

The Effect of Partial Outlet Obstruction on Collagen and Smooth Muscle Myosin in the Rabbit Urinary Bladder

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Abstract

Introduction: One of the major etiologies of obstructive bladder dysfunction is tissue fibrosis. As obstructive bladder dysfunction progresses, the replacement of functional smooth muscle with connective tissue results in progressive bladder failure. In the current study, we quantitated the collagen and smooth muscle myosin and analyzed them both by duration of obstruction and severity of contractile dysfunction.

Materials and Methods: Four groups of eight rabbits each were subjected to Partial Bladder Outlet Obstruction (PBOO) for 4, 8, and 12 weeks respectively. The control group was comprised of eight sham surgical rabbits. At the end of the experimental time period, the bladders from all rabbit groups were surgically removed under general anesthesia and 2 full thickness strips of bladder body were taken and fixed in formalin and embedded in paraffin for histology; and three full thickness strips were taken for contractile studies. The balance of the bladder body was separated into muscle and mucosal tissue, frozen and stored at -80oC for biochemical analyses. Quantitative collagen and smooth muscle myosin assays were conducted on bladder smooth muscle samples. Bladder weight was used to categorize the rabbits into mild, intermediate, and severe decompensation groups.

Results: Both collagen and smooth muscle myosin showed similar correlations with both the severity of decompensation and the duration of obstruction. Collagen levels increased significantly while smooth muscle myosin levels decreased.

Discussion: The conversion of smooth muscle to collagen was shown to be an important mechanism causing bladder decompensation.

Keywords: Bladder; Collagen; Myosin; Obstruction; Rabbit; Smooth Muscle.

Introduction

Recent studies have focused on characterizing the relationship between partial bladder outlet obstruction (PBOO) in the rabbit urinary bladder with several biomarkers and enzymes. Nitrotyrosine (NT) and Dinitrophenyl (DNP) are markers of oxidative stress [1,2]. The enzymes Superoxide Dismutase (SOD) and catalase are endogenous antioxidants that are widespread in the body, and alterations in the activities of these enzymes are also markers of oxidative stress [3,4]. Calcium activated enzymes

such as calpain and phospholipase A₂ (PLA₂) are biomarkers for intracellular calcium dysregulation resulting in increased intracellular free calcium [5-7].

In recent publications, the data for these biomarkers were grouped by both the duration and severity of bladder decompensation at the end of the experiment. The duration of the obstruction was separated into 4, 8, and 12 weeks obstruction; and the severity was separated into mild, intermediate, and severe obstruction by the contractile responses to *in-vitro* stimulation by field stimulation; carbachol; and KCl [8-10]. Perhaps the most novel and noteworthy finding from these studies was that in mild decompensation, bladders did not show increased levels of

oxidative stress as shown by the nitrotyrosine and DNP levels, but the calpain and PLA₂ levels were elevated [8]. This suggested that initial bladder decompensation may be mediated more by calcium dysregulation than oxidative stress. The reason for this may be because the cell's antioxidant defense mechanisms were able to cope with oxidative stress whereas the cell was less able to handle the increased cytosolic free calcium.

The final part of these studies was analyzing the collagen and smooth muscle myosin levels in the bladders that were exposed to PBOO. In theory, the conversion of smooth muscle to collagen should directly correlate with decreasing contractility and increasing bladder decompensation [11-13]. In other words, the increasing oxidative stress and calcium activated enzymes stimulate the conversion of smooth muscle to collagen thereby increasing the rate of decompensation [14]. In addition to analyzing collagen using a total collagen assay kit and smooth muscle myosin by Western blot analysis, we also analyzed the bladder histologically into connective tissue and smooth muscle compartments by digitally analyzing trichrome stained samples from each bladder. These biochemical and histological studies were limited to the bladder smooth muscle compartment only; the mucosa and serosa do not participate actively in contraction and thus were not part of this study.

Materials and Methods

All studies were approved by the Institutional Animal Care and Use Committee and the Research and Development Committee of the Stratton VA Medical Center, Albany, NY.

Animal Model

For this study, thirty-two New Zealand white rabbits were divided into four equal groups of eight rabbits. The first group was a control group all of which underwent a sham obstruction that caused no significant bladder decompensation. Partial bladder outlet obstructions were performed on the other three groups by loosely tying a silk ligature around the catheterized urethras of the anesthetized rabbits. The rabbits were obstructed for four, eight, and twelve weeks respectively. At those times, the rabbits were again put under anesthesia and the bladders were surgically removed. The bladder body and base were separated at the level of the ureteral orifices. Two full thickness samples of the bladder body were taken for histological studies, placed in formalin for 8 hours, and then embedded in paraffin. Three full thickness strips of bladder body were taken for *in-vitro* contractility studies. The balance of the bladder body was separated between muscle and mucosa by blunt dissection and stored in a freezer at -80° Celsius for later biochemical analyses of collagen and smooth muscle myosin.

The categories of the decompensated bladders were determined by their weight as published in previous studies

[10,15]. The increase in bladder mass correlated well with both bladder body hypertrophy and increasing bladder decompensation [10,15]. Bladders weighing less than 6 grams were considered mild, 6-20 grams were intermediate and over 20 grams were severely decompensated.

Contractility Studies

Contractility studies were conducted by placing strips of bladder tissue in baths containing warm, oxygenated Tyrode's solution (37°C) to observe maximal tension generation in response to various stimuli including Field Stimulation (FS), carbachol, and Potassium chloride (KCl). The level of contractile function (dysfunction) was calculated as the average percentage of contraction of the control tissue; thus, the greater the dysfunction, the lower the percentage of control.

Collagen Analysis

Collagen levels were determined on the muscle samples through the use of a QuickZyme Total Collagen Assay chemical kit. Tissue samples were hydrolyzed at 100 mg/ mL in 6M HCl and incubated for 20 hours in a thermoblock at 95°C. Threefold dilutions were done on all tissue samples using 4M HCl. The standard curve was prepared from a stock of 1.2 mg/ mL in 12M HCl. The curve created with dilutions in 4M HCl had values of 0.672, 0.448, 0.224, 0.112, 0.056, 0.028, 0.014 mg/ mL, and a blank. The kit required the use of clear 96 well plates and a fluorescence plate reader (SpectraMax Plus by Molecular Devices) set at 570nm.

Smooth Muscle Myosin

Frozen tissue of bladder muscle wall was homogenized on ice in homogenization buffer (50 mM Tris, pH 7.5, 5% Tiron) containing the Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL) at 100 mg/ml. After addition of SDS (final concentration, 1%), the sample was boiled for 4 min and centrifuged at 10,000 rpm for 15 min. Protein concentration in the supernatant was measured using the Pierce BCA protein assay kit. Membranes were blocked with 5% nonfat milk in 0.05% Tween 20 in PBS for 1 h at room temperature and then incubated with primary antibody, monoclonal antibody to Smooth Muscle Light Chain A; Smooth Muscle Light Chain B; and Smooth Muscle Heavy Chain. All antibodies were obtained from Sigma-Aldrich [16,17]. After treatment with the primary antibody, the membranes were washed and incubated with secondary antibody (Santa Cruz **Biotechnology**, Santa Cruz, CA). The substrate was visualized by using ECL-Plus (Amersham Pharmacia Biotech, Buckinghamshire, England) for 2 minutes and analyzed with a Kodak Image Station 440CF and Kodak 1D image analysis software (Scientific Image System, Rochester, NY).

Histological Analysis

Each paraffin block was sectioned at 5 M and 3-4 sections were placed on individual slides. Each section was de-paraffinized

by graded ethanol and stained with trichrome stain. Connective tissue stains blue while smooth muscle stains red. Smooth muscle areas of each section (minus mucosa) were digitally analyzed and the percentage of connective tissue and smooth muscle were calculated and then normalized to 100% of the area under investigation.

Results

The bladder weights and severity levels for each duration are given in Table 1. Control rabbits had a mean bladder weight of 2.6 +/- 2.0 grams. The 4-week obstructed bladders had a mean weight of 10.5 +/- 5.8 The 8-week obstructed bladders had a mean weight of 16.4 +/- 4.8 grams. The 12-week obstructed bladders had a mean weight of 27.2 +/- 6.5 grams. Control rabbits had no decompensated bladders. The 4-week obstructed bladders had 3 mild, 3 intermediate, and 2 severely decompensated bladders; the 8-week obstructed bladders had 2 mild, 4 intermediate, and 2 severely decompensated bladders. The 12-week obstructed bladders had 0 mild, 4 intermediate, and 4 severely decompensated bladders.

	Bladder Weight gm	Severity
		Number of Rabbits
Control (8 rabbits)	2.6 +/- 2.0	Mild 0
		Intermediate 0
		Severe 0
4Week Obstructed	10.5 +/- 5.8 *	Mild 3
		Intermediate 3
		Severe 2
8 Week Obstructed	16.4 +/- 4.8 *	Mild 2
		Intermediate 4
		Severe 2
12 Week Obstructed	27.2 +/- 6.5**	Mild 0
		Intermediate 4
		Severe 4

* = significantly different from control, p < 0.05
 ** = significantly different from all other groups, p < 0.05

Table 1[10]: Bladder weight and distribution of severity groups.

The contractility studies from these groups have been published previously [10]. Although contractile responses decreased in relation to both severity and duration; there was a closer correlation with severity than with duration.

The collagen concentration in mg/ g of tissue was evaluated by both severity of decompensation and duration of obstruction and presented in Figure 1A. By duration, the collagen levels of both the 8 weeks and 12 week groups were significantly higher

than control. When analyzed by severity, the mild and intermediate groups were near control levels. A major increase was seen only in the severe group which was significantly higher than both control and the other obstructed groups. In Figure 1B, the collagen levels in mg/ bladder were compared by severity and duration. When grouped by duration, the same general outline was seen as in Figure 1A. However, after severity analysis a slightly different trend emerged. This time, the collagen levels were the same in the control and mild groups, but a significant increase was seen from both mild to intermediate and from intermediate to severe decompensation.

Fig 1

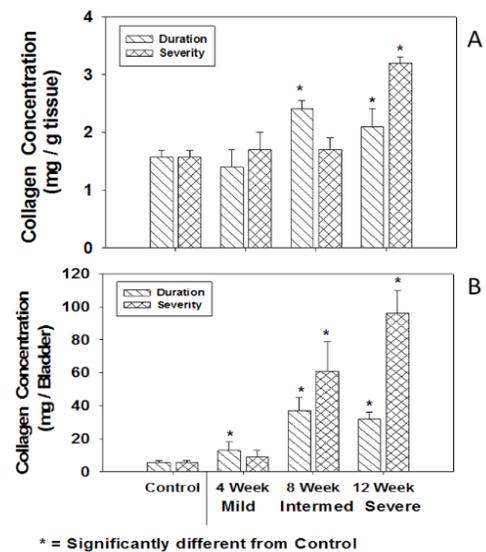


Figure 1A: Collagen concentration in mg/ g tissue following PBOO by duration and severity. Each bar for duration is the mean +/- SEM of 8 individual rabbits. Each bar for severity is the mean +/- SEM of between 5 and 11 rabbits. * = significantly different from control, p < 0.05.

Figure 1B: Collagen concentration in mg/ bladder following PBOO by duration and severity. Each bar for duration is the mean +/- SEM of 8 individual rabbits. Each bar for severity is the mean +/- SEM of between 5 and 11 rabbits. * = significantly different from control, p < 0.05.

The smooth muscle myosin heavy chain is presented in Figure 2 by severity and duration. The data was nearly identical when comparing both severity and duration with 4 weeks obstructed and mild decompensation being equal to control, 8 and 12-week obstructions; and Intermediate and severe decompensations were equal to each other and significantly reduced from control and 4 weeks obstructed.

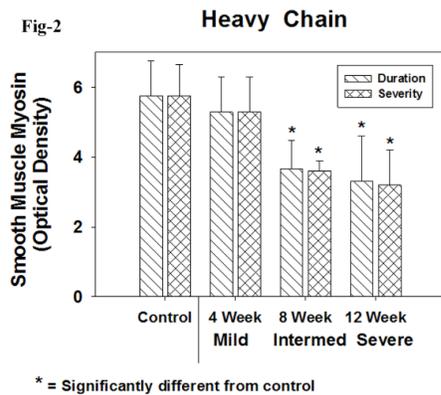


Figure 2: Smooth muscle myosin heavy chain (optical density) by duration and severity. Each bar for duration is the mean \pm SEM of 8 individual rabbits. Each bar for severity is the mean \pm SEM of between 5 and 11 rabbits. * = significantly different from control, $p < 0.05$.

Myosin light chains A and B (Figures 3A and B) showed very similar results to those shown for myosin heavy chain except that for light chain A, the mildly decompensated bladders showed significantly reduced concentrations compared to control.

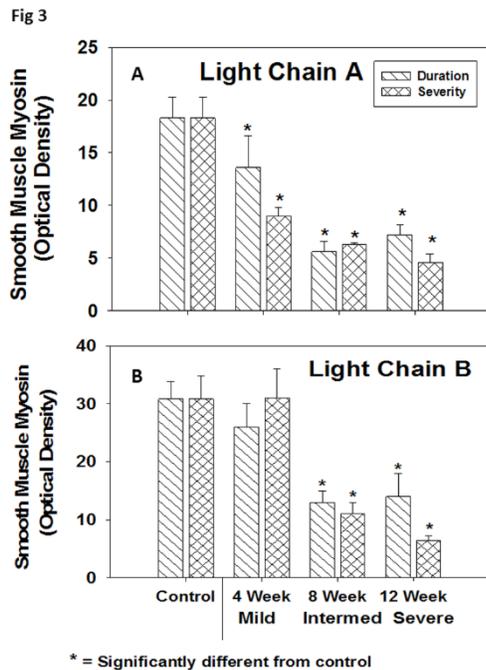


Figure 3A: Smooth muscle myosin light chain A (optical density) by duration and severity. Each bar for duration is the mean \pm SEM of 8 individual rabbits. Each bar for severity is the mean \pm SEM of between 5 and 11 rabbits. * = significantly different from control, $p < 0.05$.

Figure 3B: Smooth muscle myosin light chain B (optical density) by duration and severity. Each bar for duration is the mean \pm SEM of 8

individual rabbits. Each bar for severity is the mean \pm SEM of between 5 and 11 rabbits. * = significantly different from control, $p < 0.05$.

Figures 4-7 show representative trichrome-stained micrographs for control, 4, 8, and 12-week obstructed bladders. For quantification of connective tissue and smooth muscle, each histological section of each bladder was separated by both duration and severity, and the ratio of smooth muscle / collagen was calculated by digital analysis.

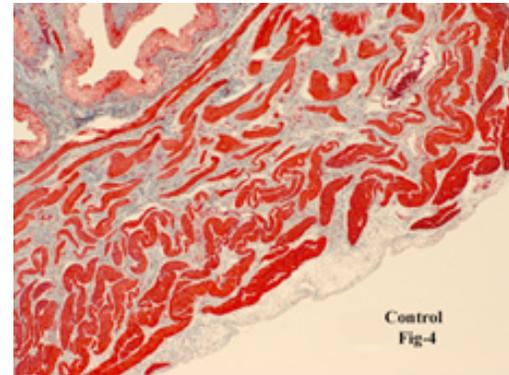


Figure 4: Representative histological section from a control rabbit.

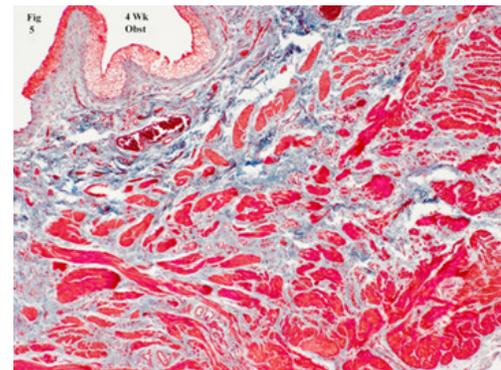


Figure 5: Representative histological section from a 4-week obstructed rabbit.

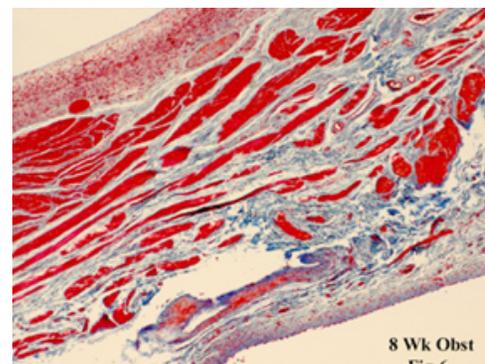


Figure 6: Representative histological section from an 8-week obstructed rabbit.

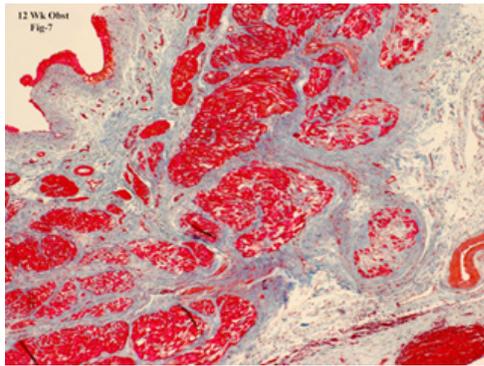


Figure 7: Representative histological section from a 12-week obstructed rabbit.

The percentages of Connective Tissue (CT) and Smooth Muscle (SM) analyzed by histology for duration and severity are presented in Figures 8A and B. By duration, the percentage of SM was significantly higher than the percentage of CT for control tissue. The percentages of CT for 4, 8, and 12-week obstructed rabbits were significantly higher than control while the percentages of SM was significantly lower than control. There were similar percentages of both SM and CT for all durations of obstruction (Figure 8A). Similar results were observed for the analysis by severity, (Figure 8B) except with mild decompensation there was an intermediate level of connective tissue between control and intermediate levels of decompensation.

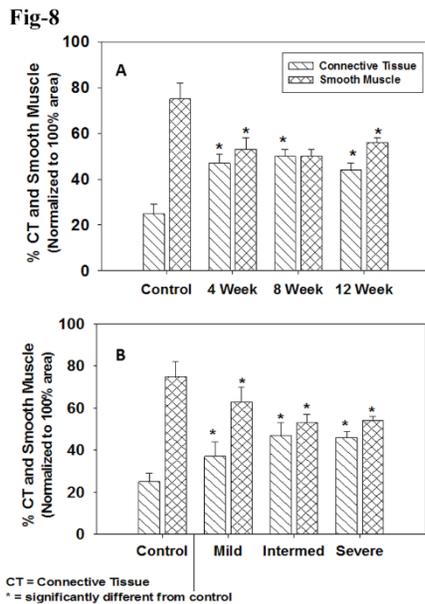


Figure 8A: Percent connective tissue and smooth muscle normalized to 100% area and grouped by duration for connective tissue and smooth muscle. Each bar for duration is the mean +/- SEM of 8 individual rabbits

* = significantly different from control, $p < 0.05$.

Figure 8B: Percent connective tissue and smooth muscle normalized to 100% area and grouped by severity for connective tissue and smooth muscle. Each bar for severity is the mean +/- SEM of between 5 and 11 rabbits. * = significantly different from control, $p < 0.05$.

Discussion

Figure 9 shows a modification of our schematic on the etiology of obstructive bladder dysfunction published in our review article: Obstructive Bladder Dysfunction: Morphological, Biochemical and Molecular Changes [18]. Based on our studies, the shift from compensated to mild decompensation involves the left branch; i.e., calcium dysregulation and the activation of calcium-dependent proteases and lipases. As decompensation proceeds (from mild to severe) free radicals are generated by the decreased blood flow, hypoxia, and ischemia due to bladder hypertrophy. Simultaneous, collagen fibrosis occurs parallel to bladder hypertrophy which adds to the severity of obstructive bladder dysfunction. Initially, the fibrosis is reversible if the obstruction is relieved [19]. Interestingly, the longer the obstruction lasts, independently of the severity the collagen (connective tissue) develops cross bridges that resists reversal; thus, the longer the duration, the greater the influence of fibrosis has on bladder function. This is not the case with either calcium dysregulation or oxidative stress where reversal depends on severity rather than on duration. These differences are true for obstructive bladder dysfunction in humans where obstructive bladder dysfunction can exist for years, and fibrosis has a greater influence on bladder dysfunction than in rabbits [20,21].

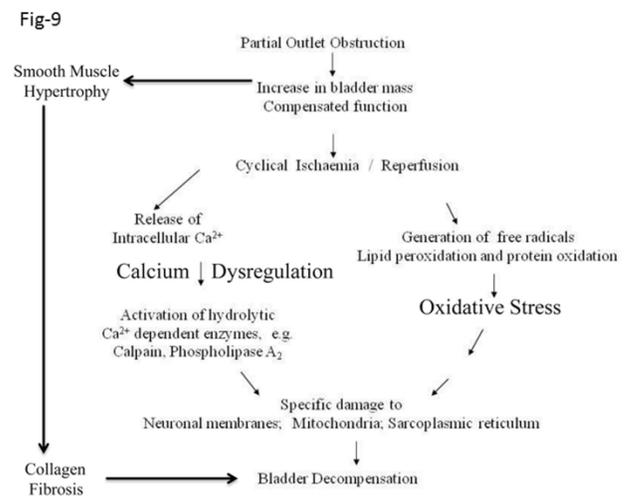


Figure 9: Schematic of the etiology of obstructive bladder dysfunction.

In a study to determine the extent that collagen deposits can influence contractile function, rabbits were divided into two groups: control and 2-week obstructed. Each rabbit was anesthetized, and

the bladder body was excised and cut into equal width strips of 0.5, 1.0, and 2.0-cm lengths [22]. The contractile responses to field stimulation, carbachol, and potassium chloride were determined. At the end of the experiment, each strip was fixed in formalin and immune-stained for collagen. The contractile responses for the control were similar for all strip lengths. In obstructed tissue, the shorter strip lengths generated significantly more tension per cross-sectional area than did the longer strips. The collagen density and distribution were significantly greater for the obstructed bladder strips than in control strips. In addition, the obstructed bladder strips had significantly increased collagen deposits between and within the smooth muscle bundles and cells.

Because the relationship between strip size and contraction were similar for field stimulation, carbachol, and potassium chloride, it was the increased density of connective tissue within and between the muscle bundles and fibers that interfered with contraction (i.e., the greater the strip length, the greater the interference and the greater the contractile dysfunction). Therefore, both functional and structural alterations in the obstructed bladder participate in contractile dysfunction [22].

As expected, the bladder mass increased with increasing duration of obstruction because of the associated increasing SM hypertrophy and mucosal hyperplasia [23,24]. In regard to contraction, oxidative stress, and calcium overload, the link is more direct between the severity of decompensation than with the duration of obstruction [8, 10]. Unlike the previous observations, the increase in collagen and decrease in SM may be more closely related to duration of obstruction than severity. When analyzed per bladder, the apparent progressive increase in collagen is probably due to the progressive increase in bladder mass, although there was a significant increase in collagen associated with severe decompensation and not with mild or intermediate decompensation. However, this trend was not observed in the histological study where all durations and severities had approximately the same decrease in SM and increase in CT. This is probably due to the fact that the histological analysis is a qualitative analysis whereas the biochemical assays were quantitative.

The durations of obstruction were closely controlled while the level of bladder decompensation was dependent on the response of the individual rabbit to the presence of the obstruction. This observation has been made previously in several models of obstruction [15,23,25]. This is similar to the response in men to obstructive uropathy secondary to BPH. That is, the severity of the obstruction is not related to the size of the prostate but to the response of the individual to the presence of the constriction [26-28].

The collagen levels did increase with increasing severity of bladder decompensation but only in the severely decompensated group. This is most likely because the collagen increase itself was

directly involved with the severe decompensation [22]. The smooth muscle myosin levels decreased similarly with increasing bladder duration and severity of decompensation. The decreased smooth muscle myosin was significant in the 8 and 12-week obstructed groups to approximately the same degree as the intermediate and severe decompensation groups. The decrease in SM was related to the increase in collagen because of the conversion of SM to collagen in the presence of obstruction in both rabbits and humans [29,30]. Since the SM allows the bladder to contract, the decrease of SM subsequently mediated the bladder decompensation. Interestingly, the smooth muscle myosin heavy chain concentration was nearly identical when grouped by both severity and duration which was not seen in the collagen or even in the smooth muscle light chains. This may be because the heavy chain converts at a more stable rate than the light chains. The increased density of the heavy chain may provide some means to allow the conversion to collagen to be more gradual causing the graphs of both duration and severity to be similar.

It is also worth noting that light chain A seemed more susceptible to PBOO than light chain B. The mildly decompensated group had much lower smooth muscle myosin levels for light chain A than control, while for light chain B, the mild group was the same as control. The interplay between smooth muscle myosin heavy chain and light chain A and B could very well be complex but also important to better understand the steps leading toward decompensation. In any case, all three types of smooth muscle myosin were significantly decreased by the time severe decompensation (or 12-week obstruction) was reached while collagen levels were significantly increased. The histological studies also would support the idea that duration is more related to the ratio of SM to CT than severity.

Conclusions

The functional decrease in contractility following partial bladder outlet obstruction is a multi-factorial process involving oxidative stress, calcium overload, changes in SOD and catalase, and the conversion of SM to collagen. Interestingly, oxidative stress and calcium overload appear to be related to the severity of decompensation to a higher degree than to the duration of obstruction, whereas the conversion of SM to collagen appears to be more closely related to duration. This may well be a function of the conversion of SM to collagen being a slow process whereas changes in oxidative stress and intracellular calcium concentrations are relatively rapid processes.

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