kinase (MLCK), which phosphorylates the regulatory myosin light chain,

that in turn results in contraction [3–5]. The rate and magnitude of pressure generation requires the activation of actin and myosin with Ca^{2+}, and net breakdown of cytosolic ATP [6].

With age, the bladder, like all vital organs, shows alteration in many functions. Some of these are a reduction in bladder capacity, increased incidence of uninhibited contractions, decreased urinary flow rate, abnormal urethral pressure profile, and increased residual volume. Few studies have focused on understanding the pathophysiologic mechanisms underlying symptoms in the aging bladder [7–9]. Yet, these natural changes with age are particularly important because the aging male bladder is also often exposed to obstruction in addition (from prostatic enlargement).

Bladder function and dysfunction is mainly mediated by cellular processes, including dysregulation of intracellular calcium storage and release from the sarcoplasmic reticulum (SR) and cellular mitochondria malfunction [10, 11]. Biomarkers for these functions are thapsigargin sensitive calcium ATPase for calcium storage and release from the SR (SERCA) [12], and citrate synthase for mitochondrial oxidative metabolism [13]. The aim of this study is to determine the effect of aging on these cellular activities after short-term PBOO.

Materials and methods

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

A total of 20 young (1 month old) and 20 mature (1 year old) male New Zealand white rabbits were divided into 4 sub-groups of 5 rabbits each. 4 rabbits in each group underwent partial outlet obstruction (2,12,13). Briefly, each rabbit is anesthetized with isoflorane and the bladder catheterized. The bladder base and urethra are exposed through a central lower abdominal incision. The fat is cleared by blunt dissection and a 2–0 silk ligature is placed under the blood vessels and around the catheterized urethra. The catheter is removed and the wound closed in layers.

The other rabbit in each group underwent sham operations and they served as the control group. The rabbits in each group were evaluated after 1, 3, 7, and 14 days of obstruction, respectively. At the end of the experiment period each rabbit was anesthetized and bladder excised rapidly and divided into body and base at the level of ureteral orifices. The animal was euthanized with 2 ml Fatal Plus euthanasia fluid (Vortech Pharmaceutical, Dearborn, Michigan) intravenously. The mucosa was separated from muscularis by blunt dissection and tissues were frozen in liquid nitrogen and stored at −70°C until analyzed. Body mucosa and muscle were analyzed separately.

Physiologic study

The bladder was opened longitudinally and 3 full thickness strips (with intact urothelium) at 2 mm × 10 mm were placed in separate organ baths containing 15 ml Tyrode's solution at 37°C, and equilibrated with a mixture of 95% O_2 and 5% CO_2. One end of each strip was connected to an isometric force transducer (Grass Instruments, Quincy, Massachusetts), and tension changes were measured and recorded with a 7D Polygraph recorder (Grass Instruments).

Each strip was equilibrated for 30 min at 2 gm resting tension. After equilibration field stimulation (FS) was applied at 32 Hz through 2 platinum electrodes set on either side of the muscle strip in the organ bath with an S-88 field stimulator (Grass Instruments) delivering 80 V square wave pulses 1 ms in duration with 20-s trains. The interval between stimulations was 3 min.

Citrate synthase assay (mitochondrial function) [13]

Frozen tissue samples were homogenized in ice-cold Tris buffer (50 mM, pH 7.6) at 50 mg/ml and centrifuged at 2,500g for 10 min to remove the cell membranes and nuclei. A sample aliquot (100 µl) of supernate was added to a 0.5 cm cuvette, along with 1.0 ml 0.05 M Tris buffer (pH 7.6), 50 µl 0.2–10 mM oxaloacetate (substrate), 30 µl 12.3 mM acetyl-coenzyme-A, 100 µl 1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and 100 µl 10% Triton X-100. The free coenzyme-A generated by citrate synthase activity reacted with DTNB to form a colored compound that was quantified at 412 nm. Absorbance was recorded every 30 s for 6 min (reaching steady state), using a Hitachi spectrophotometer. Protein concentration was determined using the Lowry method. Citrate synthase activity is given as nmoles Coenzyme-A generated per min per mg protein.

Ca^{2+} ATPase Activity (Sarcoplasmic reticulum function) [12]

SR function was evaluated by measuring thapsigargin sensitive calcium ATPase activity (SERCA) activity. Frozen tissue samples were 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 were incubated at 37°C

in TRIS buffer with 4 mM ATP (substrate) and $CaCl_2$ (1 mM). Complete details of the methods are given in the references. The reaction was measured in the presence and absence of 10 μM thapsigargin. Thapsigargin specifically inhibits the calcium-ATPase localized in the SR membrane (SERCA) and thus the activity of SERCA was quantitated by subtracting the activity in the presence of thapsigargin from the total activity.

Statistical analysis

All values are presented as the mean ± SEM with $P < 0.05$ considered statistically significant. Analysis for comparative purposes was performed by Analyses of Variance and Bonferonni test for individual differences.

Results

The data from all control animals showed no significant differences and were combined into one group. Figure 1 shows relative change of body weight/bladder weight ratios. Control young bladder weight/body weight ratio was 1.2 ± 0.3 gm/1.77 ± 0.08 kg; control old bladder weight/body weight ratio was 2.6 ± 0.5 gm/3.8 ± 0.9 kg. Bladder/body weight ratios for young rabbits increased between 1 and 7 days obstruction and came back toward control levels at 14 days obstruction. In old rabbits, bladder/body weight ratios increased after obstruction reaching a maximum at 3 days and remaining at this level throughout the 14 days. The bladder body weight ratios of the young rabbits increased at a significantly faster rate over the first 7 days than did the bladder/body weight ratios of the old rabbits. Interestingly, the actual mass of the hypertrophied bladders for both the young and old increased to the same weights, although the relative increase was significantly greater for the young rabbits.

The contractile response to FS normalized to 100% control is given in Fig. 2. The contractile responses of the young and old to 32 Hz FS were 18.1 ± 3.1 and 16.0 ± 3.0 gm/100 mg tissue, respectively. For the young and old rabbits, the responses to 32 Hz stimulation were significantly reduced at 1, 3, and 7 days of obstruction. In contrast, the response of the young rabbits increased significantly at 14 days whereas the response of the old rabbits remained low.

The basal activities of both enzymes are presented in Table 1. In general, the activities of the bladders from the old rabbits had higher activities than the bladders from the young rabbits for both smooth muscle and mucosa; and the activities of the mucosa were higher than the activities of the smooth muscle. The higher activities of both enzymes in the older rabbits compared to the younger rabbits may be a compensatory factor for the progressive decrease in contractile and metabolic function associated with ageing.

The result of the measurements of SERCA activities of bladder body smooth muscle and mucosa homogenates after obstruction are displayed in Fig. 3A and B. Values in all graphs are normalized to control = 100%. The SERCA activity of smooth muscle in the young rabbits increased by 7 days after obstruction, eventually reaching a two-fold increase at 14 days after obstruction. For the old rabbits, there was a significant reduction in the activity reaching a

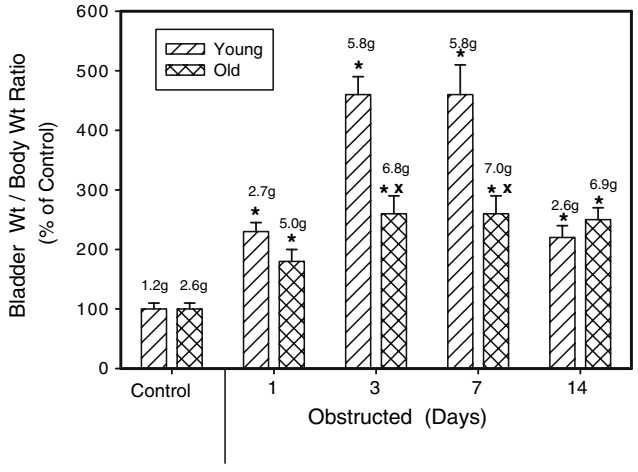

Fig. 1 Effect of PBOO on bladder–body weight ratios in the young and old rabbits. Bladder–body weight ratio is normalized to control = 100% for both young and old rabbits. Each bar is the mean ± SEM of four individual animals. * = significantly different from control; x = significantly different from young, $P < 0.05$. The actual bladder weight is given above each bar

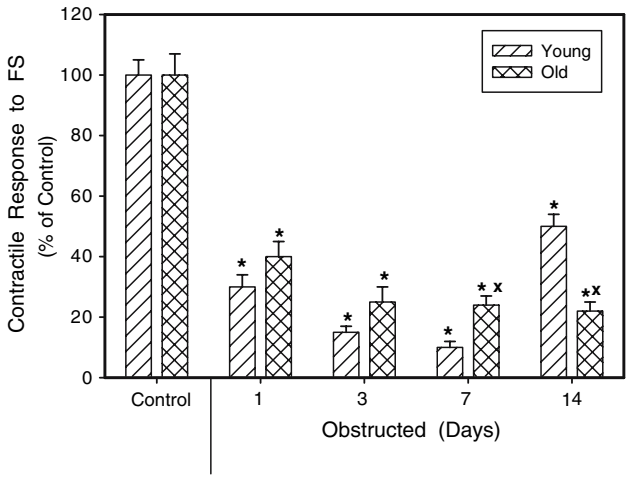

Fig. 2 Effect of PBOO on field stimulation in the young and old rabbits. The response to 32 Hz FS is normalized to 100% for comparative purposes. Each bar is the mean ± SEM of four individual animals. * = significantly different from control; x = significantly different from young, $P < 0.05$

Table 1 Baseline values of SERCA and citrate synthase

	SERCA μmolPi/mg protein		Citrate synthase nmol Co-A/min/mg protein	
	Body muscle	Body mucosa	Body muscle	Body mucosa
Young	2.6 ± 0.6	5.5 ± 0.9*	118 ± 11	192 ± 13*
Old	5.8 ± 1.3¥	7.8 ± 1.1*¥	154 ± 14¥	234 ± 24*¥

* = Significantly different from body muscle, $P < 0.05$

¥ = Significantly different from young, $P < 0.05$

minimum at 3 days and remaining at this low level at 14 days. The SERCA activity of the mucosa after obstruction showed a moderate decreased activity for both young and old rabbits.

Figure 4A and B display the effect of short term obstruction on the citrate synthase activity. The citrate synthase activity of the smooth muscle in the young rabbit decreased over the 3 days, remained low at 7 days and then increased to near control values by 14 days following obstruction. In contrast, in the old rabbits, the citrate synthase activity of smooth muscle progressively decreased over the course of the study. By 14 days after obstruction

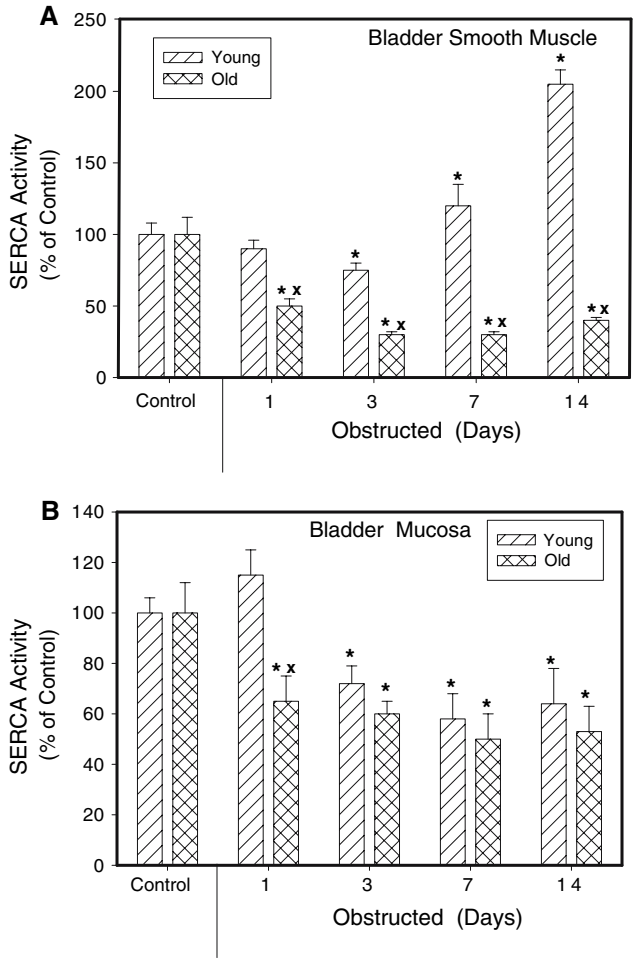

Fig. 3 Effect of PBOO on SERCA activity for bladder smooth muscle (**A**) and mucosa (**B**) in the young and old rabbits. The SERCA activity is normalized to 100% of control for comparative purposes. Each bar is the mean ± SEM of four individual animals. * = significantly different from control; x = significantly different from young, $P < 0.05$

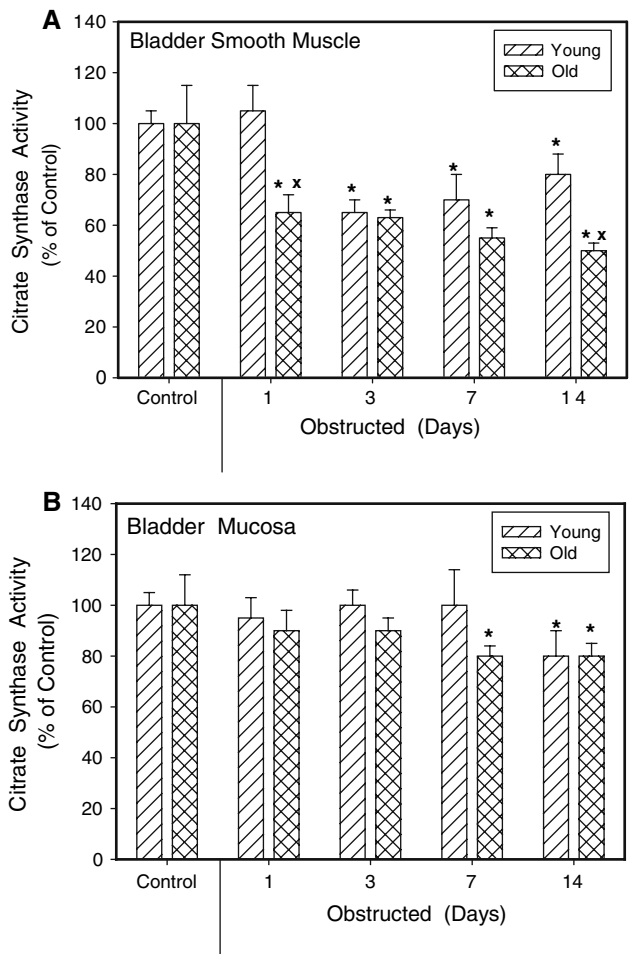

Fig. 4 Effect of PBOO on citrate synthase activity for bladder smooth muscle (**A**) and mucosa (**B**) in the young and old rabbits. The citrate synthase activity is normalized to 100% of control for comparative purposes. Each bar is the mean ± SEM of four individual animals. * = significantly different from control; x = significantly different from young, $P < 0.05$

the citrate synthase activity of the old rabbits was significantly lower than that of the young rabbits. The mucosa showed only minor decreased activities at 7 and 14 days for both the young and old animals.

Discussion

Studies in the rabbit have shown that bladder contraction can be divided in two periods. The first or phasic period is characterized by an initial increase in intravesical pressure that shapes the bladder and is associated with urethral opening. The second or tonic period displays prolonged increases in pressure during which the bladder empties [6, 14]. The phasic response depends primarily on adenosine triphosphate (ATP) present in the bladder smooth muscle and is mediated in part by the stimulated release of Ca^{2+} from the SR, whereas the tonic phase requires energy generation via mitochondrial oxidation of substrates [5, 15]. Thus, both the SR (and SERCA) and mitochondrial oxidative function (of which citrate synthase is a marker enzyme) are both directly involved in bladder emptying.

The mucosa plays an important role in bladder function. Under normal conditions, the mucosa is impermeable to the contents of the urine and thus protects the underlying tissues from the caustic effects of uric acid and other constituents of the urine [16, 17]. Most importantly, the sensory nerves lie within the submucosa, and are very sensitive to damage from ischemia, free radicals, and chemical toxins, resulting in urgency, frequency, and bladder instability. PBOO induces smooth muscle hypertrophy and mucosal hyperplasia. In addition, blood flow to both the bladder muscle and mucosal compartments decrease following PBOO and results in both hypoxia and the generation of free radicals and oxidative damage [18–20]. Studies have clearly demonstrated that the bladder mucosa has a significantly higher metabolic rate than the smooth muscle and is substantially more sensitive to ischemia and anoxia than the smooth muscle [14, 21]. These factors make it very important to study the response of the mucosa to PBOO as well as the response of the smooth muscle elements.

Based on both changes in bladder weight and contractile response to FS (which represents neurohumoral transmission), the young rabbits showed an ability to recover from the initial response to PBOO by both reducing the level of hypertrophy and by increasing the contractile response to FS.

To determine if either SERCA or citrate synthase play a role in the recovery observed in the young bladders, we evaluated the activities of both enzymes in the bladder smooth muscle and mucosal compartments. SERCA activity showed a significant increase in activity in the young rabbits throughout the 14 day obstruction period, whereas there was a marked and prolonged decrease in the old rabbits. The large increase in activity of SERCA in the young bladder smooth muscle between 7 and 14 days likely increases the muscle's ability to release calcium that should enhance contractile function.

Unlike the muscle, the response of the SERCA activity of the mucosa to obstruction was a progressive decrease in activity throughout the 14 day study such that by 14 days the activities of the mucosa of both young and old were reduced by approximately 50%. Whereas the SR in the smooth muscle is involved in contractile function, the SR/ER in the mucosa is involved in secretory function, which can modulate contractile function in muscularis [22, 23]. As mentioned earlier, the mucosa is more sensitive to hypoxia/ischemia than the muscle, and it was not surprising that the SERCA activities in the mucosa of old and young rabbit bladders decreased to approximately the same level.

Smooth muscle contractile activity is regulated by intracellular Ca^{2+} uptake and release. The SR Ca^{2+}-ATPase (SERCA), plasma membrane Ca^{2+}-ATPase, and plasmalemmal $Na+/Ca^{2+}$ exchanger are responsible for lowering intracellular Ca^{2+} following contraction which leads to smooth muscle relaxation [24]. It has been demonstrated that ischemia followed by reperfusion is an etiology for the progression of bladder dysfunction associated with PBOO [25]. Cyclical ischemia-reperfusion (I/R) leads to an increase in free intracellular Ca^{2+}. Presumably, the increased Ca^{2+} reuptake rate which can be inferred by the increased SERCA activity is consistent with the faster adaptation of young rabbit to PBOO and the recovery between 7 and 14 days. On the other hand, these results suggest that decreased SERCA activity of the bladder smooth muscle of the old rabbits may have an important role in the progressive deterioration of old bladders subjected to PBOO. Though this study does not address the question specifically, there is a strong suggestion that blood flow in the younger animal is better, leading to less I/R (or conversely that blood flow is worse in the older animals, leading to more I/R and resultant damage to SR.

Our understanding from this study is that age related changes are important in the regulation of SERCA. There is substantial evidence to implicate increased intracellular free Ca^{2+} in ischemic bladder injury via the activation of Ca^{2+}-dependent hydrolytic enzymes such as calpain, phospholipase A_2, and endonucleases [26, 27] as well as causing the generation of free radicals [28, 29], which result in damage to nerve, synaptic, and intracellular membranes. The increased SERCA activity would certainly be important in increasing the speed of the translocation of Ca^{2+} across both cellular and SR membranes, thus lowering intracellular Ca^{2+} and this regulation would be beneficial to bladder function. Of course, other buffering and transporting systems like the Na^{2+}/Ca^{2+} exchanger, Ca^{2+} channel, Ca^{2+}-binding proteins, and

intracellular Ca2+ uptake systems should be studied as well.

Clearly mitochondrial function is also more sensitive to aging. Mitochondrial function supplies the energy needed for contraction and may be injured by I/R as well as SR. Damage to mitochondria results in decreased cellular levels of high-energy phosphate compounds, including ATP and creatine phosphate, a decrease in the rate of substrate oxidation for glucose/pyruvate and lowered specific activities of mitochondrial enzymes, such as citrate synthase. We found that citrate synthase activity in the bladder body muscle decreased in the 3 and 7 days obstructed young rabbits and came back to control level at the 14 days. Contrary to this, there was a progressive decrease of citrate synthase activity by reaching a minimum level by 14 days in the old. Again the cause may be I/R, which may relate to better blood flow preservation in the young animals.

Similar to SERCA, the citrate synthase activity of the mucosa of both the young and old rabbit bladders showed a similar significant decrease by 14 days PBOO; confirming that the enzymatic changes between the old and young rabbits lies primarily within the smooth muscle compartments.

Another function of mitochondria is that they have a critical role for cleaning Ca^{2+} near the membrane and the disruption of mitochondrial function may have a negative effect on the bladder function. There are no published data comparing SERCA and citrate synthase levels in young versus old bladder tissue. But numerous studies have demonstrated an age dependent reduction in the capacity of mitochondria. For example, it has been shown that aging reduces the mitochondrial enzyme activity of the rat bladder, resulting in a lower energy-production capability [28]. Conley et al. [29] found a 50% reduction in skeletal muscle oxidative capacity between adult and elderly human groups, half of which was due to reduced mitochondrial volume density and the remaining half was due to reduced mitochondrial function. Decrease in mitochondrial oxidative capacity and respiratory enzyme activities with aging was also found in other human tissues, e.g., cardiac tissue [30].

These findings may make it possible to envision a new therapeutic approach to PBOO, targeting key mechanisms involved in intracellular Ca^{2+} handling and mitochondrial function. Controlling I/R may improve the health of SR and mitochondria, but in addition, using pharmacological agents to preserve these enzymatic functions may well lead to improvements in bladder function.

Conclusion

The smooth muscle of urinary bladder in the young rabbit has a considerable ability to adapt to increased intravesical outlet obstruction. This is not seen in older animals. Further, our studies strongly suggest that younger animals have a better ability to preserve SR and mitochondrial functions and since bladder function mirrors the function of these cellular organelles, this may be a key mechanism by which younger animals can preserve bladder function.

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