University of Alberta

Novel Mediators and Medical Prevention of Bladder Fibrosis After Partial Bladder Outlet Obstruction

by

Conrad Christopher Maciejewski

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<u>Dedication</u>

I would like to dedicate this thesis to the man who taught me to question
the workings of the world I saw around me

Waldemar B. Maciejewski

Abstract

Introduction: Partial bladder outlet obstruction (pBOO) is a significant problem that can affect renal health. We hypothesize that changes occur in small leucine-rich proteoglycan (SLRP) composition of extracellular matrix over time in this process. We hypothesize that medical treatment of this condition will improve functional and molecular outcomes.

Methods: Rats underwent surgical pBOO for 4-16 weeks time, and were compared to shams and untreated control animals. Urodynamic measurements were taken, bladders were weighed, measured for thickness, and frozen for RT-PCR and mass spectrometry.

Results: Decorin and biglycan were found to be inversely related over the course of pBOO, with decorin decreasing in RNA and protein expression, and biglycan increasing. Treatment of animals with oxybutynin or tadalafil resulted in favorable urodynamic, histologic and molecular changes.

Conclusion: SLRPs are differentially regulated in pBOO. Treatment with oxybutynin or tadalafil delayed progression of pBOO pathology. We believe that the process of pBOO is medically alterable.

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List of Abbreviations

BPH – Benign Prostatic Hyperplasia

BSA – Bovine Serum Albumin

cAMP – Cyclic Adenosine Monophosphate

cGMP – Cyclic Guanosine Monophosphate

CIC – Clean Intermittent Catheterization

CTGF – Connective Tissue Growth Factor

DNA – Deoxyribonucleic Acid

FAK – Focal Adhesion Kinase

GAPDH – Glyceraldehyde Phosphate Dehydrogenase

HIF-1α – Hypoxia Inducible Factor 1 Alpha Subunit

LUTS – Lower Urinary Tract Symptoms

MMP – Matrix Metalloproteinase

mTOR – Mammalian Target of Rapamycin

NO – Nitric Oxide

NOS – Nitric Oxide Synthetase

pBOO – Partial Bladder Outlet Obstruction

PDE – Phosphodiesterase

PVP – Photovaporization of the Prostate

RT-PCT – Reverse Transcriptase Polymerase Chain Reaction

RNA – Ribonucleic Acid

SLRP – Small Leucine Rich Proteoglycan

TIMP – Tissue Inhibitor of Metalloproteinase

TGF-β – f Transforming Growth Factor Beta

TURP – Transurethral Resection of the Prostate

VEGF – Vascular Endothelial Growth Factor

VUR – Vesicoureteral Reflux

Chapter 1: Introduction

1.1 Scope of the Problem

1.1.1 Definition

Partial bladder outlet obstruction (pBOO) is one of the most commonly seen conditions in urology. This condition itself has a nearly ubiquitous presence in older men, but can also appear in females and children. pBOO has been defined as an increase in bladder pressure required to void due to sphincteric resistance. This can occur secondary to prostatic hypertrophy, anatomical abnormalities, or congenital defects.

The severity of the obstruction depends typically on its etiology. Prostatic hypertrophy, for example, proceeds in an insidious manner and progresses with age, leading to a decreased urine flow rate. This leads to a progressive worsening in lower urinary tract symptoms, and has a profound effect on health related quality of life.

While the etiology of pBOO is directly related to an abnormal outlet and sphincter mechanism, the bladder can be thought of as an innocent bystander in the process. In its normal form, the bladder can be thought of as having two roles: the continent storage of urine at low pressure, and the voluntary emptying of urine when deemed appropriate. Storage requires a stable bladder with a closed sphincter, whereas emptying requires a contracting bladder coordinating with sphincter relaxation. If the bladder has to function and contract against an obstructed outlet, it will undergo compensatory hypertrophy, as can be expected of any muscle in

the body. With prolonged pBOO, this excess hypertrophy will eventually decompensate with a decreased ability to generate pressure, and eventual fibrosis[1]. As such, an end-stage bladder forms, where a small capacity is evident, and lacking the compliance to store urine reliably at low resting pressures.

However, the resultant increase in lower urinary tract symptoms is not the most threatening issue behind a damaged bladder. High urine storage pressures can cause backflow of urine towards the kidneys, in a process termed vesicoureteral reflux (VUR). The pressure of urine on the kidneys secondary to the presence of this condition predisposes the patient not only to recurrent infection, but most importantly carries a significant lifelong risk of renal failure.

1.1.2 Epidemiology of Male Partial Bladder Outlet Obstruction

In males, pBOO occurs almost exclusively due to benign prostatic hyperplasia (BPH). This disease process occurs in 8 percent of 40-year-old men, up to 50 percent of 50 to 60 year old men, and up to 88 percent of eighty-year-old men[2, 3]. The disease process progresses slowly, leading to the appearance of multiple abnormalities in the urinary tract, including renal failure, bladder fibrosis, bladder calculi, recurrent urinary tract infections, and hematuria[4-6]. Most apparent to the patient, however, is the onset of bothersome lower urinary tract symptoms, which have a profound impact on health related quality of life[7]. Validated patient based

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questionnaires, such as the International Prostate Symptom Score have been developed to quantify and prognosticate the progression of this disease[8]. Severe cases require surgical intervention[9-11], but the damage to the detrusor is often irreversible[12].

1.1.3 Epidemiology of Female Partial Bladder Outlet Obstruction

Female bladder outlet obstruction is a considerably less common entity than that in the male. Community studies have identified a 10.8% prevalence rate of voiding difficulty in women over sixty years of age, with 29% of these patients being identified to have BOO[13-15]. Given the absence of the prostate gland in the female, etiologies for this condition include pelvic organ prolapse, primary bladder neck obstruction, iatrogenic obstruction secondary to stress incontinence surgery, and other anatomical abnormalities, including neoplasia, diverticula, and stricture disease[15, 16]. While lower urinary tract symptoms prevail in patient presentation, and anatomical definition is mandatory, the International Continence Society emphasizes that urodynamic testing is mandatory for the diagnosis of BOO in a female, relying on elevated detrusor pressures and decreased flow rates[17]. The treatment of this condition relies on the underlying cause. Pelvic organ prolapse, anatomical abnormalities, and primary BOO are treated with surgical correction[16, 18, 19], whereas less severe obstructive symptoms can often be managed medically[20, 21]. The end result of untreated obstruction in females, however, is no different

than in males, resulting in bladder muscle hypertrophy, fibrosis, and eventual irreversible bladder damage.[22]

1.1.4 Pediatric Bladder Outlet Obstruction

Bladder outlet obstruction in children is almost always secondary to congenital abnormalities. The most frequent cause for lower urinary tract dysfunction in children is neurologic abnormalities, which can comprise up to 25% of patients presenting to a pediatric urologist's clinic[23]. These conditions lead to lifelong need for medical care and intervention, and resultant morbidities for the patient include frequent urinary tract infections, the need for multiple surgical procedures, and a lifelong risk of progression to renal failure.

1.1.4.1 Posterior Urethral Valves

Posterior urethral valves arise in 1/8000 to 1/25000 live births, and account for up to 10% of all prenatally diagnosed urinary tract obstructions[24, 25]. The valve mechanism itself appears as a sheet of tissue that extends from the urethral floor at the verumontanum, extending proximally to the bulbar urethra in one of three distinct patterns[25-27]. This tissue effectively obstructs the bladder in varying degrees. In its most severe form, fetuses are identified to have Potter's Facies, hydronephrosis, urinary ascites, oligohydraminos, leading to severe pulmonary hypoplasia and often death in neonatal life[28]. In less severe forms, the bladder develops against a continually obstructed outlet,

becoming markedly abnormal in function, able to generate extremely high voiding pressures, and as a result, massively hypertrophic. Such high voiding pressure, in the setting of chronic urinary retention with an obstructed outlet, leads to vesicoureteral reflux, predisposing the child for recurrent urinary tract infections, and renal damage later in life. Coined the Valve-Bladder Syndrome, these children may eventually progress to end stage bladders, and renal failure without careful management, which unfortunately can occur in up to 10% of cases[29, 30]. Recent literature has focused on early diagnosis of this condition, and the importance of the relief of obstruction on the developing bladder, better elucidating the natural history of this disease, and highlighting the importance of early treatment to prevent lifelong bladder dysfunction[31, 32].

1.1.4.2 Spinal Cord Dysraphism

Any clinical situation that impairs normal nervous system outflow to the bladder and sphincter complex can create a host of clinical problems. While trauma, tumors, and other insults to the nervous system can impair normal innervation and function, the most commonly seen scenario for a pediatric urologist is congenital spinal dysraphism. While this can present in an occult manner, such as a tethered spinal cord, more severe and overt bladder dysfunction is seen in the setting of myelomeningocele. With the advent of folate supplementation for gestating mothers, as well as antenatal detection in combination with therapeutic abortion, the incidence of myelodysplasia has decreased to 0.7-1.0 cases per 1000 live

births[33]. These children were historically at very high risk of renal failure, primarily due to dyssynergic bladder contractions against a fixed, tonic urinary sphincter (detrusor sphincter dyssynergia, DSD), leading to extremely high intravesical pressures, which secondarily cause the reflux of urine back towards the kidneys (vesicoureteral reflux). The bladder, contracting against a highly resistant outflow tract, becomes hypertrophied, and eventually fibrotic and non-functional[34, 35]. Progression of reflux has been documented, presenting in up to 5% of newborns, and up to 30-40% of 5 year olds in the absence of treatment[35]. These children require lifelong urologic follow up for management of incontinence and monitoring of renal function, and require significant medical management, and eventually surgical procedures to relieve bladder pressure, most often using intestinal tissue for bladder augmentation[36, 37]. Contemporary literature has emphasized the need for early and aggressive management of the problem, in an effort to prevent the progression to renal failure and need for transplant[34, 38, 39]. However, current standards of medical management are still rudimentary, highlighting the need for a better molecular understanding of the pathology of this syndrome.

1.2 Anatomy and Physiology

1.2.1 Anatomy and Neural Control of the Bladder

The bladder is an organ that possesses several functions, each of which is reflected in its unique histological structure[40]. Its role can be

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simplified to the performance of two tasks: storage of urine, and emptying of urine. The storage of urine demands that the bladder is a receptacle of adequate capacity, and able to store urine at low pressure. It must be stable at rest, lacking spontaneous contraction, and compliant to increases in pressure and volume. For attaining continence, it must face a closed sphincter. The urinary sphincter mechanism has two distinct parts. Sympathetic efferent activity originates in L2 and L3 and travels through the hypogastric nerve, first via sympathetic ganglia and through the inferior mesenteric ganglion[41]. This results in contraction of the smooth muscle of the bladder surrounding the outflow tract (the intrinsic / internal / smooth urinary sphincter). Simultaneously, β-adrenoceptor activation results in the relaxation of the remainder of the smooth muscle of the bladder. Gradual increases in urine volume are thus dynamically stored without appreciable increases in pressure. Simultaneously, somatic efferent activity, via the pudendal nerve originating in S2,3,4 activates slow twitch muscle fibres in the voluntary component of the striated / extrinsic / external urinary sphincter, producing a dynamically tight closure resulting in continence[42]. These outflow signals originate in Onuf's nucleus, located on the lateral aspect of the ventral horn of the spinal cord.

Voiding is a complex reflex, initiated in higher centres in the central nervous system, feeding to the pontine micturition centre. Initially, sympathetic outflow is inhibited, relaxing the internal sphincter.

Simultaneously, parasympathetic outflow commences, via the pelvic nerve, resulting in the activation of a complex sub-urothelial nerve network whose role is to stimulate the contraction of the bladder smooth muscle[43]. This action is mediated by acetylcholine release, primarily through the activation of muscarinic M3 receptors. While M2 receptors are abundant throughout the bladder, their role in voiding and urine storage remains unclear. Nitric oxide release follows, resulting in the relaxation of outlet mechanisms, and voiding commences[44]. Maintenance of this reflex depends on the communication between the spinal cord and the pons, as well as the periaqueductal gray matter. Overall, the entire storage and voiding mechanism is a complex interplay of central function, an intact spinal cord, and coordinated sympathetic and parasympathetic efflux[45, 46].

1.2.2 Physiology

They key aspect to safe storage of urine relies on the bladder's ability to accommodate to volume changes, a property referred to as compliance. While part of this is dependent on intact neural networks, the histologic structure of the bladder is equally important. A transitional epithelial layer lines the internal lumen, with a multilayered epithelial cell architecture designed to allow for stretch[43]. The muscle layer is a three-layer smooth muscle sheet, which with filling reorganizes to a network of randomly oriented concentric fibres primed for contraction and emptying. The stroma itself contains a complex extracellular matrix, including collagen types I, III, and IV, fibroblasts, and elastin[47, 48]. This stromal layer

remains passive in normal situations, allowing for maximal tissue stretch and compliance. Stress situations cause alterations in its composition, leading to the pathophysiology of bladder decompensation.

1.2.3 Pathophysiology

A considerable amount of animal model work has been performed to elucidate the pathologic mechanisms that occur in pBOO. The detrusor muscle has stress placed on it during the voiding process, stemming from increased intravesical pressure required to pass urine through the partially obstructed bladder outlet. As with any muscle, this chronic process results initially in hypertrophy, improved perfusion, and an ability to generate considerable intravesical pressure[49]. This process is thought to occur secondary to multiple growth factors and hormones that affect bladder tissue growth. As the muscle wall continues to hypertrophy in the setting of cellular stress, a loss of contractility is observed, with concomitant changes in collagen deposition and changes to the extracellular matrix[50, 51]. Ultimately, the bladder becomes noncompliant and acontractile, and this unable to perform its functions of storage and transport of urine.

Smooth muscle hypertrophy in this setting not only results in the ability to generate increased pressure, but also leads to an unstable bladder, where uninhibited contractions occur during the filling process. For the patient, the clinical result is the onset of sudden urge incontinence.

1.3 Current Management

The current management of pBOO relies on a combination of medical and surgical management[52]. While the relief of obstruction is the ultimate goal, this is often not attainable, particularly in the setting of congenital defects such as myelomeningocele, where the sphincter is tonically fixed[53]. A secondary outcome is to prevent and modulate unfavorable changes that will inevitably occur to the bladder tissue, which becomes a priority in patients with uncorrectable defects, or those in a late stage of the disease process.

1.3.1 Diagnosis

While useful information can be garnered from patient history, serum investigations, imaging, and physical examination, the primary tool used to diagnose pBOO is urodynamic testing[6, 54]. It serves as a cornerstone of measurement for assessment of bladder capacity, compliance, stability, and voiding pressure. Early studies have provided a threshold of 40 cm of H_2O pressure as being associated with renal injury[55], although many clinicians now use lower levels to initiate therapy[56, 57]. However, in the setting of a high-pressure bladder with an uncorrectable resistant outflow tract, the pathologic process behind bladder hypertrophy and eventual fibrosis progresses silently, and will inevitably lead to a patient with end stage bladder, and potentially renal failure[58, 59].

1.3.2 Surgical Relief of Partial Bladder Outlet Obstruction

Adult partial bladder outlet obstruction occurs primarily in the setting of benign prostatic hypertrophy. Those who fail medical management require surgical intervention[60, 61]. The current gold standard for the relief of BPH related obstruction is through endoscopy, utilizing a number of procedures to open the bladder outlet. These include primarily transurethral resection of the prostate (TURP), photovaporization of the prostate (PVP)[62]. Other options include thermoablative therapies, such as needle ablation[63], microwave ablation[64], or high intensity focused ultrasound[65, 66]. Finally, intraprostatic stents are available for use in selected patients[67].

In the setting of pediatric patients with posterior urethral valves, management is guided by age. Older boys who present with a diagnosis of a valve can successfully be relieved of obstruction by endoscopic incision or resection[28, 32, 68]. Placement of an indwelling catheter remains the first step in management, however if serum creatinine fails to normalize, a cutaneous vesicostomy is created. The bladder lumen is brought up to the skin to create an incontinent conduit for the drainage of urine, resulting in extremely low resistance to emptying[69, 70]. This treatment modality has been demonstrated to have benefit in terms of bladder drainage and upper tract drainage, with a limited complication profile[71-73].

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1.3.3 Medical Treatment of Partial Bladder Outlet Obstruction

The advent of effective medical therapy for benign prostatic hyperplasia has significantly changed the practice of urology. In the past, obstructive voiding symptoms secondary to BPH were addressed only using endoscopic resection. While a TURP is still one of the most frequently performed procedures in urology, it places the patient at risk of multiple perioperative complications[74]. Caine and colleagues first studied alpha-adrenergic antagonist therapy in 1976[75, 76]. The premise of this medical therapy is to target smooth muscle fibres in the prostate stroma, leading to prolonged relaxation of the gland and subsequent opening of the prostatic urethra. Subsequently, 5-α reductase inhibitors were developed to reduce outlet resistance using a separate biochemical mechanism[77, 78]. The Type-II isoform of 5-α reductase is an intracellular enzyme within the prostate, which converts circulating testosterone to its active form, dihydrotestosterone[79]. In the absence of testosterone, prostate growth is halted, and the gland itself will shrink[80]. Multiple large-scale studies have been conducted on the use of these two classes of medications, often in conjunction with one another[81, 82], demonstrating a significant benefit in symptom reduction, as well as prevention of the sequelae of bladder outlet obstruction. While these medications can target the offending pathologic mechanism, they do not have any ability to directly target or reduce the compensatory changes that occur in the bladder itself.

One significant change in bladder function stems from smooth muscle hypertrophy. A thicker more muscular bladder wall is often more unstable, and more likely to engage in spontaneous uninhibited contractions, resulting in either urinary incontinence or elevated bladder pressures[83-85]. An unstable bladder is commonly seen in neurologic conditions, such as following a stroke[86], or from a congenital abnormality to bladder innervation, as is seen in spina bifida[87]. Oxybutynin was the first anticholinergic medication to be introduced to prevent bladder contraction[88], and to this day remains a mainstay in urologic therapy. It functions directly on the smooth muscle of the bladder wall, blocking acetylcholine binding to muscarinic receptors, which thereby prevents muscle depolarization and contraction[89-91]. However, its nonspecific binding nature results in multiple other side effects for the patient, including dry mouth, constipation, blurry vision, flushing, and mental status changes, particularly in the elderly[92, 93]. More specific isoforms of this medication have been developed, with improved side effect profiles[94-97], but compliance remains an issue not only due to residual side effect, but also for cost[98, 99].

1.3.4 Clean Intermittent Catheterization

No medication is able to stimulate controlled bladder contraction and sphincteric relaxation, and therefore a mechanical form of assistance is required. This can be achieved with a urinary catheter. Prolonged bladder catheterization can lead to multiple deleterious complications[100,

[101]. Indwelling catheters are associated with an increased rate of urinary tract infection[102]. Prolonged pressure on the urethral meatus can lead to necrosis, resulting in urethral erosion[103]. Finally, continuous drainage of a previously normally functioning bladder leads to the absence of normal cyclic bladder contractions associated with voiding, which can lead to urinary retention from detrusor laxity. Clean intermittent catheterization (CIC), first described in 1972[104], allows for the avoidance of many of these issues, by allowing for regular drainage of a bladder without a continuous indwelling foreign body. Its use is indicated in patients unable to otherwise void, or having a diagnosed high-pressure bladder on urodynamics. Its complications include urinary tract infection, trauma to the lower urinary tract, and epididymo-orchitis[105-108]. While the therapy is generally well tolerated, particularly in patients who lack perineal sensation, its invasive nature can often lead to a delay of its institution[109]. As a result, many of these patients have high urine storage pressure, and increased risk of renal injury.

1.3.5 Lower Urinary Tract Reconstruction

Conservative measures for the management of partial bladder obstruction will fail in 1 out of 5 patients, in whom the bladder continues its process of hypertrophy, leading to a stiff fibrotic bladder with poor capacity and compliance[110]. Eventually, bladder pressures will rise to an unsafe level, requiring surgical intervention. Bladder augmentation relies on the inverse relationship between pressure and volume. Historically, many

procedures have been attempted for bladder augmentation, ranging from autoaugmentation to grafting of synthetic material. Today, the most commonly performed procedure for bladder augmentation relies on readily available tissue from the gastrointestinal tract, the most common of which is ileum.

In an ileocystoplasty[111], a detubularized segment of ileum is folded on itself in a U or W configuration to form a 'sheet' of tissue, which is then sewn onto the anterior surface of the bladder. This effectively increases capacity, and increases compliance due to the introduction of distensible tissue. While this can effectively relieve high intravesical pressure, complications of this procedure are significant and not uncommon[112].

1.3.5.1 Gastrointestinal Complications

An ileocystoplasty requires a lower midline incision and entry into the intraperitoneal and extraperitoneal space, and small bowel resection[113, 114]. As a result, adhesions following this procedure are common, and bowel obstructions have been reported in up to 3% of patients[115]. Ileum is also the sole site of vitamin B12 absorption in the body, and if the terminal 15-20 centimetres of ileum are used, deficiency will occur[116].

The bowel will continue to secrete mucus after placement in the urinary tract. As such, mucus buildup can interfere with urinary drainage during voiding or catheterization, and daily bladder irrigation is

recommended to minimize its presence[117, 118]. Excess mucus in the bladder can serve as a nidus for both stone formation and infection[119].

Finally, the bowel itself can perforate, leading the spillage of urine into the intraperitoneal or extraperitoneal space. This complication can be fatal, as these patients often have decreased sensation and present in severe sepsis. While the etiology of delayed perforation is unclear, it has been suggested that it may be related to catheterization trauma, fixed extravesical adhesions, and chronic overdistension[120]. In the Wayne State University series, perforations were noted in 9.6% of patients, averaging 4.3 years following their initial operation[115]. Early recognition and surgical correction of this fatal complication is key to ensuring patient survival.

1.3.5.2 Metabolic Complications

The exposure of functional small bowel to urine is an abnormal situation, and can cause multiple metabolic abnormalities, resulting from a functional piece of bowel with intact absorptive mechanisms being exposed to urine and the resultant absorption of ammonium ion. Most frequently, when ileum or colon is used, these abnormalities present in a hypercholoremic metabolic acidosis[121-124], which is exacerbated with any impairment of renal function. This chronic excess of hydrogen ion leads to bone demineralization, in a physiologic attempt to buffer the blood pH, and eventually leads to osteoporosis and the impairment of linear growth.

Furthermore, a chronic acidosis leads to a decreased urinary excretion of citrate ion, which is a potent inhibitor of urinary stone formation. Drug absorption can be increased for drugs relying on renal filtration, such as Dilantin, making close monitoring of levels a necessity[125].

1.3.5.3 Infectious Complications

Nearly all patients following ileocystoplasty will have chronic bacteriuria, thought to be related to the use of clean intermittent catheterization and mucus accumulation. Recurrent symptomatic cystitis has been reported in 23% of patients following this procedure, with 13% having a febrile infection[126]. Such high rates of infection can lead to further morbidity for these patients.

1.3.5.4 Malignancy

Urine exposure to bowel, in the setting of ureterosigmoidostomy, has been estimated to increase the risk of development of adenocarcinoma by 7000 fold, compared to age matched controls[127, 128]. The etiology is thought to be related to chronic inflammation and carcinogen exposure, although the exact etiology is unknown. Adenocarcinoma, transitional cell carcinoma, and anaplastic lesions have been described following ileocystoplasty, with latent periods ranging from 3 to 53 years[129].

1.4 Current Research

1.4.1 Molecular Pathways

While we have a crude understanding of the histologic and clinical effects of pBOO on the bladder, we require a better understanding of the molecular pathways involved before new therapies can be targeted and developed. The pathways under analysis involve mediators of cellular stress and inflammation. The signal transduction cascades in question involve transforming growth factor β (TGF-β), nitric oxide (NO), the mammalian target of rapamycin (mTOR), and many of their downstream targets. From an extracellular point of view, other enzymes associated with the construction and degradation of extracellular matrix are under examination, such as the family of matrix metalloproteinases (MMPs) and the regulation of their inhibitors (TIMPs), connective tissue growth factor (CTGF), insulin-like growth factor (IGF), and multiple other cytokines and chemokines.

1.4.1.1 TGF-β

The transforming growth factor β (TGF-β) family comprises a large number of structurally related peptides that globally control a wide array of cellular processes[130]. These growth factors have been implicated in proliferation, adhesion, migration, and death. Its action, on a more systemic scale, has been implicated in wound healing, angiogenesis, and carcinogenesis[131, 132]. Clearly, this is a cellular signal that regulates multiple cellular processes, and depends not only on the cellular

microenvironment, but also on associated signals enhancing or counteracting its actions.

Three major isoforms have been identified in mammalian species – TGF-β1, TGF-β2, and TGF-β3[133, 134]. Each of these ligands has its own set of specific TGF-β receptors that reside on the surface of the cell membrane[134, 135]. The binding of this ligand leads to the dimerization of two TGF-β type I receptors and two TGF-β type II receptors, resulting in a tetrameric protein which in turn activates intracellular signaling mainly through the phosphorylation of SMAD proteins[133, 136, 137]. While the protein itself is produced in an inactive form (pre-pro-TGF-β), a complex series of intracellular processes lead to the binding of a processed but inactive form to latency associated peptides (LAP), the dissociation of which allows for either a soluble or membrane-bound TGF-β to exert its action[131].

SMAD is a direct substrate for the protein serine kinase activity of the activated TGF-β receptor[133]. Upon phosphorylation, activated SMAD protein complexes move to the nucleus and contact specific gene promoters, thus activating transcription. Multiple isoforms of SMAD have been identified, each with unique function. SMAD-3 deficient mice, for example, have been shown to generate a significant pro-fibrotic response, markedly accelerated wound healing, and a greatly elevated rate of carcinogenesis[138].

One of the major discoveries in elucidating the role of TGF-β relates to its key role in the epithelial-mesenchymal transformation[139]. Multiple cell types are involved in inflammation and fibrosis, where the primary cell is the myofibroblast. Its differentiation from epithelial cells is crucially dependent on TGF-β[140]. Further research of this pathway demonstrated TGF-β's role in metastasis and carcinogenesis, where malignant cells are often deficient in inhibitory signals of the TGF-β cascade[132, 141, 142].

In the process of bladder hypertrophy and fibrosis, TGF-β's role has been well studied but is far from understood[143, 144]. Its RNA signals have been shown to be upregulated in acute overdistension of the bladder, but subsequently downregulated in chronic obstruction[145-149]. Exposure of bladder smooth muscle cells to TGF-β decreases key cellular adhesion molecules, which is thought to be part of the pathogenesis of poor bladder contractility[150-153]. When the TGF-β type II receptor is absent, in the setting of a knockout mouse model, bladder fibrosis is inhibited[149]. Current research has shown us that TGF-β has a crucial role in the initial steps of the pathologic process of bladder hypertrophy and fibrosis, but its role past these initial steps remains a mystery.

1.4.1.2 Nitric Oxide

Since the discovery of nitric oxide (NO) as endothelial derived relaxation factor (EDRF) in the early 1980s[154], a significant amount of

research has been performed in elucidating its synthesis, regulation, and role, particularly in the setting of smooth muscle relaxation. While its function systemically can be summarized as the induction of smooth muscle relaxation, much still remains to be gained in terms of therapeutic potential in the modulation of its function[155]. Furthermore, it has been identified as a primary neurotransmitter in non-adrenergic non-cholinergic neurons (NANC)[156, 157], whose role not surprisingly stems around muscle relaxation. Recent evidence and research have identified a role for NO release in the urinary tract[158], initially in the setting of erectile dysfunction, and these smooth muscle relaxation mechanisms are only starting now to be therapeutically exploited for the relief of LUTS and pBOO.

NO is produced by several isoforms of nitric oxide synthetase (NOS)[159]. At present, three classes of NOS have been identified: an inducible form, relying on calcium ion release and calmodulin activation (iNOS), and endothelial form (eNOS), and a neuronal form, associated with the NANC system (nNOS)[160]. Once nitric oxide is synthesized from its substrate, L-arginine, it its released into the circulation or cellular microenvironment. Its interaction with guanydyl cyclase results in the intracellular formation of cGMP from GTP, resulting in multiple downstream effects. In muscle tissue, cGMP can induce the opening of calcium channels, and the activation of protein kinase A_2 and G, all of which collectively relaxes smooth muscle by disruption of actin-myosin bridges[161-163].

The activity of these cyclic nucleotide second messenger systems is modulated by a large family of enzymes that catalyze its hydrolysis: phosphodiesterases (PDE). At present there have been over 50 isoenzymes identified in various tissues[164].

Inhibitors of phosphodiesterases were initially developed for the treatment of pulmonary hypertension, through the augmentation of NO effects on endothelium. It was found that their effect could be used for other smooth muscle dependent processes, namely erectile dysfunction[165].

PDE inhibition for erectile dysfunction led to an unusual discovery. It was found that men who took this class of medications also experienced an improvement in voiding symptoms[164]. Experimental work has previously shown that nitric oxide can induce detrusor muscle relaxation, as well as urethral relaxation[161, 166, 167]. Mapping studies have identified a significant NANC contribution to the neuronal control of micturition[158]. PDE's have been identified now in nearly all tissues pertaining to the lower urinary tract, from the distal ureter, to the bladder itself, the prostate, and the urethra[168-171]. Multiple studies have now been conducted using PDE inhibitors to assess their impact on voiding symptoms, each of which has yielded a significant positive result[172-178]. While the exact biochemical mechanism for PDE inhibition's role in the relief of LUTS secondary to pBOO has yet to be elucidated, the role of nitric oxide and cGMP on the relaxation of the lower urinary tract cannot be ignored.

The role of NO in the inflammatory response, primarily through the induction of iNOS, has been well studied. Similarly, the role of iNOS in the pathophysiology of pBOO is becoming clearer[169]. BOO induction has been shown to induce mRNA transcription of iNOS, and the absence of this enzyme in a knockout model has been shown to ameliorate the changes associated with chronic obstruction[179, 180]. Similarly, iNOS inhibition has been shown to reduce bladder contractility and fibrosis[166]. Furthermore, elevated levels of NO can diminish the pro-inflammatory effects of TGF-β on bladder tissues[181]. Given these important experimental results, it has been shown that treatment of obstructed mice with sildenafil can decrease detrusor overactivity, hypertrophy, and fibrosis[182]. As such, the modulation of the NO response lends itself to be a prime therapeutic target in pBOO pathology.

1.4.1.3 mTOR

The mammalian target of rapamycin (mTOR) is a central, evolutionarily conserved signaling molecule that can control multiple cellular processes[183]. The inhibition of this molecule's action has shown to be immunosuppressive, as is seen in the setting of organ transplantation[184]. Similarly, administration has been shown to have strong antiproliferative effects, evident in the setting of delayed wound healing following transplant[185, 186]. Its use has been seen in scar modulation[187, 188], and more recently for the inhibition of transplant arteriosclerosis[189], and in coronary artery stents for the prevention of

intimal hyperplasia and stent failure[190, 191]. We are only recently beginning to understand the role of this molecule in carcinogenesis[192, 193], and its inhibition has been investigated in lung cancer[194, 195], urothelial cancer[196-198], and hepatocellular carcinoma[199-201]. This molecule is a strong mediator of cellular proliferation, and its selective inhibition may prove useful in a number of clinical scenarios.

Activation of mTOR[183] happens through a complex multistep pathway that begins with upstream activation of phosphinositide-3-kinase (PI3K) and AKT activation, leading to inactivation of the tuberous sclerosis complex 1 and 2 (TSC1 / TSC2) heterodimer[202]. The inactivation TSC1 / TSC2 leads to the release of inhibition of Rheb, with subsequent activation of mTOR through Rheb GTPase activity. From there, mTOR can associate with one of two molecules, Rictor and Raptor, leading to multiple downstream effects[203, 204]. Association with Rictor activates AKT, SGK1, and PKCα, leading to actin cytoskeleton modification, cellular proliferation, and survival[205]. Association with Raptor activates S6K1, 4EBP1, and HIF1-α, leading to a coordinated promotion of protein and lipid synthesis, and subsequent cell growth and proliferation[206, 207].

Given that the pBOO initially involves a hyperplastic and proliferative phase, it would seem to follow that inhibition of cellular processes that drive hyperplasia might be beneficial to preventing pBOO pathology. There has been to date one combined *in vitro* / *in vivo* study on the role of mTOR inhibition on bladder smooth muscle cells (BSMC)

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and in an obstructed rat model[208]. Administration of rapamycin to stressed BSMCs inhibited proliferation, de-differentiation, and activation of downstream targets of the mTOR pathway. *In vivo* analysis showed a decrease in smooth muscle actin, and maintenance of MMP-7 activity, both in keeping with antiproliferative effects. These data suggest that the mTOR pathway is activated in pBOO, likely through mechanical stretch and possibly hypoxia[208]. At present, we have no indication of what early administration of mTOR inhibitors to an obstructed bladder can achieve, both from a clinical / urodynamic perspective, as well as a histopathologic one.

1.4.1.4 Fibrocytes

While the concept that circulating fibroblast-like cells are directly involved in the wound healing response, more conclusive evidence for the existence of these cells has surfaced in recent years. These cells, known as fibrocytes, appear to be a bone marrow derived and spleen matured circulating cells that home to sites of tissue injury throughout the body[209]. The can serve as a potent antigen presenting cell, can activate T-cells, and produce multiple cytokines, chemokines, and extracellular matrix proteins involved in fibrogenesis and wound healing[210, 211]. In a normal homeostatic state, fibrocytes tend to comprise between 0.1%-0.5% of cells in the circulation. However, in circumstances of severe systemic injury, such as burns, their prevalence in the circulation can increase to nearly 10%[212]. While initial descriptions of these cells was limited primarily to

scar formation and liver fibrosis, newer evidence suggests that this population of cells may be implicated in fibrotic processes throughout the body.

Multiple combinations of unique surface markers have been used to characterize these cells, the most commonly accepted ones are CD45, CD34, CD11b, collagen I, and LSP-1[213]. Their cellular ultrastructure appears as a spindle-shaped cell, unique from both a fibroblast and a leukocyte, with multiple pseudopodia and microvilli. Following their activation by T-cells, in the presence of TGF-β, they hone to injured tissue, express collagen I & III, PDGF, IL-1, MIP, and α-SMA[214]. As such, wound healing and scarring appears to be partially dependent on this unique population[215].

Initial reports discovered the presence of these cells in normal[216] and hypertrophic scars[217, 218], as well as in liver fibrosis[219]. New studies have identified the presence of fibrocytes in lung fibrosis[220], as well as kidney fibrosis[221]. The mouse model of unilateral ureteral obstruction has served as a very useful clinical model of renal fibrosis, particularly when considering that the contralateral kidney can serve as an endogenous control[222]. Studies on the fibrotic process on these animals have identified the presence of fibrocytes in fibrotic kidneys, and further elucidated nuances in the T-cell / chemokine / fibrocyte interaction that leads to activation of the fibrotic process and collagen deposition[223-227]. Interestingly, the first report of fibrocytes being present in human renal

biopsy studies from patients with renal fibrosis became available earlier this year[228].

Fibrocytes, as yet, have never been described directly in the bladder outside of chronic inflammation and urothelial carcinoma[229]. To date, only one study has shown the presence of bone marrow derived cells in an pBOO mouse model[230]. Unfortunately, no further immunohistochemical analysis was performed in this setting, and the cellular identification of these cells has yet to be elucidated. Given our understanding of fibrocyte differentiation and involvement in many fibrotic processes, it would not be unreasonable to consider the possibility that fibrocytes could be involved in the process of bladder fibrosis as well.

Nephrogenic systemic fibrosis is an unusual clinical entity that was identified in a small subset of patients undergoing gadolinium-enhanced magnetic resonance imaging[231]. While gadolinium was initially thought to be safe to use in patients with impaired renal function, the presence of this crippling fibrotic syndrome in gadolinium-exposed patients has challenged that notion. Current research into this syndrome has revealed that monocyte and macrophage interacting with both chelated and unchelated gadolinium leads to their activation, and activated fibrocytes have been identified in the scars that occur in this syndrome[232-236]. While the precise etiology and predisposing factors to the initiation of this systemic inflammatory cascade is unclear, this serves as a model for what can happen when fibrocyte activation is out of control.

Studies into unilateral ureteral obstruction and nephrogenic systemic fibrosis have yielded several important results in terms of immune modulation. Kidney fibrosis is greatly decreased with either blockade of TGF-β or SMAD, either directly or in a knockout model[224, 237]. Kidney fibrosis and T-cell activation is greatly decreased with administration of rapamycin, and subsequent mTOR blockade[222]. Furthermore, nephrogenic systemic fibrosis can be arrested or alleviated with early administration of rapamycin[238].

1.4.2 Regenerative Medicine

The very concept of regenerative medicine is a departure from much of current surgical practice. Rather than looking at the modulation of pathophysiologic processes, the promise of regeneration involves the repair or replacement of abnormal or dysfunctional tissues. Attempts have been made for multiple cell types and organs, including bone, liver, heart, and even kidney. However, the first publication and introduction of a commercially available regenerated organ into clinical trials is a bladder[239]. While significant hurdles remain in the incorporation of this technology into mainstream urologic practice, the current outlook of the field is astoundingly promising.

1.4.2.1 Unseeded Scaffolds

The first attempts at bladder augmentation with artificial or biosynthetic tissues involved the incorporation of a scaffold in place of a

bladder augment. The choice of material is critical in this circumstance. Non-absorbable materials in the urinary tract become a nidus for stone formation. Attempts were first made using collagen, acellular matrix, alginate, small intestine submucosa (SIS), and polyglycolic acid polymers[240-245]. The latter materials showed most promise. In select circumstances and reports, these materials were found not only to be organically and immunologically inert, but in in some cases even allowed for the growth of multilayered bladder tissue, consisting of muscle and urothelial layers[246]. Problems remained, that while a transitional epithelium was successfully expanded onto the graft, the muscle layer remained abnormal, disorganized, and poorly vascularized. Eventually, the graft itself would fibrose, leading to a decrease in bladder capacity. Although neovascularization can be improved using porous graft materials, this is not compatible with the concept of a watertight bladder[247]. While reports of the success of these materials were variable, both in the initial success and incorporation of the graft, as well as its longevity and freedom from fibrosis, they served as a proof-of-concept that this technology could eventually succeed.

1.4.2.2 Seeded Scaffolds

In an attempt to create a more physiologic bladder replacement material, attempts were made using cell seeded acellular matrix grafts[248-250]. The success that was achieved by Atala in constructing a regenerated neobladder relied on this concept[239]. The concept begins with obtaining a

bladder biopsy from a patient, and separately expanding both the urothelial cells and muscle layers *in vitro*. Once adequate cell numbers are obtained, these are seeded onto a hemispherical collagen or collagen / polyglycolic acid scaffold, and allowed to culture for several weeks. The seeded scaffold is then sewn onto the bladder, acting as an augmentation, and wrapped in omentum for vascular supply. This procedure was initially performed on beagles[251], and subsequently on humans. The most important success that can be identified from these studies is that bladder regeneration is possible, the serving as proof of concept. Criticism of this study, however, lies mainly in the choice of scaffold material. While muscle and urothelial layers in this model do grow in a physiologic fashion, the compliance is unchanged. As such, urodynamic data from these grafts shows primarily an increase in capacity, but not in compliance. While promising, this does not fit the representation of a true replacement of bladder tissue.

Other cells have been used in the seeding of grafts. Specifically, bone marrow derived mesenchymal stem cells (BSMC) have been used with successful seeding on grafts[252-255]. Given these cells are multipotent, their use has been seen in tissue engineering for vascular grafts, cartilage, and neural materials. Their ability to differentiate into smooth muscle, as demonstrated with vascular regeneration, has shown promise in the field of bladder regeneration.

The search for a new scaffold material resulted in the discovery of poly(1,8-octanediol-co-citrate) (POC)[256, 257]. This is a biodegradable polymer that is immunologically inert with a short half-life, has a customizable elastic modulus, and can serve as a scaffold material with no ill effects on cells. These properties alone make POC a more ideal scaffold material than the previously used collagens.

Combining a POC scaffold with BSMC has yielded the most promising application of bladder tissue regeneration to date[258]. Sharma's group successfully created a POC scaffold with similar elastic modulus to native bladder tissue, with successful seeding of BSMCs. Their results showed a new graft that develops typical bladder tissue architecture, with retention of normal bladder muscle formation and expression of normal contractile proteins. Muscle to collagen ratios in the BSMC seeded grafts were nearly physiologic, in comparison to smooth muscle seeded POC grafts. This technology, while awaiting proof in a human host, appears to have the greatest promise for the partial regeneration of bladder tissue.

While each of the new scaffolds developed has shown biocompatibility and similar histology to normal tissue, the only functional data available indicate an augmented bladder capacity. The remaining characteristics of the graft, specifically contractility and muscle stability, remain unknown. Further research will be required before human application of these novel biosynthetic tissues.

1.5 Conclusions and Formulation of the Research Problem

The translation of the considerable amount of research performed in this field will ultimately be the future challenge in its clinical application. While not for shortage of avenues to exploit, many of these need further exploration in animal models prior to application in humans. The availability of an inexpensive and easily reproducible rat model for pBOO will be essential in allowing this translation to occur.

Today's current research environment has provided us with a considerable amount of information regarding the inflammatory cascade, from the cellular level to the molecular pathways that modulate it. Pharmacologic research has presented several medications, which can affect the pBOO pathway. While excellent work has been done in the clinical use of α-blockers and 5-α reductase inhibitors to treat benign prostatic hypertrophy, there has yet to be an attempt at translating the use of immune modulators to treat the diseased bladder. Given the promise shown in the use of mTOR inhibitors and PDE inhibitors in other clinical settings, their use to treat pBOO may prove clinically relevant.

We will attempt to modulate the pathophysiologic process of partial bladder outlet obstruction by oral medical therapy. Sprague-Dawley rats will undergo surgical partial bladder outlet obstruction. Three cohorts of rats will be created, based on daily oral therapy with either rapamycin, tadalafil, or oxybutynin, as well as a control arm over a 16 week

timeframe. We hypothesize that the established process of pBOO from inflammation, to hypertrophy, to fibrosis will be diminished by each of these medications. Tadalafil, by increasing intracellular cGMP levels and inhibiting phosphodiesterase activity, may help relax the bladder through this process, thereby preventing the cascade of inflammation and hypertrophy. Rapamycin, by its immune modulatory and antiproliferative effects, could prevent the inflammatory phase of obstruction, and this prevent the fibrosis seen in late pBOO. Oxybutynin will be administered at high doses to these rats, as it is the current gold standard therapy. Use of these medications to inhibit pathophysiologic decompensation is entirely novel, and based on our understanding of molecular biology and pharmacology, these agents could prove to be effective.

The potential for treating pBOO is remarkable, and a considerable amount has been learned to this point. With a validated model now in place, we can now begin to further investigate the medical treatment of pBOO, from both a functional and molecular biology aspect. We can attempt new tissue engineering procedures, and assess the functional improvements they portend. This foray into the research of bladder outlet obstruction will provide key information for the future translation of these therapies to improve our patient's well being.

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Chapter 2 - Experimentation: Differential Expression of Class I Small Leucine-Rich Proteoglycans in an Animal Model of Partial Bladder Outlet Obstruction

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2.1 Introduction

Partial bladder outlet obstruction (pBOO) is a significant and common problem in urology, resulting in sequelae seen in both adult and pediatric patients. Recent experimental work has demonstrated the progressive deterioration of the bladder in an animal pBOO model though a process of initial inflammation, followed by hypertrophy, and ending in fibrosis[1]. The end-stage of the obstructive pathology involves fibrosis from deposition of collagen in the bladder wall[2]. How this process occurs and is regulated remains to be elucidated.

Small leucine-rich proteoglycans (SLRPs) are a series of structurally and functionally related extracellular matrix molecules that are present in connective tissue and regulate key steps in cell adhesion, gene expression, and growth factor activity[3]. The process of collagen fibrillogenesis is thought to be regulated by a tightly controlled and orchestrated sequence of SLRPs binding to collagen fibrils, resulting in a correctly synthesized matrix with specifically determined physical properties. SLRPs are divided into four classes based on their temporal

expression[3]. Prior work has shown in models of pulmonary fibrosis[4], liver cirrhosis[5], and hypertrophic scar[6] that the expression and synthesis of two specific SLRPs is pathologically altered: decorin tends to be downregulated, and biglycan tends to be upregulated.

Decorin and biglycan are class I SLRPs. Decorin has multiple high affinity binding sites to collagen, and has been shown to regulate the diameter of collagen fibrils so that resultant helices are thinner and more regular in diameter[7]. Biglycan has two separate glycosaminoglycan chains, both of which are thought to be critical in its binding mechanism to the forming collagen helix. Biglycan has been shown to be important in regulating collagen cross linking without having an effect on fibril diameter[8].

SLRPs have not been described in fibrosed bladder. Prior work has demonstrated that the pathology behind the process of pBOO is heavily dependent on immune system activation[9]. We hypothesized that the scarring process in this model should provide a similar SLRP profile as other previously studied scar models, in that pBOO would show a progressive decrease in decorin, alongside an increase in biglycan.

2.2 Materials & Methods

2.2.1 Animal Use & Surgical Partial Bladder Outlet Obstruction

Full approval from the University of Alberta Animal Care and Use Committee was obtained prior to commencing research. Adult female

Fisher rats underwent a pBOO procedure as previously described[10]. Anesthesia was induced with isoflurane, and the bladder exposed through a lower midline incision. An 18 gauge angiocatheter was introduced into the bladder through a superior cystotomy and advanced to the urethra. Urodynamics were performed at an infusion rate of 0.1 cc per minute, with pressure measurements being obtained by a transducer at 15 second intervals. Infusion was halted once leakage was visually detected at the urethral meatus.

Following conclusion of urodynamic measurements, the catheter was advanced antegrade into the urethra, and dissection of the bladder neck was undertaken with a right angle clamp. The clamp was passed around the urethra immediately below the level of the ureters, and a 2-0 silk suture was passed around and gently tied, using the angiocatheter as a calibration stent. The catheter was then removed and the cystotomy closed. The abdomen was closed in two layers.

Rats were sacrificed at 4, 8, and 12 week time points, alongside a sham group at 11 weeks. A total of three (n=3) animals were included in each time point, resulting a total of 12 animals. In the sham, the surgical procedure was identical aside from the passing of a silk suture around the urethra. Animal weight was obtained prior to surgery, and followed over the course of the experiment as a measure of animal health.

At sacrifice, urodynamics were performed and organs harvested.

Harvested tissue was either snap frozen in liquid nitrogen for subsequent protein and nucleic acid analysis or fixed in 4% paraformaldehyde as well as Shandon Cryomatrix (Thermo Scientific, UT). Paraffin and Cryomatrix blocks were mounted and sectioned.

2.2.2 RT-PCR

Total RNA was extracted from snap-frozen tissue specimens. Tissue was deep frozen in liquid nitrogen, then homogenized using a Micro-Dismembrator (B. Braun Biotech Inc., Allentown, PA). The powder was reconstituted in Trizol solution. Total RNA extracted using RNeasy spin columns (Qiagen, Mississauga, ON, Canada). Contamination from genomic DNA was removed by DNAse digestion for 60 minutes. First strand cDNA was synthesized using random primers in a first strand synthesis kit (Sigma, Oakville, ON, Canada). Real-time RT-PCR was conducted using Power SYBR Green PCR Master Mix (ABI, Foster City, CA) in a 25 µL tube for a total reaction volume of 25 µL containing 1 µL of first strand product and 0.2 µM of gene specific upstream and downstream primers for decorin and biglycan. For control reactions, GAPDH primers were used. Amplification and analysis were performed using an ABI 7300 real-time system (Applied Biosystems, Foster City, CA). Cycling conditions were an initial denaturation at 95°C for 3 minutes, followed by 40 cycles consisting of a 15 second denaturation interval at 95°C, and a 30 second interval for annealing and primer extension at 60°C. GAPDH amplification was used for standardization of the amplification curves.

Primer sequences for biglycan were GATGGCCTGAAGCTCAA for the forward sequence, and GGTTGTTGAAGAGGCTG for the reverse. Primer sequences for decorin were TGGCAGTCTGGCT for the forward sequence, and ACTCACGGCAGTG for the reverse. Primer sequences for GAPDH were CCTGGAGAAACCTGCCAAGTAT for the forward sequence, and CTCGGCCGCCTGC for the reverse.

2.2.3 Immunofluorescent Staining

Using Cryomatrix blocks, sections at 8 µm were mounted and initially fixed in acetone for 10 minutes, then permeabilized using Triton-X 100 solution at 0.5% for 10 minutes. Slides were then washed with PBS, and blocked using BSA 5% for 1 hour at room temperature. Incubation with primary antibodies was done at 4°C temperature overnight at 1:200 dilution in a solution of PBS containing 1% BSA.

Primary antibodies used were sourced from Santa Cruz Biotechnology (Santa Cruz, CA), including mouse anti-human decorin (SC-73896) and rabbit anti-human biglycan (SC-33788). Secondary antibody incubation was then performed using Alexa-fluor conjugated antibodies sourced from R&D Systems (Minneapolis, MN), including anti-rabbit-594 and anti-mouse-488, for 1 hour at room temperature. After washing the sections with PBS, DAPI staining and coverslip mounting was performed using ProLong Gold Antifade reagent (Invitrogen, ON, Canada). Control immunostaining was performed using an appropriate

nonimmune serum resulting in negative staining results (data not shown). Images were captured using Carl Ziess Laser Confocal Microscope equipped with LSM5 software (Carl Ziess MicroImaging Inc., Thornwood, NY). Image pixel intensity was measured using ImageJ software (National Institutes of Health, USA). A total of four images were captured per slide.

2.2.4 Statistical Analysis

Data are presented as mean (standard deviation), and for parametric statistical analysis an unpaired two-tailed Student's t-test was performued using STATA Version 10.0 software (StataCorp, College Station, TX). A p-value of <0.05 was considered to indicate statistical significance.

2.3 Results

2.3.1 Animal Health

All animals used in our data collection survived to experimental endpoints in good health. Analysis of body weight revealed no significant difference between obstructed and sham animals.

2.3.2 Immunofluorescent Staining for Decorin

A clear trend for decreased fluorescent intensity over the experimental time course was evident on anti-decorin stained sections (Figure 2.1). Anti-decorin staining was localized to the interstitial areas surrounding muscle fibers, with minimal staining in urothelial layers. Quantitation of pixel intensity in ImageJ revealed a decrease in intensity

spanning across the experimental time course, with a statistically significant difference compared to sham appearing at 12 weeks (45.7 +/- 5.6 vs 16.3 +/- 1.7 intensity units, 2.76 fold, p=0.02) (Figure 2.2).

2.3.3 Immunofluorescent Staining for Biglycan

Image intensity was clearly augmented when examining anti-biglycan fluorescence over the experimental time course. Once again, staining was localized to the interstitial areas surrounding muscle fibers with minimal staining in the urothelial layers (Figure 2.3). Quantitation of pixel intensity in ImageJ revealed an increase in intensity across the experimental time course, with a statistically significant difference compared to sham at 12 weeks (65.5 +/- 2.48 vs 35.9 +/- 1.38 intensity units, p=0.02) (Figure 2.4).

2.3.4 Realtime Quantitative PCR

Quantitative PCR revealed a progressive downregulation of decorin mRNA over the experimental time course. This was most significant at the 12 week time point compared to sham (Mean CT Fold Change 0.45 +/- 0.24 vs 2.54 +/- 0.34, p=0.02), and also significant at the 8 week time point (p=0.01) (Figure 2.5).

There was a simultaneous upregulation of biglycan mRNA observed over the experiment. This was most pronounced at the 12 week time point (Mean CT Fold Change 2.04 +/- 0.33 vs .82 +/- 0.43, p=0.08) (Figure 2.6)

2.4 Discussion

While the end stage of pBOO remains a scarred, fibrotic bladder both clinically and in animal models, the process leading to the development of this fibrosis remains a mystery. Recent studies have shown SLRPs to be essential to the process of normal collagen fibrillogenesis in other anatomic sites and pathologic processes. However, the pattern of SLRP expression in an abnormal bladder has yet to be described.

We present the first description of differential SLRP expression in a model of an abnormal bladder. While SLRPs, specifically decorin, have been identified in bladder tissue in the past[11], they have yet to be described in a model allowing for temporal examination of the pathologic process. Other anatomic sites have shown that as abnormal collagen is synthesized and scar is laid down, biglycan tends to be more present than in normal tissues, and decorin tends to be more absent[6, 12]. Our results in this paper confirm these findings in the setting of bladder inflammation and fibrosis.

The aim of this study was to confirm altered SLRP regulation in an obstructed bladder. We found a downregulation of decorin mRNA over the time course of the experiment, and upregulation of biglycan mRNA. These results were significant at the 12 week time point compared to sham, but the trend was visible over the duration of the experimental time course. Immunofluorescent staining revealed the same trend in these molecules. These results provide evidence of consistency of the altered

regulation of these molecules on both a message and protein level. Decreased levels of decorin have previously been linked to development of scars, with reduction of scar mirroring the return of decorin to normal levels[12]. Furthermore, a diminished amount of decorin leads to the synthesis of collagen fibrils that are irregular in diameter, and thus show impaired tensile strength[13]. In a tissue that continually cycles, stretches, and contracts, it is not inconceivable that weak and continually damaged collagen may lead to a further progression of local inflammation. Furthermore, decorin has been shown to have a key role in the induction of apoptosis[14]. Its absence early in the process of pBOO could help contribute to the muscle hypertrophy and hypercellularity that was previously described by our group. Altogether, an abnormally diminished level of decorin in this setting may contribute to the development of scarring in our model, this accelerating the development of an end-stage bladder.

The role of biglycan in the inflammatory process only beginning to be understood. Prior work has shown that it may portend a role in inflammatory signaling, where in a renal obstruction model, biglycan levels were found to increase prior to macrophage infiltration[15]. Other work has shown that soluble biglycan can interact with Toll-like receptors on macrophages[16]. Furthermore, biglycan deficient mice in a unilateral ureteral obstruction model demonstrate impaired mononuclear cell infiltration[16]. Biglycan deficient mice have been shown to have a survival

benefit in lipopolysaccharide induced sepsis, with lower circulating levels of inflammatory cytokines[17]. It is becoming clear that this is a key molecule in the activation of the immune system, and not merely a bystander in the building blocks of the extracellular matrix. The increase we demonstrate in biglycan levels correlates with the induction of an inflammatory response. Our prior work demonstrated a strong and prominent inflammatory infiltrate in pBOO-affected bladders, which correlated with the upregulation of multiple pro-inflammatory markers. As biglycan appears to be crucial in the functioning of the inflammatory response, the robust and early increase we see in biglycan levels in this study would corroborate these results.

SLRPs appear to have an important role in immune system signaling and regulation as well. Studies have shown that decorin and biglycan, both in *in-vitro*[18] and *in-vivo*[4] models, can bind to TGF-β1, which is a key fibrotic mediator and involved in multiple systems of cellular signaling, proliferation, and inflammation. These studies suggest that there exists an inverse relationship between TGF-β1 signalling and resultant fibrosis, and SLRP expression.

Our group has previously reported the natural history of pBOO in this model[1]. We identified that these animals demonstrated from an initial inflammatory phase to smooth muscle hypertrophy at 4 weeks, and eventual fibrosis by 8-13 weeks. Urodnamic changes mirrored this process, with an initial increase in bladder capacity followed by a

decrease, as well as progressive increases in pressure . Collagen levels increased throughout the process. Our demonstration of the temporal relatinoship of SLRPs correlates with this data, giving further evidence that the pathway to fibrosis begins early after pBOO.

The promise of understanding the function and regulation of SLRPs could hold considerable therapeutic potential. It has been previously shown that augmenting decorin levels in scar models can lead to a favorable scarring outcome[19, 20]. The regulation of biglycan can theoretically lead to the development of a novel systemic or local anti-inflammatory strategy. Such treatements could potentially delay or prevent the onset of bladder fibrosis and development of the "end-stage" bladder . Garnering a better understanding of how to control the molecular mechanisms of inflammation in the bladder can potentially prevent multiple urologic morbidities, delay need for bladder augmentation, and potentially even improve quality of life by maintaining voiding function.

Our study has several important limitations that must be considered. Our numbers in this study are limited with 3 animals per experimental time point. The time course of the experiment also only progresses to 12 weeks, which potentially is not long enough to see further changes in bladder function and SLRP expression. While these are important limitations to consider, we feel that they do not impede the interpretation of the trends present in our data.

We present the first demonstration of differential SLRP expression in a model of a fibrotic bladder. The results we present here provide a new avenue for future therapeutic development for the prevention of bladder fibrosis in a clinical setting. A better understanding of the regulation of these important molecules and their exact roles in collagen fibrillogenesis can provide key information for the treatment of long term partial bladder outlet obstruction.
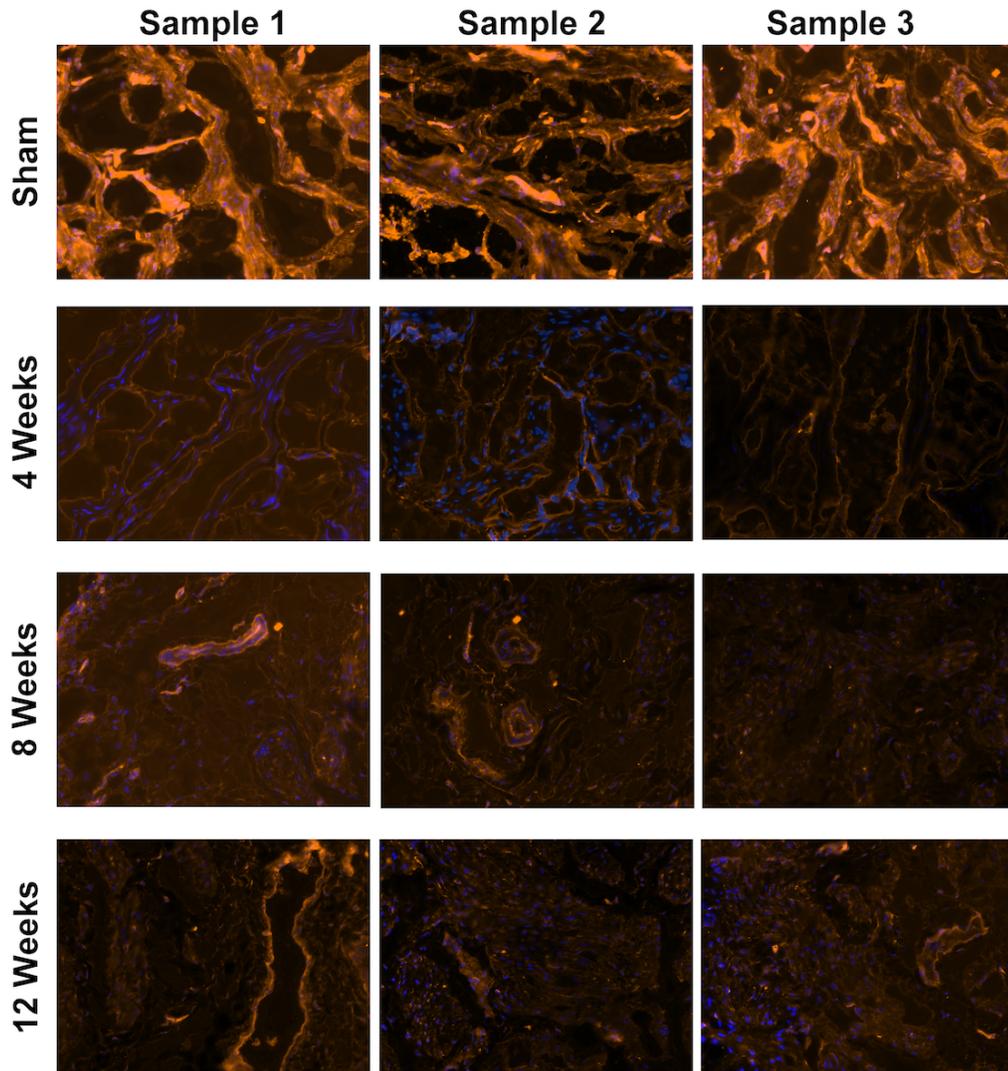
2.5 Figures

Figure 2.1: Immunofluorescent staining for decorin. Staining is localized to the interstitial areas of the bladder wall. A decrease in pixel intensity is evident over the experimental time course.
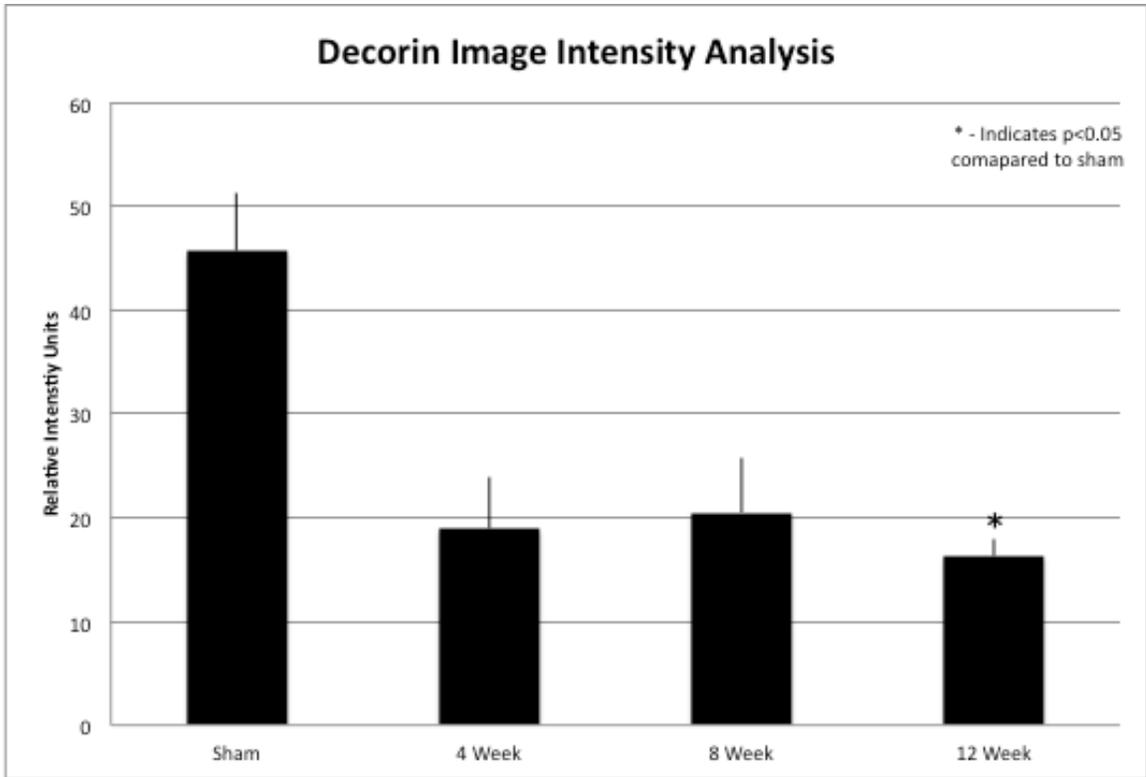
Figure 2.2: Image intensity analysis for decorin immunofluorescent staining.

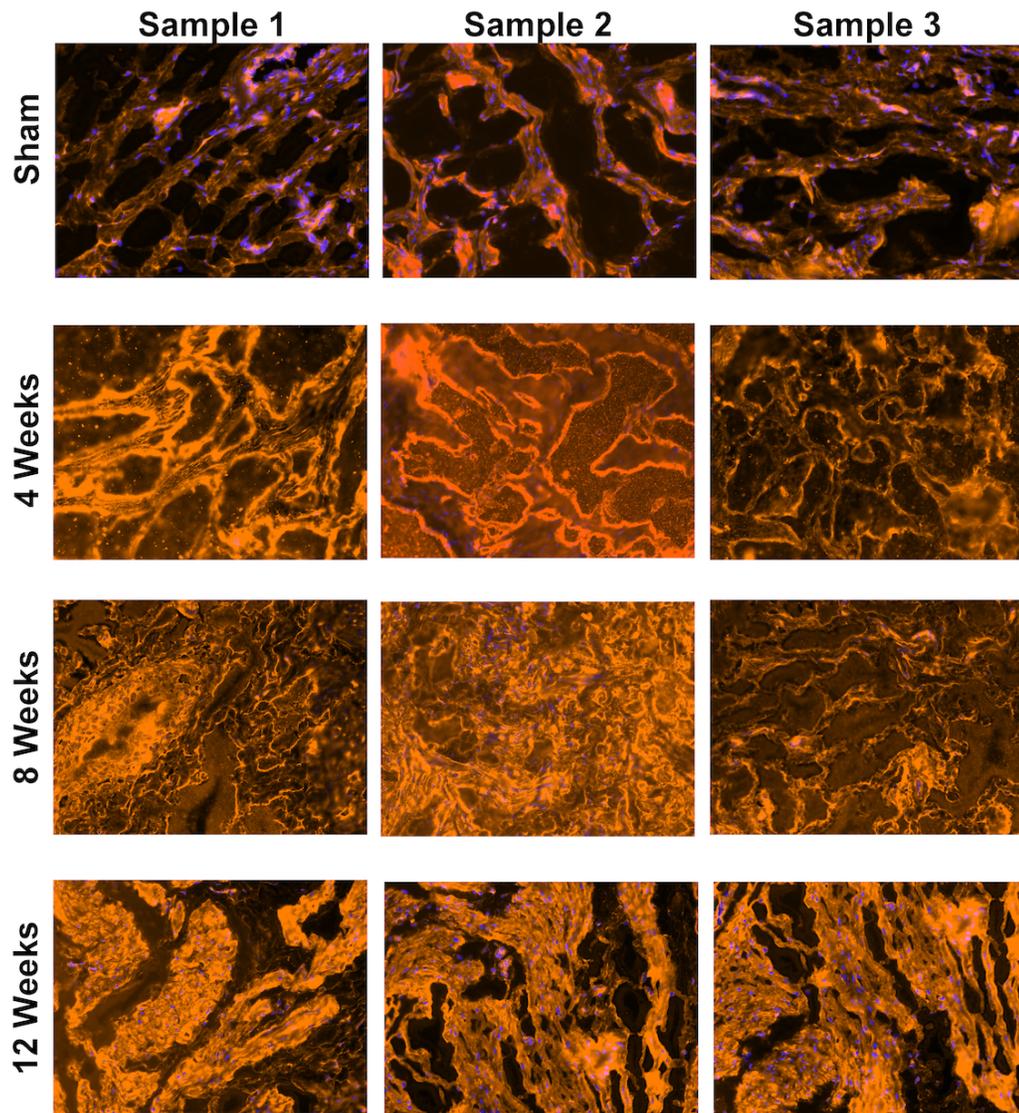A clear decrease in pixel intensity is seen over the experimental time course.

Figure 2.3: Immunofluorescent staining for biglycan. Staining is localized to the interstitial areas of the bladder wall. An increase in pixel intensity is evident over the experimental time course.
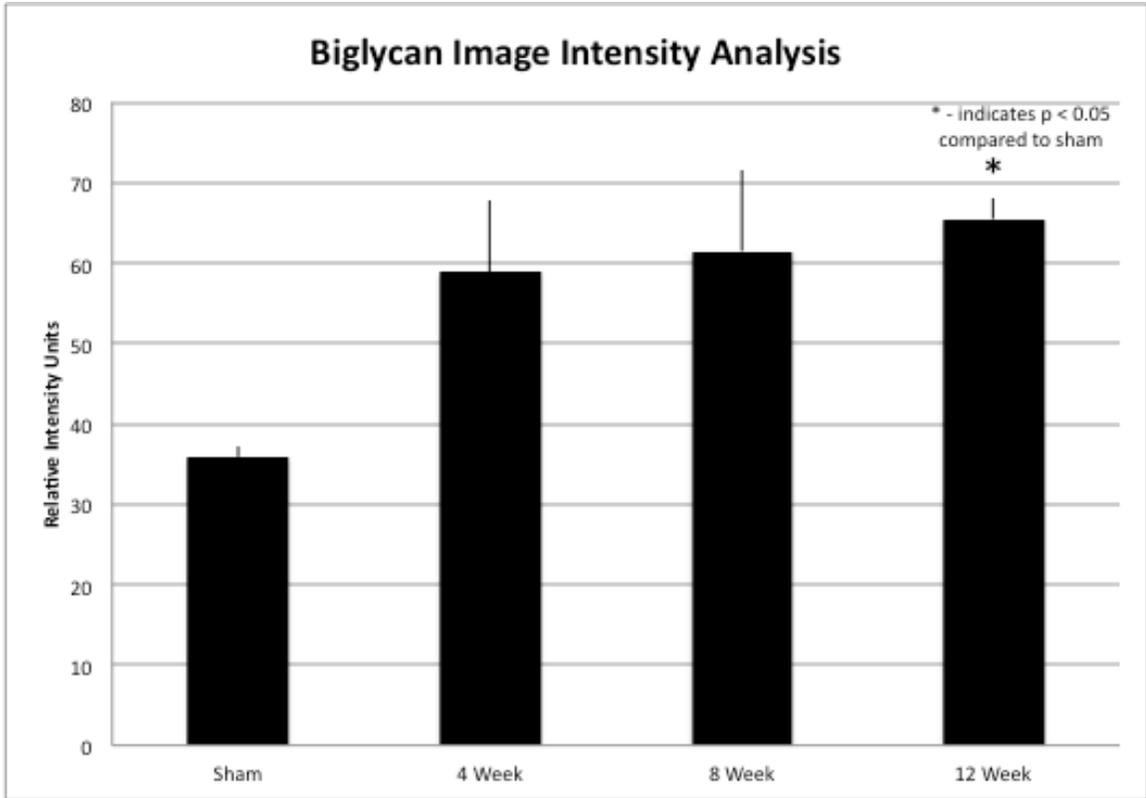
Figure 2.4: Image intensity analysis for biglycan immunofluorescent staining.

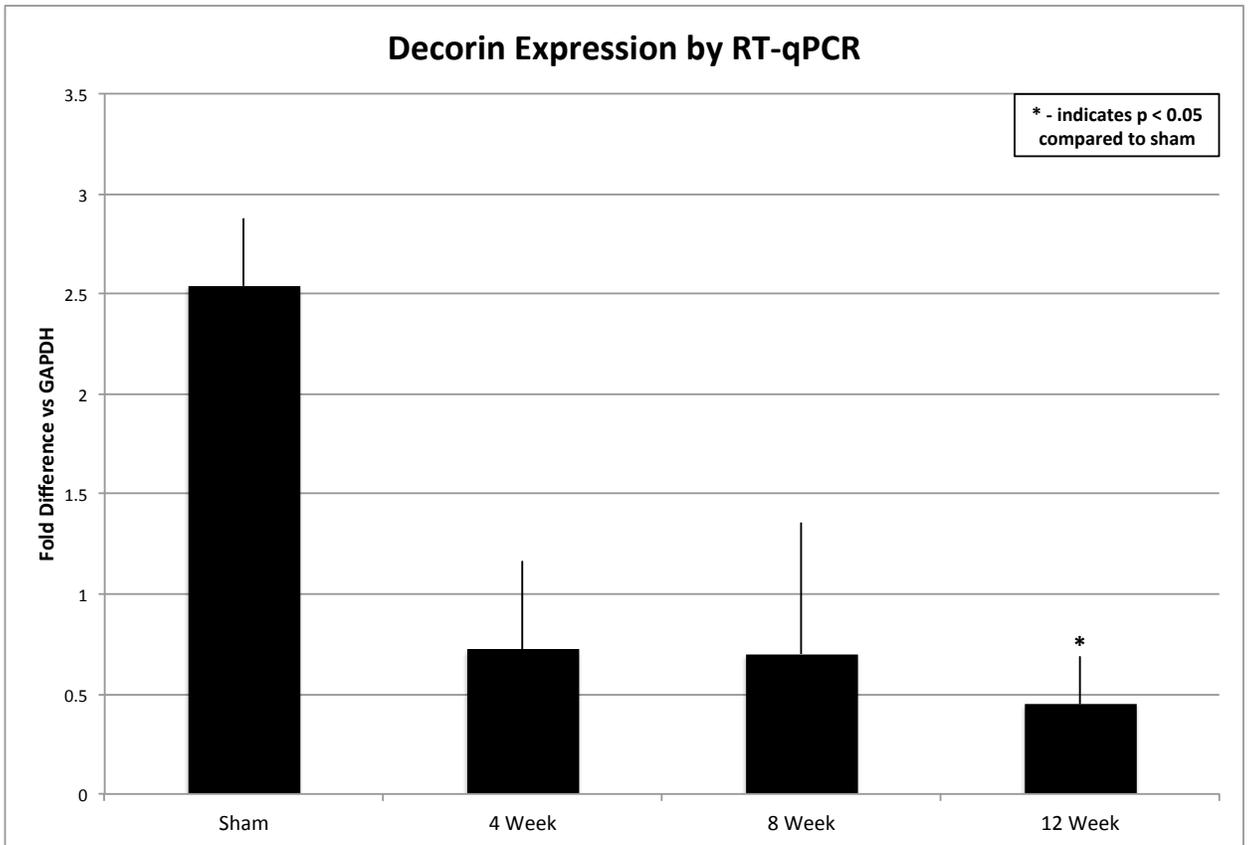A clear increase in pixel intensity is seen over the experimental time course.

Figure 2.5: Decorin expression by RT-qPCR.

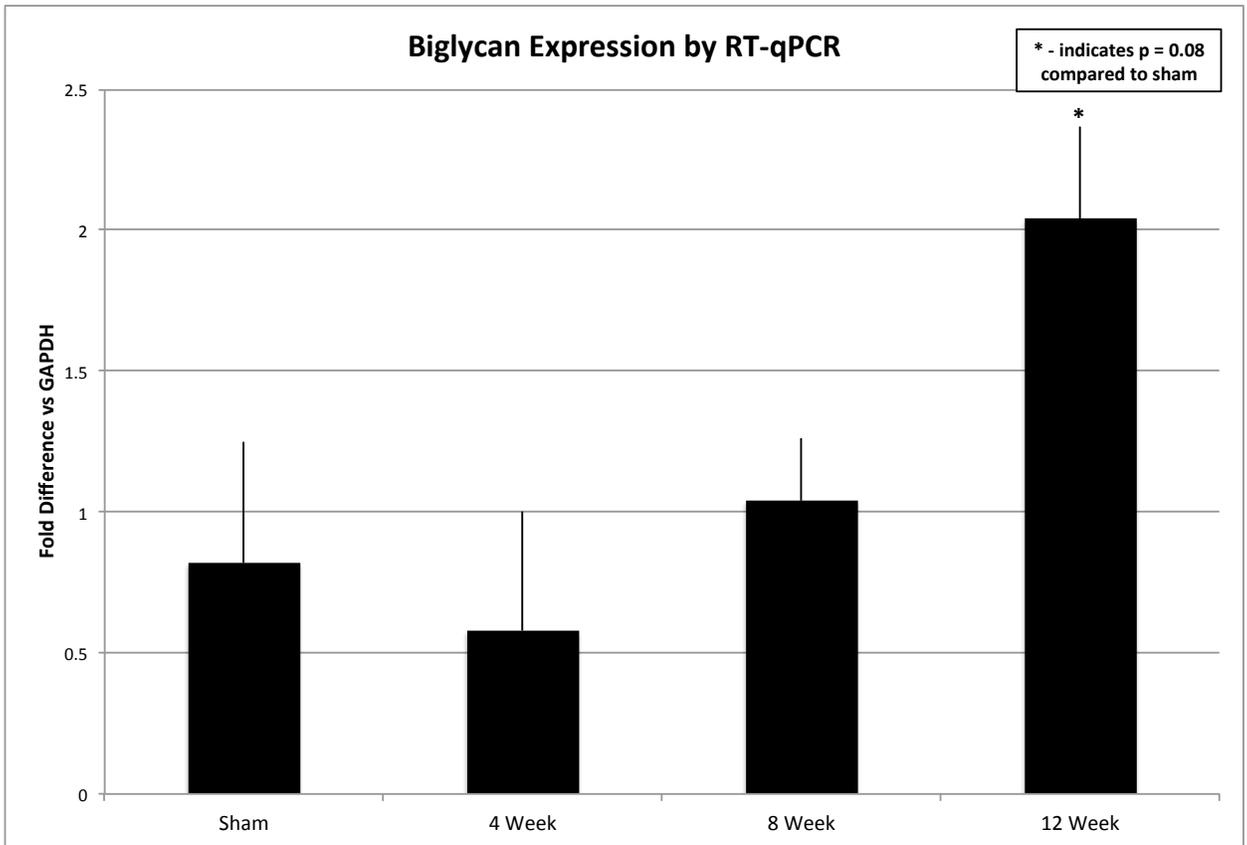Decorin is consistently downregulated over the experimental time course.

Figure 2.6: Biglycan expression by RT-qPCR.

Biglycan is consistently upregulated over the experimental time course, and approaches statistical significance at 12 weeks.

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Chapter 3: Experimentation – Novel Oral Medical Management of Partial Bladder Outlet Obstruction in an Animal Model

3.1 Introduction

Partial bladder outlet obstruction (pBOO) is a problem seen in nearly all disciplines of urology, ranging from males with benign prostatic hyperplasia or urethral stricture, to children with posterior urethral valves or spinal cord dysraphisms[1]. Clinically this presents in a slow insidious process, where the bladder initially can compensate for increased voiding pressure requirements, however this eventually progresses to a thick-walled and fibrosed organ that lacks adequate contractility or compliance[2]. While a great deal of clinical knowledge has been accrued regarding the natural history and management of this condition, the mechanisms behind bladder decompensation remain poorly understood.

The mainstay of treatment of pBOO involves three targets: relieving obstruction, maintaining low voiding pressures, and maintaining adequate bladder capacity. In the myelodysplasia cohort, where hypercontractile denervated bladder contracts against a dyssynergic sphincter, aggressive management is undertaken with anticholinergic therapy and clean intermittent catheterization. While this is effective, many of these patients still develop high storage pressures, and as such require surgical intervention[3].

An animal model for pBOO has been previously described in a rat, and found to mimic the clinical scenario well[4]. This model demonstrates discrete temporal changes that occur in the bladder secondary to the induction of obstruction. Initially the bladder becomes hypertrophic and large, accommodating for the increased voiding pressure requirements. As time progresses, the bladder decompensates and voiding pressures increase. Analysis of this model showed changes in multiple inflammatory cytokines, indicating that this deterioration happens in specific phases. As such, we postulated that alteration of specific molecular signaling pathways should be able to alter the process of bladder decompensation.

We identified three medications to assess in our animal model of pBOO: oxybutynin, rapamycin, and tadalafil. Oxybutynin is the current standard-of-care anticholinergic medication used to treat overactive and obstructive bladders. Its mechanism of action centers on targeting muscarinic receptors in the bladder, and inducing relaxation of smooth muscle[5]. Rapamycin is a potent inhibitor of the mTOR pathway, and has been shown to be antifibrotic and antiproliferative in both clinical[6] and in-vitro[7] studies. As multiple inflammatory cytokines related to mTOR had been identified in our previous work, we postulated that rapamycin treatment could have an effect on this process. Phosphodiesterase-5 inhibitors were initially developed for treatment of pulmonary hypertension, and are commonly used in urology for treatment of erectile dysfunction. They have been found to concurrently improve lower urinary tract

symptom scores[8], presumably by targeting PDE-5 enzymes found in the lower urinary tract and relaxing the bladder[9]. Tadalafil has the longest half-life of the PDE-5 inhibitors, and as such it was felt to be good candidate for treatment of this condition in this study.

We hypothesized that treatment of an obstructed bladder in an animal model with oxybutynin, rapamycin, or tadalafil would provide an improved urodynamic profile in comparison to untreated obstructed controls undergoing the natural disease history.

3.2 Materials & Methods

3.2.1 The Animal Model

Prior to commencing experiments, full approval was obtained from the University of Alberta Animal Care and Use Committee. Female Sprague-Dawley Rats at 200-225 grams underwent pBOO induction surgery[4]. Female rats were specifically used due to favorable anatomy for the obstruction procedure. Anesthesia was induced using isoflurane. The animal was positioned supine, shaved and sterilized using Germi-Stat solution. A lower midline incision was used to expose the bladder, and an 18-gauge angiocatheter introduced through a superior cystotomy and advanced to the urethra. The urethra and ureters were dissected bluntly, and a 4-0 silk tie passed posterior to the urethra. The silk tie was then tied gently to induce partial obstruction around the bladder neck, using the

angiocatheter as a calibrating stent. The catheter was then removed, and the cystotomy closed using a 4-0 vicryl suture. The abdomen was closed in layers, anesthesia was reversed, and animals were allowed to recover.

A total of five experimental animals were used for each medication at specific experimental time points, defined as 4, 8, 12, and 16 weeks post-pBOO surgery. Three controls, which underwent pBOO induction but did not receive medication were used at each time point.

3.2.2 Animal Health

Postoperative monitoring was done in a twice-daily fashion for the first three postoperative days. Animals were assessed for overall appearance, grooming, and porphyrin staining. Using these variables, pain scores were calculated using a standard scoring sheet. Morphine was administered at these assessments as required. Following this initial postoperative period, animals were assessed during daily gavage, and weighed weekly.

3.2.3 Medication Administration

One week following surgery, medication administration was commenced using oral gavage on a daily basis. Control animals received gavage of 1cc saline, in order to account for potential stress effects of the gavage procedure, while experimental animals received 3 mg/kg oxybutynin, 2 mg/kg tadalafil, or 2 mg/kg rapamycin.

3.2.4 Urodynamic Testing, Animal Sacrifice, and Tissue Collection

Once the animals reached experimental end points, urodynamic testing was performed under anesthesia. An 18-gauge angiocatheter was introduced into the bladder dome and secured with a purse-string suture. The bladder was completely drained. A y-connector with tubing was attached to the angiocatheter with one end at a saline pump, and the other to a Digi-Med LPA 400 System (Micro-Med Inc, KY, USA), set to read pressure measurements once per second. Infusion of saline was the commenced at 0.1 cc / minute. Peak bladder capacity was determined when leakage of urine was first seen at the urethral meatus.

After obtaining urodynamic measurements, animals were sacrificed using anesthetic overdose and exsanguination. Bladders and kidneys were harvested, and inspected for presence or absence of hydronephrosis and bladder stones. Bladder wet weight was obtained. Paraffin sections were obtained by placing tissue in foam blocks for support and minimization of contraction in 4% PFA solution. Frozen sections were obtained by embedding tissue in Shandon Cryomatrix (Themo Electron Corp, PA, USA). The remainder of tissue was snap frozen in liquid nitrogen and stored at -80°C. Paraffin blocks were created from the paraformaldehyde fixed specimens and sectioned to a thickness of 5 μm.

3.2.5 Histologic Analysis

Paraffin sections were stained with hematoxylin and eosin (H&E) or Gomori Trichrome stain. Photomicrographs were taken using a digital camera (Nikon Coolpix 4500) mounted on a microscope (Nikon Optishot 2) using 20x optical magnification. All photographs were taken with identical lighting and exposure settings alongside a micrometer to ensure comparability.

3.2.6 Real-Time Quantitative PCR

Total RNA was extracted from snap-frozen tissue specimens. Tissue was first deep frozen in liquid nitrogen, then homogenized by shaking to powder using a Micro-Dismembrator (B. Braun Biotech Inc., Allentown, PA). The powder was reconstituted in Trizol solution. Total RNA was extracted using RNeasy spin columns (Qiagen, Mississauga, ON, Canada) following the manufacturers recommendations. Contamination from genomic DNA was removed by DNAse digestion for 60 minutes. First strand cDNA was synthesized using random primers in a first strand synthesis kit (Sigma, Oakville, ON, Canada). Real-time RT-PCR was conducted using Power SYBR Green PCR Master Mix (ABI, Foster City, CA) in a 25 µL tube for a total reaction volume of 25 µL containing 1 µL of first strand product and 0.2 µM of gene specific upstream and downstream primers for decorin and biglycan. GAPDH was used as a housekeeping gene. Amplification and analysis were performed using an ABI 7300 real-time system (Applied Biosystems, Foster City, CA). Cycling conditions were an initial denaturation at 95°C for 3 minutes,

followed by 40 cycles consisting of a 15 second denaturation interval at 95°C, and a 30 second interval for annealing and primer extension at 60°C. GAPDH amplification was used for standardization of the amplification curves. Species-specific primer sequences were used for decorin, biglycan, mTOR, TGF- β, HIF-1a, and CTGF, and are outlined in Table 3.2.

3.2.7 Hydroxyproline Analysis

Tissue collagen content was determined using liquid chromatography / mass spectrometric analysis of 4-hydroxyproline from snap frozen samples[10]. An internal standard (*N*-methyl-*L*-proline) was added to freeze-dried bladder tissue, then hydrolyzed overnight at 115$^{\mathrm{O}}$C. The *O*-butyl ester derivatives were then prepared using 10% BF$_2$ ethanol for 30 minutes at 120$^{\mathrm{O}}$C after drying the hydrolysate. Liquid chromatography mass spectrometry analysis was performed using a Hewlett-Packard mass selective detector monitoring for ions at m/z 186 and 188. Results are expressed as µg hydroxyproline per mg of dry sample weight.

3.2.8 Data presentation and Statistical Calculation

Parametric statistics were performed throughout using a 2-tailed unpaired Student t-test using STATA statistical software. A threshold of $p < 0.05$ was set to determine statistical significance. Graphs were

constructed using mean values and error bars representing the standard error of the mean.

3.3 Results

3.3.1 Animal Health

Rats included in the analysis remained healthy to all experimental endpoints. In these animals, there was no statistically significant difference in weight either pre-pBOO or among animals at specific time points. Animal survival is outlined in Table 3.1. Of note, only one of five animals in the 16-week rapamycin treated animals survived. These animals were found to exhibit physical signs of dehydration and lethargy, and had evidence of pyocystis on autopsy. Together, these findings are compatible with a septic cause of death. Hydronephrosis was present in two animals; one in the 4-week oxybutynin group, and another in the 12-week oxybutynin group.

3.3.2 Urodynamic Outcomes

Urodynamic data were obtained to demonstrate functional changes elicited by medical therapy in the obstructed animals. These were compared to controls for representation of longitudinal changes in an obstructed bladder.

3.3.2.1 Bladder Capacity

A smoothed curve of bladder capacity measurements for control and treated animals was plotted and analyzed (Figure 3.1). Control animals had an initial rise in bladder capacity at 4 weeks at nearly 250% of baseline, followed by a steady decline as time progressed. Oxybutynin treatment resulted in an initial rise in bladder capacity at 4 weeks ($p<0.05$), followed by a decrease at 8 weeks, followed by an increase at 12 weeks ($p<0.05$). Tadalafil provided a similar trend to oxybutynin, with an initial increase at 4 weeks ($p<0.05$), followed by a smoother more gradual decrease in capacity prior to a late increase.

3.3.2.2 Bladder Pressure

A smoothed curve of measured bladder pressure revealed that oxybutynin treated bladders had the highest pressures overall throughout the study, particularly at the 12 week time point ($p<0.05$) (Figure 3.2). Tadalafil treated bladders had pressures that did not differ significantly from controls, as did rapamycin treated bladders. Rapamycin treated bladders, however, demonstrated the lowest measured pressures of the group, and were significantly lower than oxybutynin treated bladders at the 8 and 12 week time points ($p<0.05$).

3.3.2.3 Bladder Compliance

Smoothed curves of calculated bladder compliance revealed that rapamycin treatment offered little advantage to control (Figure 3.3). Oxybutynin treatment resulted in an initial increase in compliance,

followed by a decrease, and a significant increase at 12 weeks (p<0.05). Tadalafil treatment resulted in a similar pattern to oxybutynin, although with a greater time interval to a drop in compliance.

3.3.3 Organ and Tissue Analysis

3.3.3.1 Bladder Weight

In early phases of obstruction at 4 weeks, oxybutynin and rapamycin treated bladders were heavier than control bladders (p<0.05). Tadalafil treated bladders at the 4-week time point were significantly lighter than controls (p<0.05). At the 12 week time point, tadalafil treated bladders were still significantly lighter than controls (p<0.05), and oxybutynin treated bladders were heavier (p<0.05).   While this trend persisted at the 16-week time point, it was not statistically significant. (Figure 4)

3.3.3.2 Bladder Thickness

Bladders were thickest at the 4-week time point on H&E sections measured with a micrometer. Tadalafil bladders were significantly thinner than controls (p<0.05), and demonstrated a more ordered tissue architecture and less smooth muscle hypertrophy than other groups. This effect was durable up to the 16-week time point. In the 16-week time point both oxybutynin and rapamycin treatment provided a thinner bladder wall, but this effect was not seen in earlier phases (p<0.05) (Figure 3.5) (Figure 3.6) (Figure 3.7).

3.3.4 Real Time RT PCR

RT-qPCR was performed for decorin, biglycan, mTOR, TGF-β, HIF-1α, and CTGF. No statistically significant changes in mRNA expression were seen past the 4-week time point. However, there were multiple notable differences seen at 4 weeks. Both decorin and biglycan were upregulated compared to controls, as was TGF-β. HIF-1α was seen to be decreased in the rapamycin treated animals, as well as tadalafil. No differences were seen with mTOR and CTGF (Figure 3.8).

3.3.5 Hydroxyproline Analysis

Control animals demonstrated a decrease in hydroxyproline levels followed by a sharp increase as the experiment progressed. Oxybutynin treated animals demonstrated fluctuating levels, which failed to reach significance. Rapamycin treatment resulted in a progressive increase in hydroxyproline levels, reaching a peak at 12 weeks ($p<0.05$). Tadalafil treatment resulted in a progressive decrease in hydroxyproline, reaching a nadir at 16 weeks with the lowest hydroxyproline levels seen in the course of the experiment ($p<0.05$) (Figure 3.9)

3.4 Discussion

Partial bladder outlet obstruction (pBOO) is perhaps the most commonly seen condition in urology. Current mainstays of therapy involve medical treatment to relieve obstruction or protect the bladder with anticholinergic drugs, but surgical treatment in many patients is a

requirement for maintenance of renal health [11-13]. In this study, we aimed to demonstrate that medical therapy with novel treatments can have positive effects on urodynamic parameters, histologic outcomes, and biochemical profiles.

A need exists for better medical management of pBOO prior to surgical intervention. While clinical evidence has shown that aggressive management of pBOO in pediatric patients leads to improved outcomes, progression to high-pressure bladders and renal risk remains significant[14]. By instituting novel medications that could delay this process, either alone or in conjunction with other medical therapy, the need for surgical intervention in many cases could be delayed significantly.

The availability of a reproducible animal model is critical in the search for novel medical treatments for pBOO. Our group has previously developed such a model in the rat, showing that pBOO progresses in this model in a similar fashion to what is seen clinically. Obstructed bladders pass though phases of inflammation, hypertrophy, and finally fibrosis. Most importantly, this model is readily available and reproducible, without compromising animal health [4]. This lends an ideal scenario to the investigation of new therapies.

In this study, our untreated control animals developed urodynamic profiles that parallel our prior work. There was an initial increase in bladder capacity with the induction of obstruction, followed by a decrease.

Bladder pressures in our controls rose over the course of the study. Bladder compliance initially increased, and decreased as time passed. These findings confirm the reproducible nature of our model, and reinforce the natural history of an untreated, obstructed bladder.

We have demonstrated that the changes that occur in an untreated obstructed bladder are pharmacologically alterable. Both oxybutynin and tadalafil had significant positive effects on increasing bladder capacity and compliance throughout the experiment. Each of these medications preserved the initial increase in capacity and compliance longer than in controls, and afforded a longer time to deterioration. While tadalafil had a similar effect to oxybutynin in this regard, the effect appears to be longer lasting and thus more durable. Oxybutynin bladders had the highest pressures throughout the study, which in conjunction with their histologic thickness and evidence of muscle preservation, could be due to preserved detrusor contractility. Rapamycin treated bladders did not demonstrate improvements in either capacity or compliance, but did have the lowest pressures recorded, and were statistically different than the high pressures seen with oxybutynin treatment. These effects, however, were at a dose that resulted in increased mortality. Tadalafil had the least hypertrophy noted, with thinner and lighter bladders, and least measured collagen deposition.

The urodynamic results presented show that oxybutynin and tadalafil afford a more favorable urodynamic profile than no treatment or

rapamycin. Our histologic and molecular results corroborate these results. H&E stains demonstrate that, particularly in the initial inflammatory and hypertrophic phases of obstruction, tadalafil and oxybutynin have a protective effect on bladder wall thickness. Particularly, the tadalafil treated bladders have less evidence of muscle hypertrophy, and this effect is maintained throughout the experiment. These findings are paralleled in bladder wet weight measurements.

The use of tadalafil or other PDE-5 inhibitors in treatment of bladder outlet obstruction is a novel concept. With initial use in men with erectile dysfunction, it was found that voiding symptom scores were concurrently improved [15]. Further research has demonstrated that PDE-5 mRNA is expressed in high levels in the bladder and lower urinary tract [9]. PDE-5 inhibitors have been used in pBOO models previously, and these have shown preservation of muscle function on contractility assays [16][17], as well as increases in cAMP and cGMP [18]. Also, iNOS has been shown to be critical for the prevention of pBOO mediated bladder deterioration [19]. Our work, demonstrating that tadalafil preserves bladder capacity and compliance, while not having a significant effect on bladder pressure, corroborates such prior findings showing improvements in bladder contractility following chronic PDE-5 inhibitor administration.

Recent studies have shown that hypoxia is likely to be a critical component of the process of bladder deterioration in pBOO [20]. In clinical settings, non-invasive oximetry has been used to map bladder

oxygenation [21,22]. Hypoxia has been demonstrated to be present in obstructed bladders experimentally, leading to multiple cellular stress mechanisms and induction of apoptosis[23]. In animal models, it has been shown that vardenafil improves bladder tissue oxygenation[24]. Our tadalafil treated bladders demonstrated less hypertrophic change, better capacity, better compliance, and more ordered tissue architecture on histologic stains. Given the critical role of oxygenation and hypoxia previously demonstrated in pBOO induced bladder deterioration, it is possible that tadalafil supports vascular flow in the obstructed bladder through NO-mediated vascular relaxation. As such, the cascade of hypoxia-triggered events leading to hypertrophy and fibrosis could be delayed, as demonstrated by our experimental results.

HIF-1α is a critical transcription factor involved in the acute inflammatory response and the cellular response to hypoxia. It is activated by mTOR kinase and has previously been described to be upregulated in obstructed bladders and in fibrosis models. We found that HIF-1α transcription was downregulated on RT-PCR at 4 weeks in the rapamycin and tadalafil treated animals. This is an expected result of rapamycin treatment, and suggests adequate dosing. Tadalafil has been previously described to reduce HIF-1α levels to normal in urinary tract tissues in hypertensive rats[9], but this has not been described in an obstructed model. Furthermore, HIF-1α has been shown to be a critical mediator of fibrotic pathways[25], and we found tadalafil treated rats to have

lowest levels of tissue hydroxyproline at late phases of the study. Together, this suggests that tadalafil, by altering the HIF-1α pathway, has an anti-fibrotic effect. Treatment with PDE-5 inhibitors in other anatomic sites has indeed shown similar effects. Inhibition of muscle hypertrophy has been seen in models of ischemic and obstructed cardiac flow, pulmonary arterial hypertension, and heart failure[26-28]. Clinically it has been shown to have antifibrotic effects in post-prostatectomy erectile dysfunction[29] and scleroderma[30].

HIF-1α is considered a master regulator of the hypoxic response. Following its activation, multiple effects are seen on a cellular and global level[31]. Glycolytic enzymes are induced, VEGF transcription is induced, iNOS is activated, and erythropoietin is synthesized. Through induction of glycolysis, angiogenesis from VEGF activation, and iNOS induced vasodilation, HIF-1α allows for protection of the cell from long term hypoxic damage. HIF-1α has also been shown to phosphorylate focal adhesion kinase (FAK), resulting in smooth muscle cell migration, proliferation, and adhesion during the remodeling response[32]. Furthermore, activation of the PI3K/Akt, mTOR, and HIF-1α pathways in response to hypoxia results in alterations in integrin expression, leading to further remodelling of the extracellular matrix[33-35]. Integrin expression and signaling, alongside FAK activation as well as TGF-β signaling, have been shown to induce myofibroblast differentiation[36]. FAK activation as well has been shown to be dependent on mechanical stretch via integrin pathways,

and results in further mTOR complex activation[37]. As such, a combination of hypoxia, mechanical stretch, HIF-1α activation, TGF-β signaling, FAK activation, integrin pathway activation, and mechanical stretch are regulated in a complex interplay, with fibroblast activation and tissue remodelling being the end result. Indeed, all of our obstructed animals had elevated levels of TGF-β, residing high on the hypoxia pathways described above, but the best histologic outcomes were seen with decreased levels of HIF-1α. Given the complexity of these pathways, and the sheer number of stimulatory, inhibitory, and effector molecules, it is highly likely that pBOO pathology and its partial inhibition through medical therapy depends on other cytokines which were not measured in our analysis.

Our results show decreased activation of hypoxic pathways with tadalafil and rapamycin treatment reflected in HIF-1α levels. However, tadalafil treatment resulted in an initial inhibition of bladder smooth muscle hypertrophy compared to no treatment, whereas rapamycin treatment did not. It is possible that tadalafil provides obstructed bladder tissues with better vascular supply, leading to less activation of hypoxic pathways, resulting in less downstream activation of myofibroblasts. Rapamycin, inhibiting the mTOR complex, resulted in decreased HIF-1α expression. However, hypertrophy and fibrosis proceeded in these tissues in a manner not significantly different than control. This suggests that mTOR inhibition, and thus decreases in its downstream effector molecules, are not the

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exclusive regulators of the fibrotic response in obstructed bladder tissue. Given the complex interplay of inflammatory cytokines, mechanical stretch, cell adhesion, and hypoxia previously described, it is conceivable that multiple pathways are activated in this pathology.

In our study, we found rapamycin to have a significant deleterious effect on animal health. While a known side effect of rapamycin is a chronic wasting syndrome secondary to inhibited cellular proliferation, we did not find significant differences in animal weight. Rather, the late-term 16-week obstructed animals became sick, dehydrated, lethargic, and required emergency euthanasia. The autopsy showed evidence of pyocystis, and a presumed cause of death of sepsis. We suspect the immunosuppressant nature of this medication exacerbated the chronic infection present in the obstructed bladder, leading to overt sepsis and compromise of health. Given that in clinical use, such a medication would be required to be given in a chronic fashion, a risk of septic complications would not be acceptable, particularly considering the limited urodynamic and histologic benefit of this therapy.

We demonstrated elevated TGF-β expression levels in all treatment groups compared to control. Simultaneously, and increased expression of decorin and markedly increased expression of biglycan was seen. Absence of decorin has been shown in our previous work to be related to onset of fibrosis. As such, an increased level of decorin in treated bladders indicated that antifibrotic pathways, to some degree, are being

activated. Furthermore, TGF-β has been shown to be bound by decorin and result in its inactivation and sequestration[38-40]. The elevated levels of decorin seen in our treated animals could indicate the activation of antifibrotic pathways. Furthermore, biglycan synthesis has been shown to be TGF-β dependent[41,42], and deficiency of biglycan can lead to myofibroblast differentiation[43]. This supports our conclusion that some degree of antifibrotic pathway activation is occurring resulting with medical treatment with either oxybutynin, tadalafil, or rapamycin.

Our study has several important limitations. While we attempted to best replicate a situation of clinical pBOO, and dosing animals via daily oral administration, this model does not include clean intermittent catheterization (CIC) as part of therapy. The importance maintaining low bladder volumes via CIC in this setting have been demonstrated in multiple clinical studies, and our model does not include such therapy. We did not perform bladder muscle contractility assays as well. We feel that our urodynamic testing is a reasonable surrogate for bladder contractility, but this does not provide an exact representation of muscle function as contractility testing would. Our tissue histology and thickness measurements lend to a natural degree of variability. The bladder wall, due to its elastic nature, will stretch and thin naturally, as well as contract and thicken. To minimize this variability, tissue sections were mounted on foam blocks prior to immersion in PFA solution. Gross weight, on the other hand, can be variable due to the hydration status of tissue that is

being weighed. When considering both bladder wall thickness and gross weight together, the same trends were seen across treatment groups and time points, thus indicating that taken together these variables describe the pathologic changes of muscle hypertrophy and fibrosis in a reliable fashion. Finally, our animals were treated at onset of pBOO induction. In the clinical scenario, there is typically a lead time to diagnosis of pBOO, leaving the bladder straining against an obstructed outlet and leading to progression of pBOO pathology. One future direction of study would be initiation of medical therapy at a later time point, to see if pBOO changes can be halted after progression, or if they remain permanent.

We have demonstrated that bladder decompensation in pBOO follows a discrete, programmed process. We have demonstrated in this study that bladder decompensation if treated early and aggressively with rationally chosen medications can have positive effects on urodynamic, histologic, and molecular outcomes. Together, these data suggest the importance of early aggressive medical therapy in this condition, validate the clinical use of oxybutynin as an agent to protect the obstructed bladder, and suggest tadalafil to have a potential therapeutic role in the management of pBOO.
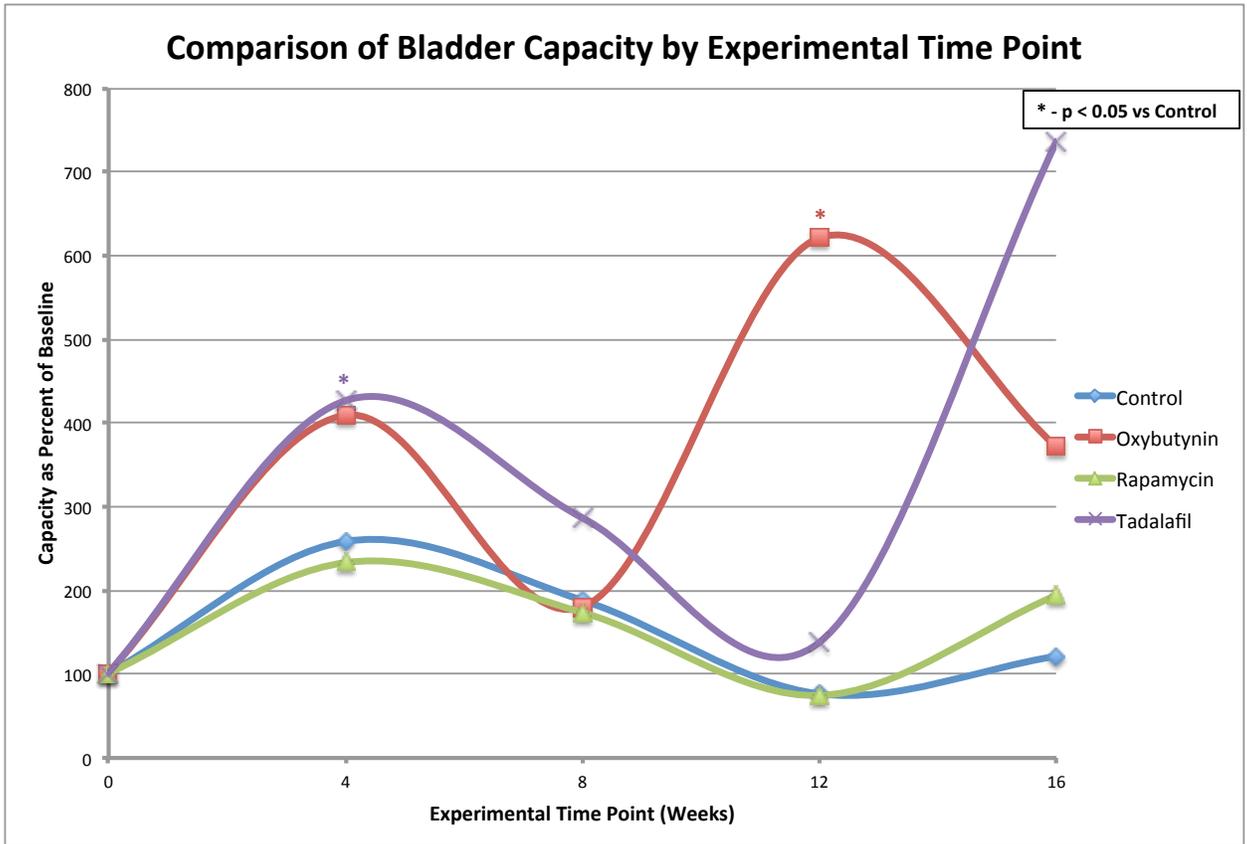
3.5 – Figures

Figure 3.1: Smoothed curves for maximal bladder capacity
measurements.

Control and rapamycin capacities mirrored each other closely, while
tadalafil and oxybutynin capacities were increased. Error bars have been
omitted from this graph due to their size, and statistical significance
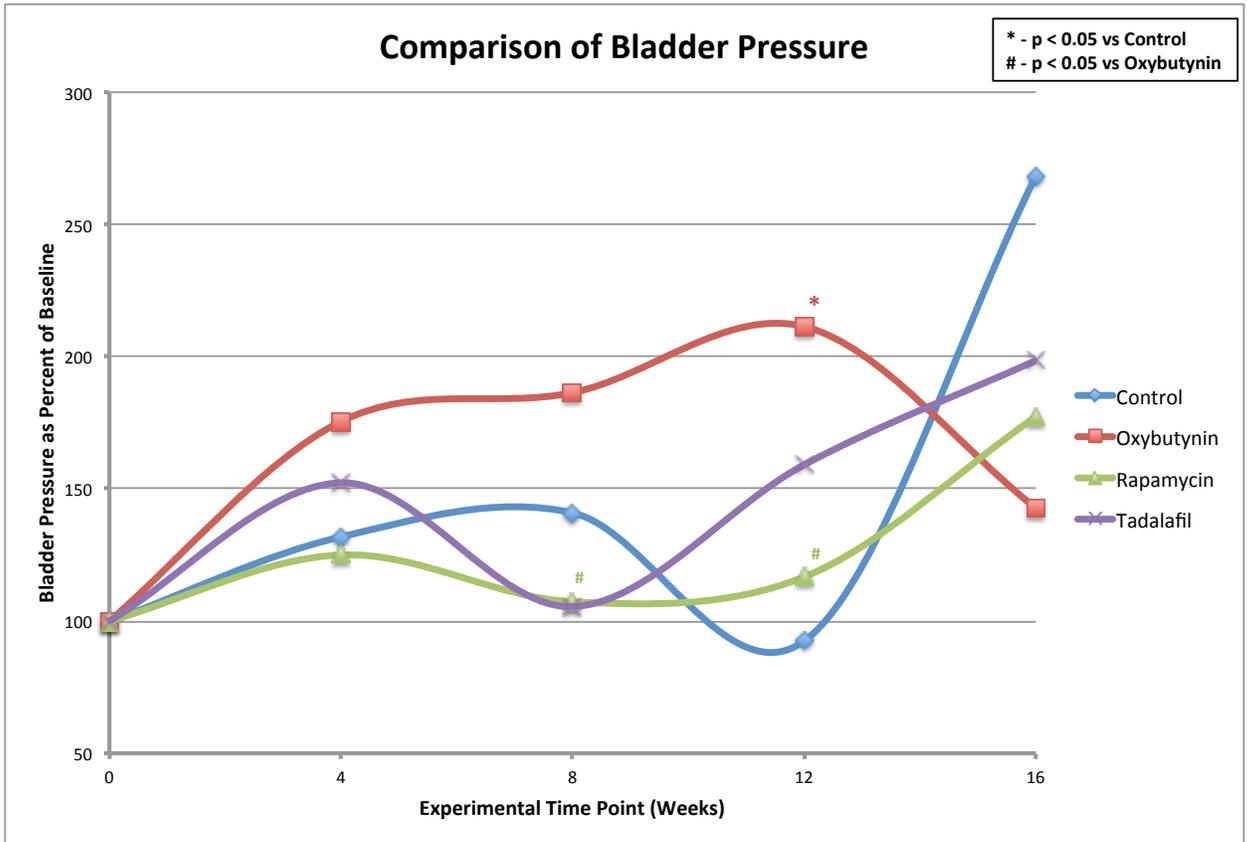compared to control is indicated with the asterisks.

Figure 3.2: Smoothed curves representing bladder pressure measurements.

Oxybutynin treated bladders had the highest recorded pressures, while rapamycin and tadalafil treatment did not differ significantly from control. Rapamycin had signficiantly lower pressures than oxybutynin. Error bars have been omitted from this graph due to their size, and statistical significance is indicated with asterisks and hash marks as indicated above.
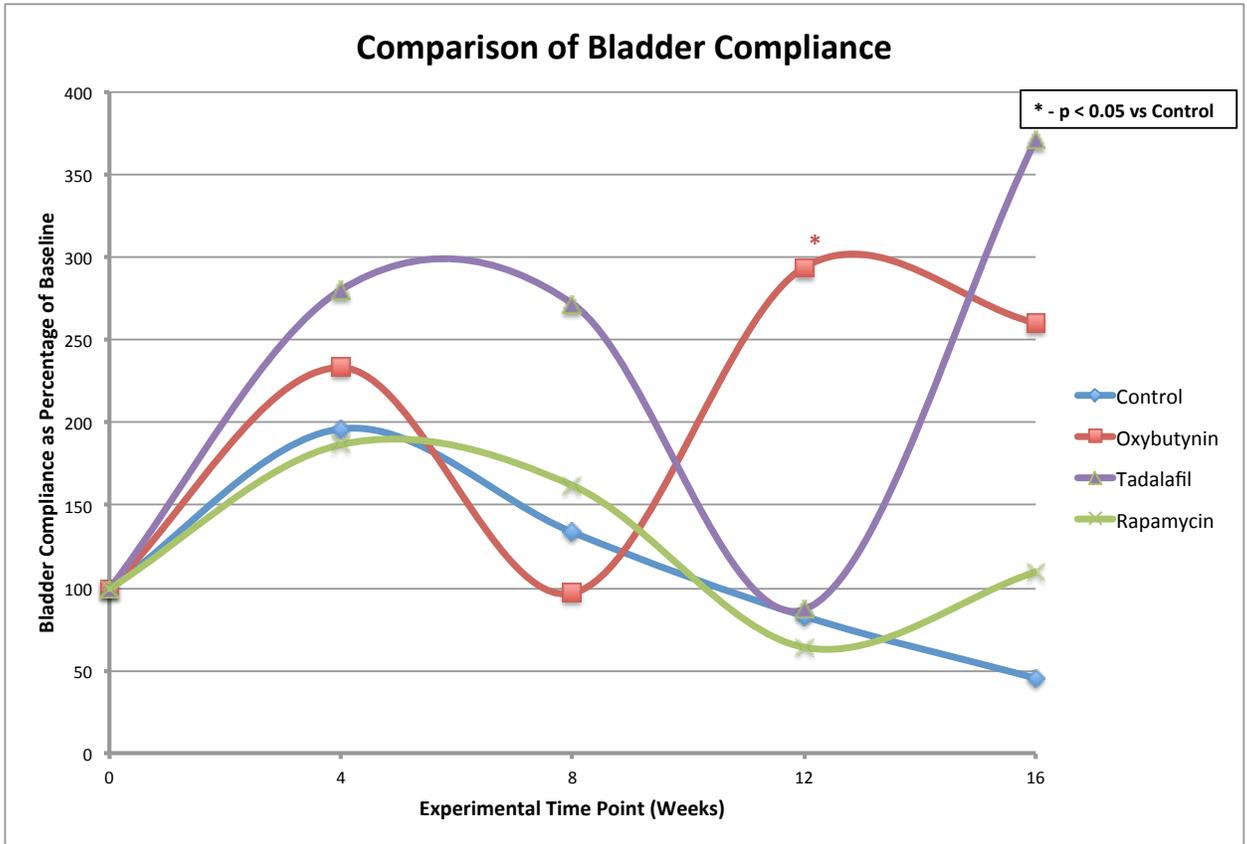
Figure 3.3: Calculated bladder compliance represented as smoothed curves.

Control and rapamycin treatments had a consistent decrease in compliance, while both oxybutynin and tadalafil had improved compliance. Error bars have been omitted due to their size, and statistical significance is indicated by the asterisk.
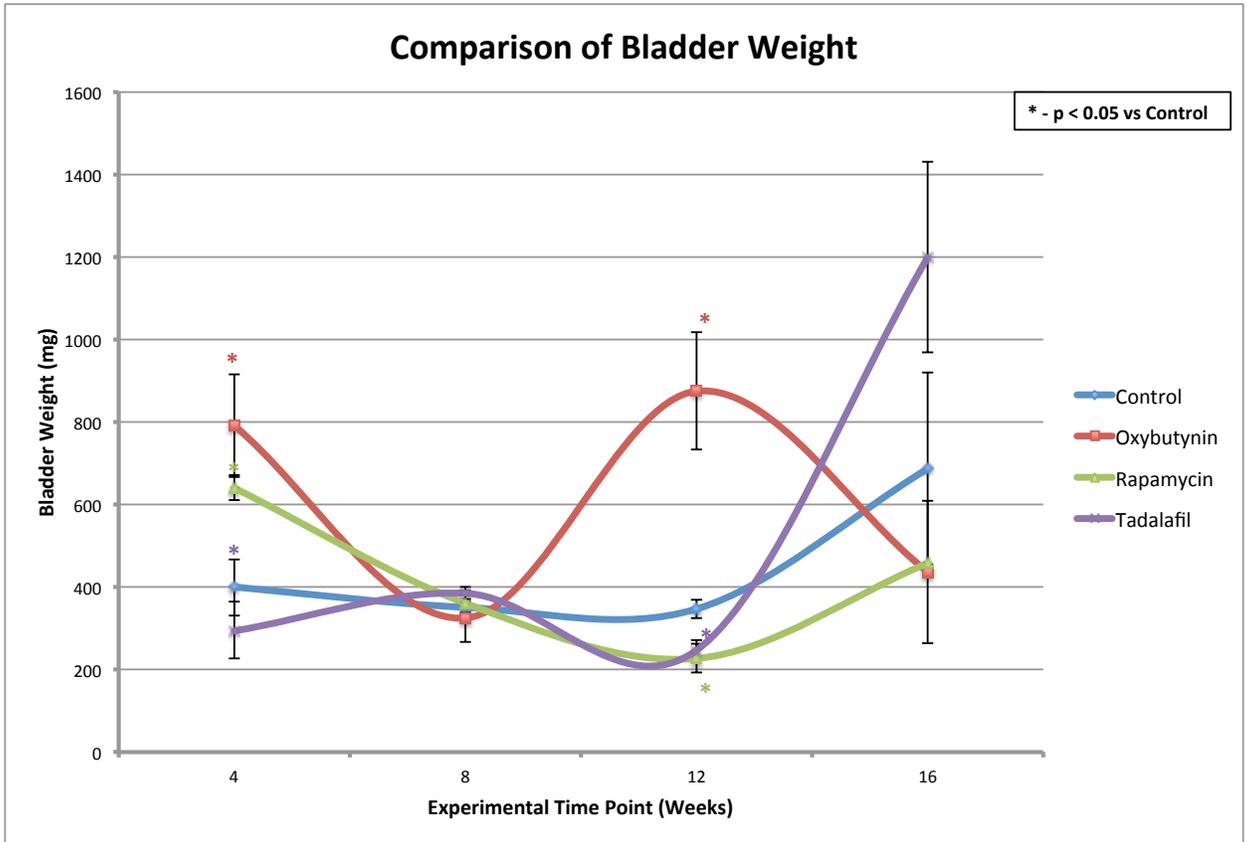
Figure 3.4: Bladder weight measurements at time of sacrifice.

Both oxybutynin and rapamycin treated bladders had significant increases in bladder weight at 4 weeks compared to controls, while tadalafil bladders were lighter at this phase. Tadalafil treatment had a protective effect on bladder weight up to the latest experimental time point. Statistical significance is indicated with the asterisk.
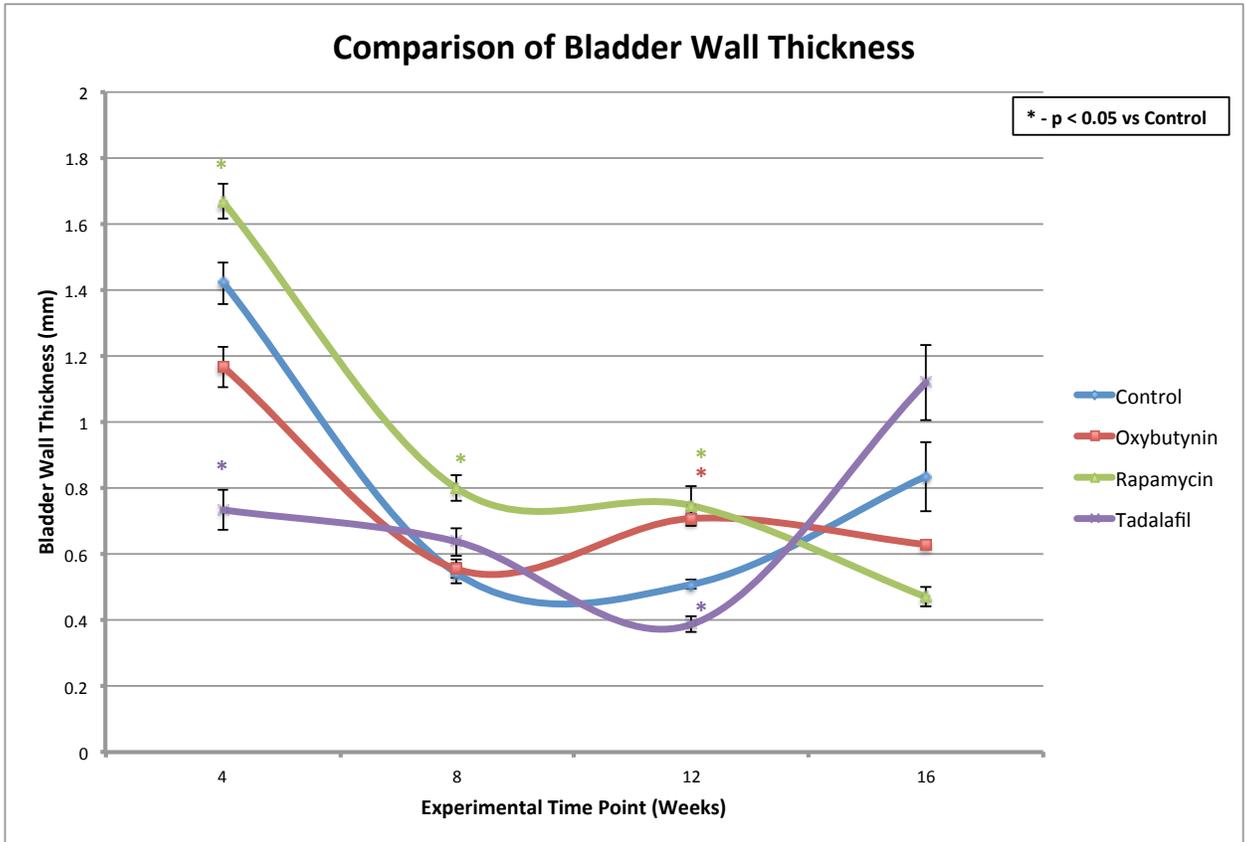
Figure 3.5: Bladder wall thickness measurements at time of sacrifice.

Bladder wall thickness measurements revealed dramatic differences at 4 weeks, where rapamycin treated bladders were significantly thicker than control, and tadalafil treated bladders were significantly thinner. Tadalafil had a consistent significant protective effect on bladder wall hypertrophy unitl the latest time point. Statistical significance is indicated by the asterisk.
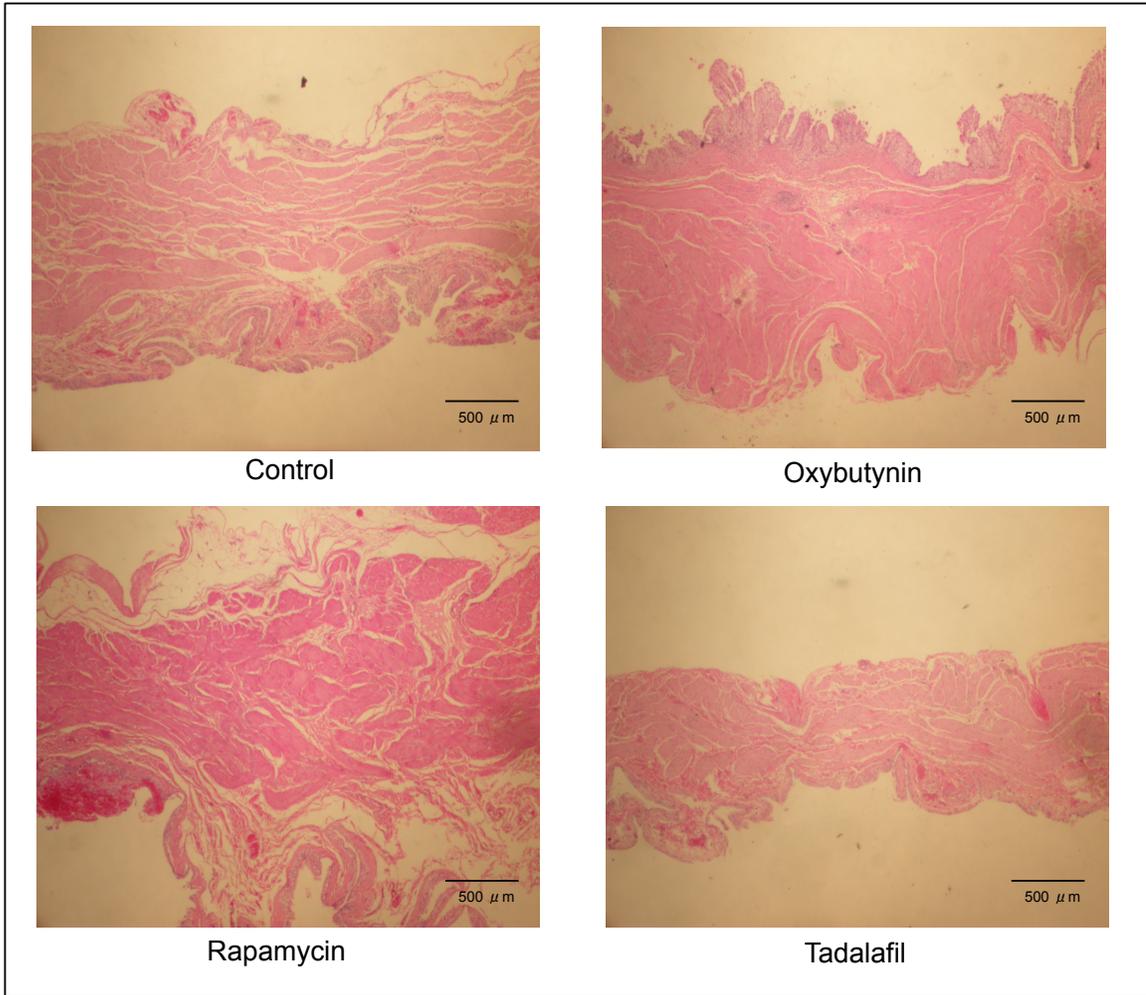
Figure 3.6: Hematoxylin and Eosin stained sections following 4 weeks of obstruction.

Representative H&E sections of bladder tissue after 4 weeks of obstruction. Control, rapamycin, and oxybutynin specimens show significant smooth muscle hypertrophy, which is absent in tadalafil sections.
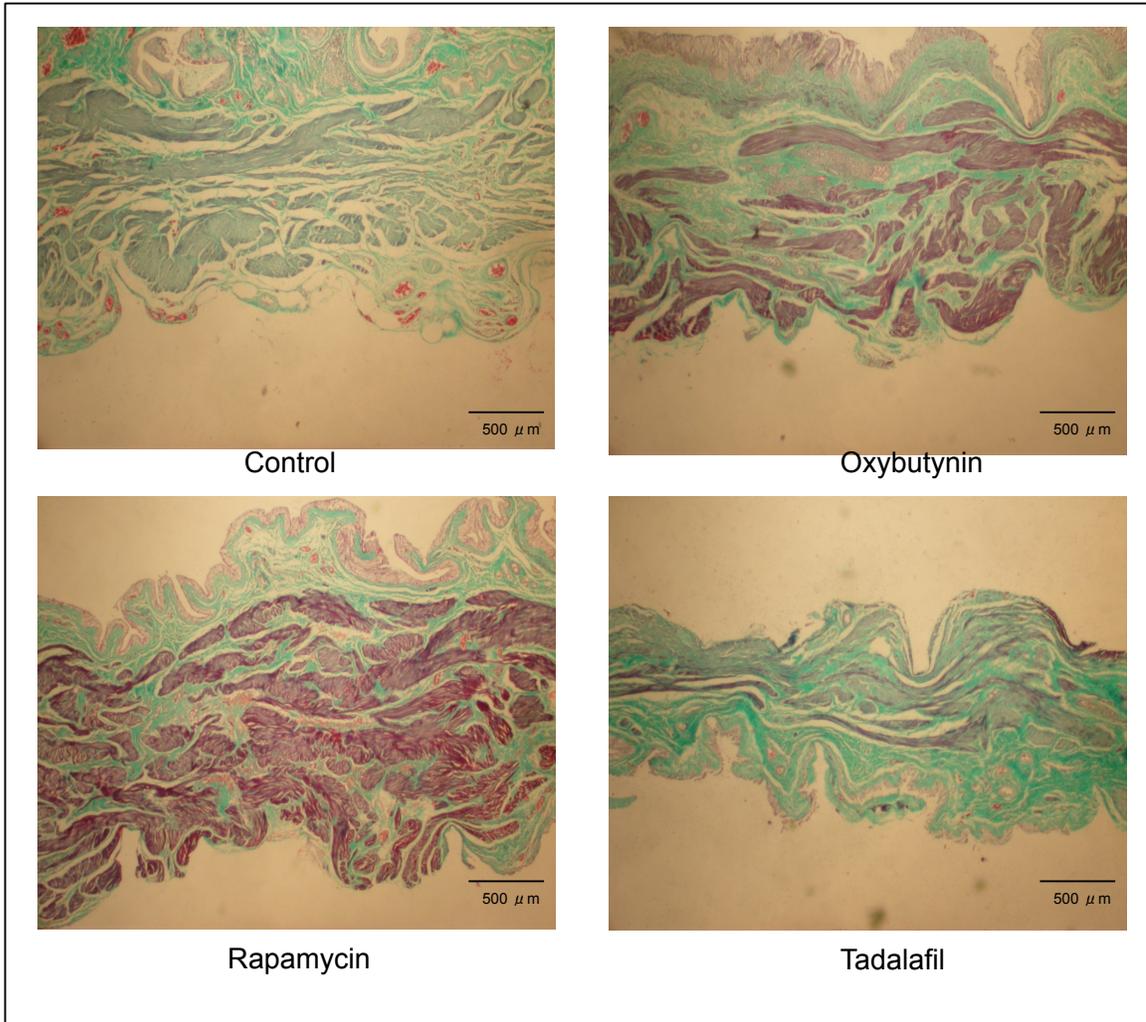
Figure 3.7: Gomori Trichome stained sections following 4 weeks of obstruction.

Representative trichrome stained bladder tissue sections after 4 weeks of obstruction. Similar to H&E sections, there is less muscle hypertrophy evident in tadalafil treated bladders as compared to other groups.
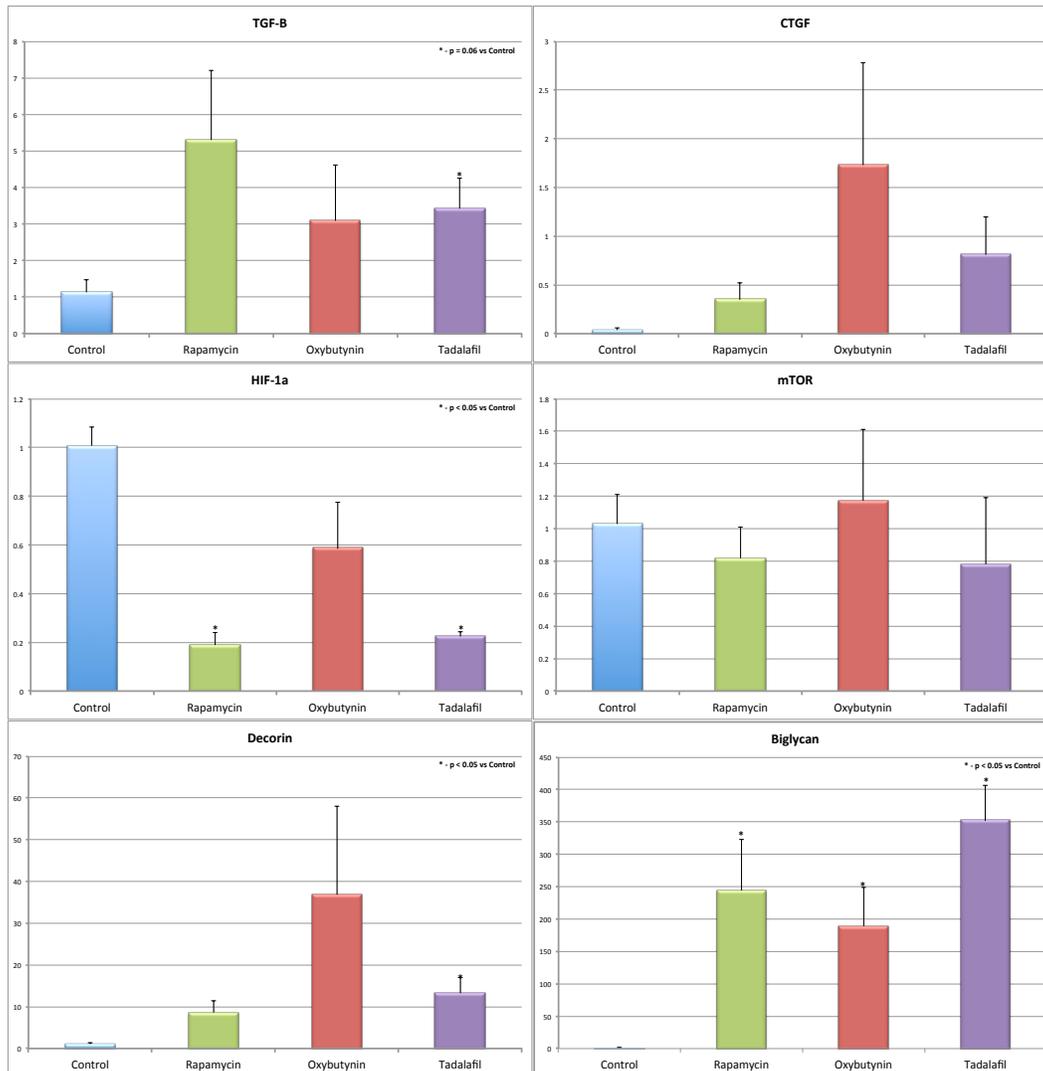
Figure 3.8: RT-qPCR results after 4 weeks of obstruction.

Both rapamycin and tadalafil treatment resulted in significant downregulation of HIF-1α, while tadalafil also afforded upregulation of decorin and biglycan. Statistical significance is indicated by the asterisks.
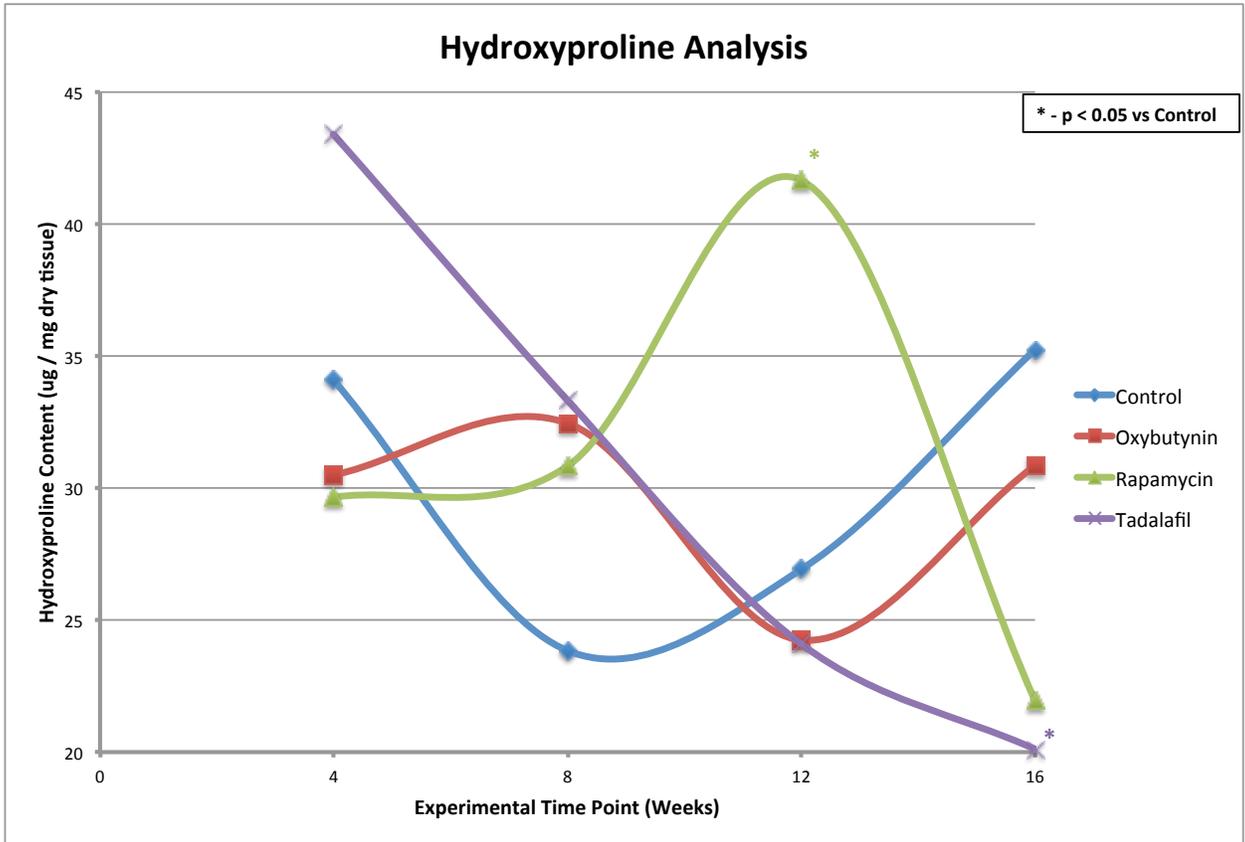
Figure 3.9: Hydroxyproline mass spectrometry to assess collagen content.

Rapamycin treatment led to a significant increase in hydroxyproline content at 12 weeks, while tadalafil treatment at 16 weeks had an objective and significant decrease in hydroxyproline content. Error bars have been omitted due to size, and statistical significance is indicated by the asterisks.

3.6 – Tables

Table 3.1: Mortality Results

| Oxybutynin				Rapamycin				Tadalafil			
4 Week	8 Week	12 Week	16 Week	4 Week	8 Week	12 Week	16 Week	4 Week	8 Week	12 Week	16 Week
0	0	2	0	1	0	2	4	1	0	2	2

Observed animal mortalities divided by experimental treatment and time group. 4/5 animals in the 16 week rapamycin group succumbed to sepsis. No mortalities were seen in the controls.

Table 3.2: PCR Sequences

Target	Forward Sequence	Reverse Sequence
Decorin	5'-TGCAGTCTGGCT-3'	5'-ACTCACGGCAGTG-3'
Biglycan	5';-GATGGCCTGAAGCTCAA-3'	5'-GGTTGTTGAAGAGGCTG-3'
mTOR	5'-GGCTTCTGAAGATGCTGTCC-3'	5'-GAGTTCGAAGGGCAAGAGTG-3'
TGF-β	5'-GGACTACCTACGCCAAAGAAG-3'	5'-TCAAAAGACAGCCACTCAGG-3'
HIF-1α	5'-TTGCTTGGTGCTGATTTGTGA-3'	5'-GGTCAGATGATCAGAGTCCA-3'
CTGF	5'-CGGAATTCGCTGTGCGTCCTCCTGCCG-3'	5'-CGGGATCCGAGTTCGTGTCCCTTACTCC-3'
GAPDH	5'-CCTGGAGAAACCTGCCAAGTAT-3'	5'-TGGCAGTCTGGCT-3'

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Chapter 4: Future Directions

4.1 Future Directions

The treatment of obstructed bladders in both neuropathic and non-neuropathic settings has progressed considerably in recent years[1]. In-vitro studies have demonstrated new mechanisms of bladder injury and helped elucidate the cellular response involved[2-5]. The availability of a reliable and easily reproducible model will have profound implications on developing new medical and surgical management strategies in this field, particularly by demonstrating the effects of new medical interventions, as well as the efficacy of new surgical options by serving as a platform for testing and development of biosynthetic grafts.

The field of regenerative medicine has grown substantially in recent years, and potential for application to urology is highly promising. Engineered bladder tissue has several important biomechanical parameters that must be achieved. Firstly, it must have elastic and mechanical properties that closely replicate the nature of the native bladder wall. Secondly, it must serve as a watertight, solute impermeable barrier to ensure storage of urine. In normal bladder tissue, these properties are achieved by integration of urothelial cells, smooth muscle cells, and a highly specialized connective tissue matrix. As such, developing such tissue poses a significant challenge[6,7].

The first bioengineered organ was developed by the Atala group in 2006, with human in-vivo application of biosynthetic bladder scaffolds[8]. The results from this initial effort have yet to be followed up in their cohort, and have generated a significant degree of controversy. Other groups have developed new grafts, however all preliminary testing at this point has been done in animal models with healthy, functioning bladders. The application of these new tissues to an obstructed, diseased bladder in any model has yet to be performed.

Initial efforts at bladder tissue engineering involved the development of tissue grafts that involved a synthetic polymer seeded with cells, creating a multilayered structure[9,10]. More recent efforts have focused on scaffoldless assembly of these tissues. The Bolduc group at Université Laval has developed a new tissue that relies on a self-assembly technique, involving a layer of dermal fibroblasts, and a layer of urothelial cells[11,12]. The graft proliferates in culture based on skin biopsies and bladder biopsy, and is ready for use after several weeks. Initial studies on the biomechanical properties of this new tissue have shown excellent results. This group has also developed a unique tubular graft that is grown under conditions of dynamic flow and pressure. Such 'cycled' culture conditions yield superior biomechanical properties than static ones. While this particular approach is best tailored to ureteral or urethral replacement, similar attempts have been made to engineer bladder tissue, with superior results compared to static culture[13,14].

Our model provides a unique platform for further development of biosynthetic grafts. We are able to reproducibly induce bladder outlet obstruction with minimal animal morbidity, demonstrate reproducible pathology, and do so at a minimal cost. As such, the implantation of a graft into our animals would be technically feasible, and would yield critical new information regarding the urodynamic performance of these grafts in a diseased bladder.

We have demonstrated with our study changes that occur in SLRP expression profiles in obstructed bladder extracellular matrix. This field of study is relatively new, and the function of these molecules is only starting to be understood[15-17]. We have shown the changes that occur with decorin and biglycan expression in normal, obstructed, and medically treated settings. Such knowledge can be applied to tissue engineering, where efforts can be made to modulate the expression and translation levels of such genes and proteins. One study relating to tendon tissue engineering has assessed the effect of addition of decorin at specific molar ratios to electrochemically aligned collagen fibrils, and found a significant positive effect in ultimate stress, strain, and thermal stability[18]. Given that the addition of decorin in this scenario had positive and desirable overall effects, we hypothesize that a graft with more physiologic levels of SLRPs in its matrix will have more normal biomechanical and urodynamic characteristics compared to a graft lacking in such composition.

Gene knockout animals can be a critical source of understanding the function of specific genes in an *in-vivo* model. Knockout studies have been performed on SLRPs in the past[16]. Decorin knockout mice are found to have fragile skin, weak tendons, decreased airway resistance, and poor wound healing. Biglycan deficient mice have been shown to have reduced bone mass, aortic ruptures, and weak tendons. Overall, these animals show a picture of a specific pattern of connective tissue dysfunction based on the absence of SLRP genes. Urodynamic testing has never been performed such animals, particularly not in the setting of pBOO, and could yield critical information for the regulation of partial bladder outlet obstruction.

Early screening and identification of disease remains a cornerstone of many treatment approaches, including pBOO. Our current approach relies on urodynamic measurements and serum creatinine levels over long term clinical follow up. The use of novel biomarkers could enhance detection of deleterious changes in the bladder. Both decorin and biglycan have been identified previously in human urine, in a study performed on patients with chronic kidney disease, and increased urinary decorin was associated with decreased renal function[19]. Measurement of urinary levels of SLRPs in unobstructed, obstructed, and medically treated animals could lead to the development of a new biomarker indicating adverse change.

Another promising area of treatment for pBOO involves the use of stem cell therapy. The Bhomwick group recently presented a study where bone marrow derived mesenchymal stem cells (BMD-MSCs) were administered to obstructed mice[20]. They demonstrated recruitment of these cells to the bladder, and also demonstrated decreased hypoxia, hypertrophy, and fibrosis. While the results are preliminary, this study demonstrated the potential of stem cell therapy for bladder outlet obstruction. Our animal model could easily be used for further understanding of the mechanisms by which BMD-MSCs have an effect on preserving bladder oxygenation and architecture in the process of obstruction, and further elucidating the molecular effects that such therapy provides.

Of the novel therapeutics used in our study, tadalafil had the most favorable urodynamic and molecular effects. Current indications for its urologic use include erectile dysfunction, as well as concurrent relief of lower urinary tract symptoms, and it has not been tested clinically for treatment of the neuropathic bladder. Our study demonstrated that this medication offers a similar urodynamic effect to current gold-standard anticholinergics, inhibition of early muscle hypertrophy, as well as a measurable antifibrotic effect. In human use, tadalafil has a fairly benign side effect profile. Its major side effects include headache, nasal congestion, dyspepsia, flushing, myalgia, and back pain[21]. Rare but more serious side effects include hearing loss (27 cases reported) and non-

arteritic ischemic optical neuropathy (1/1,000,000) [22], and these primarily

occur in patients with cardiovascular risk factors. Overall, the side effect

profile of tadalafil is fairly benign. Given that anticholinergic medications

can induce side effects that affect drug tolerability and compliance (dry

mouth, mental status changes, and flushing), the use of tadalafil

concurrently or in replacement for oxybutynin could be better tolerated. Its

effects on the obstructed bladder suggest that it would have positive

effects in human clinical use. As such, a clinical trial could be established

in a neuropathic bladder cohort to assess the urodynamic advantages this

medication could provide, with minimal risk.

One further area of study that our model would allow with medical

therapy is the timing of initiation. While it has been established that early

and aggressive medical treatment is essential in neuropathic bladder

populations, often these medications are administered once bladder

damage has already commenced. With our model, the timing of initiation

of medical therapy could be studied, allowing for a delayed start once

hypertrophic or fibrotic changes start to occur. This would create a model

scenario that closer mimics the onset of medical therapy in patients later

in the natural history of pBOO, and could yield more vital clinical

information. A study on the efficacy of delayed medical intervention in this

model would yield information that most closely parallels existing clinical

treatment, and would provide information on the ability of medical

treatment to salvage the damaged bladder and alter progression to end-stage fibrosis.

4.2 – Summary

The field of research in partial bladder outlet obstruction is rapidly advancing. Understanding the molecular mechanisms in place and their ability to be modulated by medical therapy will allow for the translation of new treatment strategies to clinical practice. Our model allows for an excellent platform for development of new therapeutics, new cell-based therapies, and new tissue engineering strategies. We have demonstrated that our model allows for reproducible pathologic effects, which can be mitigated by the administration of medications, as well as identification of new biomarkers that can indicate adverse pathologic change. Further study will allow for the development of future treatment strategies, and the refinement of existing approaches.

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