University of Alberta

The Proteome of Partial Bladder Outlet Obstruction & The Therapeutic Role of Mesenchymal Stem Cells

by

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Dedication

To who taught me devotion to work, dedication to details, and ambition for success.

Noura N. Albelaihed

Abstract

Introduction:

Partial bladder outlet obstruction (pBOO) is a common problem in urology. It passes through several pathological phases before progressing to bladder fibrosis. Herein, we investigated the proteomic profile of the urinary bladder in the early phase after pBOO, and examined the therapeutic role of mesenchymal stem cells (MSCs) in this pathology.

Methods:

Proteomic analysis of bladder tissue was performed using liquid-chromatography – mass spectrometry. The effects of intravenous injection of MSCs were examined on bladder function and gene transcription of previously described factors and newly detected proteins after proteomic analysis.

Results:

pBOO induced an increase in inflammatory, hypoxic, and cellular stress markers. Treatment with MSCs improved these molecular changes and helped maintain normal bladder capacity.

Conclusion:

Pathological changes happen early after pBOO. Inflammation, hypoxia, and mechanical strain seem to be the main factors in initiating the pathological cascade. Treatment with MSCs was successful in halting the inflammatory and hypoxic changes in the early phase after pBOO.

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List of Abbreviations

4-BPA	4-phenylbutyrate
5-AR	5-α reductase
BFGF	Basic fibroblast growth factor
BPH	Benign prostatic hypertrophy
CIC	Clean intermittent catheterization
CTGF	Connective tissue growth factor
DAPI	4',6-diamidino-2-phenylindole
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERS	Endoplasmic reticulum stress
GFP	Green fluorescent protein
GRP78	78kDa glucose-regulated protein
GTP	Guanosine triphosphate
HIF1α	Hypoxia-inducible factor 1α
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LAP	Latency associated peptide
LUTS	Lower urinary tract symptoms
MLC	Myosin light chain
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
nNOS	neuronal nitric oxide synthase
NO	Nitric Oxide
NOS	Nitric oxide synthase
OAB	Overactive bladder
pBOO	Partial bladder outlet obstruction
PDE	Phosphodiesterase enzyme
PDGF	Platelet derived growth factor
PG	Prostaglandin
POC	Poly(1,8-octanediol-co-citrate)
PUV	Posterior urethral valve
PVR	Post void residual
Qmax	Maximum urinary flow rate
RhoA	Ras homolog member A
ROCK	RhoA kinase
RT-PCR	Real-time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SLRP	Small leucine-rich proteoglycan
SMAD	Transforming growth factor β ligand transducers
SMC	Smooth muscle cell
TGFβ	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinase
TNFα	Tumor necrosis factor α
TURP	Transurethral resection of the prostate
UPR	Unfolded protein response
UTI	Urinary tract infection
VEGF	Vascular endothelial growth factor
VUR	Vesicoureteric reflux

Chapter 1

Introduction

1.1 Overview of The Problem

Partial bladder outlet obstruction (pBOO) is a common problem in urological practice. It results from a variety of causes which all have an increase in the pressure normally required for voiding, to overcome the partial blockage of the bladder outlet. It affects a spectrum of age ranges, from benign prostatic hypertrophy (BPH) in adult males, to pediatric patients. Pediatric population suffer from this problem secondary to congenital defects such as posterior urethral valve and spina bifida[1], which can lead to the unfortunate end result of renal injury[2].

The urinary bladder functions essentially as a compliant reservoir of urine, and a contractile organ when proper emptying is required. This seemingly simple task is well regulated in the normal circumstances with more complex mechanisms and mediators, to provide protection for the kidneys and upper tract from urinary reflux and high pressures, and to allow for emptying in an appropriate manner without resistance when needed.

Anatomical compositions of the bladder and urethra, and neuronal pathways controlling the lower urinary tract are all implicated to achieve the physiological micturition process, and maintain a healthy functioning system to meet the physiological needs of storage and proper unidirectional emptying. The process of voiding has multiple steps and interactions, exhibiting the beauty and complexity of physiological processes.

Relaxation of the urethral sphincter should occur, followed by contraction of the bladder smooth muscles, leading to an increase in the intravesical pressure and forcing urinary flow through the bladder neck[3]. Partial obstruction at the bladder neck interferes with the normal expected flow of urine, requiring an increase in the intravesical pressure to overcome the partial obstruction; a task achieved by stronger contractions of the detrusor smooth muscle fibers.

Initially, the bladder will tolerate the obstruction, and compensate to generate higher pressures with subsequent smooth muscle hypertrophy. However, detrusor compensation will not survive chronic and lengthy exposure to obstruction. Prolonged exposure to high pressure leads to a sequence of pathological processes, and through a complex, well regulated biochemical pathways, an end-stage, fibrotic, non-compliant bladder ensues[1]. The effect of such changes is not limited to the bladder and its function, but elevated pressure ultimately affects the upper tract resulting in renal injury, and eventually, end-stage kidney disease, requiring renal replacement therapy, with the associated morbidities and implications on patients lives[2,4,5].

1.2 Etiology of pBOO

pBOO can result from a variety of conditions, and each etiology is somewhat unique to specific age ranges and gender. BPH is a more common problem in adult males and its incidence increases with age. Whereas posterior urethral valve is a pediatric problem, and again, affecting males only. Urethral obstruction is less commonly seen in females, and the etiology in this group is

different than what was described for males. Pelvic organs prolapse, primary bladder neck obstruction, iatrogenic obstruction from stress incontinence surgeries, as well as pelvic neoplasia compromise the lesser, rarer etiologies of bladder outlet obstruction in females. However, because they are much more encountered in the urological clinical practice, we will focus the light on BPH, posterior urethral valves (PUV), as well as neuropathic bladder outlet obstruction, and elaborate further on some of their characteristic features and presentations, as well as the pathology behind each problem.

1.2.1 Benign Prostatic Hypertrophy

BPH is a frequent problem in the aging male, and is the most common cause of pBOO [6]. Prostatic epithelial and stromal cells undergo hyperplasia as men age, and this is the main etiology of this disorder [7]. The prostate gland is situated to surround a small, but important segment of the urethra, namely, the prostatic urethra. As the prostatic volume increases, it exerts an external pressure on this part of the urethra and hence, partially obstructing the urinary bladder outlet.

Several pathophysiological mechanisms are proposed to explain the reason behind this late-onset disturbance in growth, and it is believed that collective effects of multiple mechanisms are responsible, and are mediated by a complex molecular, signaling and cellular processes. It has been proposed that impaired apoptotic process is the main reason of cellular hyperplasia, but this finding is yet to be investigated [7]. Other studies has suggested that the hyperplastic process is a result of an abnormally re-launched embryonic proliferative growth of dormant

stem cells within the gland, based on the observation of histologic similarity of the prostatic stroma to the embryonic developmental mesenchyme [8].

Hormonal changes has been also involved in the pathogenesis of BPH [9]. Androgens play a major role in the pathology of BPH, and it has been classically thought to be the sole endocrinological factor mediating prostatic growth. However, recent observations and studies are putting less weight on single hormonal level changes, and more on imbalances of hormonal ratios. It is suggested that it is not the sole effect of androgens that induces the pathology of BPH, but the ratio of androgen to estradiol is involved in this process. As men age, testosterone to estradiol ratio declines [10], which has been induced and replicated experimentally on animals, and this manipulation was observed to facilitate and expedite the development of prostatic glandular enlargement in these animals [11].

BPH is a prevalent disease, affecting up to 80% of 80-year-old men [12]. Glandular and stromal prostatic hyperplasia is histologically detected in approximately 20% of men in their 40s, and up to 60% in their 60s [9]. BPH has been classically associated with the bothersome lower urinary tract symptoms (LUTS), which include a delay in the initiation of micturition, incomplete voiding, dribbling, urgency, frequency and nocturia. Furthermore, BPH has been also noted to be associated with erectile dysfunction, and chronic pelvic pain syndrome [5,13,14]. The severity of LUTS does not correlate with the severity of obstruction, as symptoms are perceived differently from person to person. However, with time, symptoms will progress and become a major concern to the

worried patient.

As a mean of objectively following up the progression of BPH, urodynamic studies come in handy for the urologist. One of the indicators of disease progression is the maximum urinary flow rate (Qmax), as it tends to decline with time if no intervention has been initiated. It is thought that the reduction of maximum flow rate is dependent on the degree of obstruction, but it has more meaning to it than a simple physical indicator. It gives the physician an insight about the bladder's functional status and contraction ability over time. Nevertheless, the reduction in maximum urinary flow rate is not solely due to obstruction. Several factors may contribute to diminished urinary flow rate, such as age-related detrusor changes [9].

Obstruction is very relevant in patients diagnosed clinically to have BPH, and estimated to be present in 80% of patients with maximum flow rate less than 15 ml/s. [15]. Prolonged obstruction will lead to an increase in the post void urinary residual, which on the long run will result in an increase in the intravesical pressure, affecting the upper tract and causing renal failure. After such changes, relieving the obstruction might not regain normal detrusor function, as a prolonged obstruction may render the harm to the detrusor to become irreversible.

Urinary stasis in the bladder exposes the patient to multiple urinary tract infections and bladder stones formation, which places even more burden on the stressed detrusor.

1.2.2 Posterior Urethral Valves

PUV is a common cause of bladder outlet obstruction in male infants. The incidence is estimated to be around 1:5000 to 1:8000 live male births, and may also result in neonatal demise [16]. Antenatal detection of PUV is currently feasible, and usually require prompt intervention[17]. PUV continues to pose a significant morbidity and mortality upon male infants and children, and counts as a cause for ongoing renal damage [18]. pBOO in children is commonly due to PUV, however, other etiologies, such as myelomeningocele and spinal cord dysraphism, are also seen.

PUV consists of a thin membrane in the posterior urethra that acts as an obstructing barrier to normal urinary flow. This membrane is thought to be formed at 4 weeks of gestation, when Wolffian duct fuses with the developing cloaca [19]. The results of urinary obstruction does not affect only the developing bladder and kidneys, but severe oligohydraminos may ensue as a result of reduced urinary output, deforming the soft tissue of the growing fetus, and interfering with lungs development causing pulmonary hypoplasia, with its subsequent deleterious consequences [20].

Antenatal interventions are currently being performed in selected cases. Vesico-amniotic shunt is an option, but carries risks of several complications, and renders unsatisfactory outcomes overall [19]. Despite early and more invasive interventions, prognosis is still poor and progression to end-stage kidney disease is still prominent [21]. Finding a therapeutic approach to the pathology of pBOO, will definitely improve the survival of these young patients by avoiding the

serious and life threatening complications of prolonged exposure to renal replacement therapy.

1.2.3 Neuropathic Bladder

Unlike the previously mentioned examples of pBOO where there is an abnormal physical barrier to urinary flow, abnormal neuronal innervation to the urinary bladder and urethra may result in pBOO as well. In congenital and acquired situations where spinal cord is affected, abnormal innervation to the urinary bladder and sphincter can produce an obstructive picture to the lower urinary tract. Spinal cord dysraphism, spinal cord trauma, and tumors can all produce functional obstruction. Spinal cord dysraphism, which can present in an occult fashion, is the most commonly seen form of neurogenic bladders in pediatric urology. It results from a congenital defect, which results in a tethered cord to the meninges. The incidence is in decline currently with the advertisement for folate supplements which aid in normal spinal development. Antenatal detection has also helped reduce its incidence with the option of therapeutic abortion.

Spinal injury can also result form myelitis, arteriovenous malformations, peripheral vascular diseases, and multiple sclerosis [22]. Aberrant innervation to the urinary sphincter can result in tonic spasms in face of bladder contractions (detrusor sphincter dyssenergia). In this case where the sphincter contracts abnormally with the contraction of the bladder, high pressures are induced. As with all the causes of pBOO, obstruction in neuropathic bladder can result in all

the complications mentioned, i.e. vesicoureteric reflux (VUR), urinary tract infections (UTIs), bladder stones, renal injury, and progression to end-stage bladder fibrosis.

1.3 Anatomy and physiology

Normally, the urinary bladder is able to store urine at low pressure, due to the unique bladder wall structure. Internally, the bladder is lined with transitional epithelium resting on a thin basement membrane. Deeper to the basement membrane lies a fibroelastic connective tissue layer, i.e lamina propria, which aids in bladder wall compliance due to its considerable elasticity [23]. This layer contains numerous blood vessels and a fine layer of smooth muscles, the muscularis mucosa. Smooth muscles of the bladder wall are arranged in three interlaced layers, inner longitudinal, middle circular, and outer longitudinal fibers.

The anatomical constitution of the bladder is of much value to aid in its two main functions; continent low-pressure storage of urine, and efficient emptying. To achieve proper storage, bladder should accommodate a satisfactory volume without a significant raise in the intravesical pressure. The elasticity and components of the bladder wall and matrix aid this function. The bladder is equipped with several layers of transitional epithelium, which are designed to accommodate distention[24], in addition to the unique smooth muscle orientation, which allows for a smooth filling without significant resistance.

The extracellular matrix (ECM) is composed of multiple components that are of important significance. Collagens I, II, IV, and elastin, all constitute the

complex ECM, and accommodate situations of stretch [25,26]. However, exposure to prolonged elevated pathological pressures, such as in pBOO, alters the ECM in several ways that contribute to the pathological complexity of end-stage bladder disease. [1]

Neuronal innervation to the bladder is autonomic in origin. Sympathetic innervation arises from the lumbar nerve roots L2, L3 and reaches the sacral plexus through the inferior mesenteric ganglia[27]. Sympathetic innervation causes contraction of the bladder neck and internal sphincter, and helps in bladder wall relaxation by activating α and β adrenoreceptors[7]. Parasympathetic supply, originating from the sacral nerve roots of S2, S3, and S4, is mainly responsible for bladder contraction, via the muscarinic M3 receptors, and exerts a relaxing effect on the internal sphincter [28,29].

The urinary sphincter is composed of two distinct groups of muscles. The inner sphincter is composed of smooth muscles, and controlled involuntarily by the autonomic nervous system, while the external sphincter, constructs of striated muscles and under voluntary control by somatic nerves. The pudendal nerve conveys somatic efferent signals from S2, S3, and S4 to contract the external sphincter, aiding in the continence mechanisms. [30].

1.4 Pathophysiology of pBOO

With pBOO, detrusor muscle is required to generate increasing amounts of pressure to overcome the resistance. Initially, these changes are tolerable and attuned by compensatory mechanisms. These changes have been reproduced and

studied in animal models [1]. An increase in bladder capacity and wall thickness and mass, smooth muscle hypertrophy, and bladder wall hypoxia, are all part of the initial inflammatory compensated phase [1,31-33].

Nevertheless, with sustained obstruction, signs of decompensation become evident. Smooth muscles fail to generate higher pressures, and diffuse changes in the extracellular matrix take place with high collagen deposition and a significant decrease in smooth muscle cells (SMC) to collagen ratio[1,34,35]. These phases are characterized with temporal variable changes in several mediators, such as transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF), hypoxia inducible factor 1-α (HIF-1α), and platelet derived growth factor (PDGF)[1]. Another possible factor that might contribute to the bladder dysfunction is bladder wall hypoxia, probably caused by high intravesical pressure compressing bladder wall capillaries[36-38].

Ultimately, the bladder transforms into a small, fibrotic, noncompliant organ which is deprived of essential basic functions; i.e. proper storage and emptying [1]. Hence, normal urinary volumes can not be stored properly, and VUR ensues alongside with urinary leakage and incontinence [39].

1.5 Current Management of pBOO

1.5.1 Diagnosis

Some of the etiologies of BOO have an insidious onset, making it difficult to be detected early on the obstructive process, and patients may present initially

with obstructive renal injury [40]. To avoid that, every possible effort should be done to detect such pathology as early as possible. Careful history taking, with a focus on possible predisposing conditions should be elaborated. Physical examination aiming to detect a palpable obstructed bladder, and signs of underlying etiologies, such as an enlarged prostate on digital rectal examination.

Clinical history and physical exam do not always reveal the presence of lower tract obstructive uropathy, and more investigations are rendered mandatory. A simple, noninvasive bedside test is the measurement of post-void residual (PVR) using a specific ultrasound bladder scan. A high PVR coupled with symptoms of lower urinary tract obstruction are highly suggestive of BOO[41,42]. However, functional studies remain the gold standard in the diagnosis of BOO, i.e. urodynamic studies. Showing bladder capacity, compliance, pressure and flow rates, urodynamic studies are the most helpful tool towards the diagnosis[43,44]. Furthermore, it is a helpful tool towards future follow-up of the progression and effects of BOO and timing of intervention [45,46].

1.5.2 Surgical Relief of pBOO

Benign prostatic hypertrophy, being the most common cause of pBOO, it is not surprising that transurethral resection of the prostate (TURP) is one of the most common procedures in adult urology[6]. Surgical interventions in BPH are utilized after failing medical therapy in most circumstances [47,48]. Several endoscopic procedures are being done to alleviate the obstruction, with different indications and techniques. TURP remains to be the benchmark of surgical

options for BPH [49]. Other transurethral options include holmium laser ablation of the prostate (HoLAP), holmium laser enucleation of the prostate (HoLEP), and photoselective vaporization of prostatic tissue (PVP) [49]. Other less invasive methods include thermal needle ablation and microwave ablation[50,51]. Open prostatectomy, being more invasive, is generally reserved for prostate volumes more than 80 mL [49].

Posterior urethral valves in pediatric patients can be managed in different ways. Endoscopic resection of the valve procedures are readily used in the appropriate age and clinical setting with high success rates [52,53]. In premature neonates, endoscopic access is less feasible and an indwelling foley catheter is needed to drain the bladder. In the cases when long term drainage is required before surgery can be safely done, a cutaneous vesicostomy is performed [54,55]. Vesicostomies carry the risk of herniation if the stoma is of a big size, and a risk of stenosis if small, but the overall success rate in relieving vesico-ureteric reflux secondary to obstruction has been reported to reach 90% [56]. Intrauterine prenatal interventions are also performed under specific situations, where a shunt is placed to drain the bladder into the amniotic fluid [19]. However, shunt patency is limited to 1- 2 weeks and migration is common [19].

1.5.3 Medical Approaches

1.5.3.1 Drug Therapy

Drug therapy is very effective in the management of BPH [57]. α-adrenergic

blockers were first studied for their therapeutic effects on BPH in 1976 [58]. Inhibition of α-adrenergic receptors, and specifically α-1A, relaxes the smooth muscles of prostatic stroma, and subsequently reduces the prostatic urethral resistance to flow. Examples to α-1A blockers include Tamsolusin and Silodosin. Silodosin has been evaluated and shown to significantly increase maximum flow rate (Qmax) after several months of treatment [59]. Furthermore, urodynamic evaluation has shown an improvement of obstruction in 56% of patients, and reduction in bladder overactivity in 40% of patients on therapy [57,59].

5-α-reductase inhibitors, often used in conjugation with α-adrenergic blockers, help reduce obstruction through an intracellular enzymatic modification[60]. By inhibiting the enzyme 5-α reductase, formation of the active form of testosterone, i.e. dihydrotestosterone, is reduced. [61]. The use of 5-α reductase (5-AR) inhibitors decreased the incidence of invasive therapy for symptomatic BPH in 64% [62]. In a large-scale long term comparative trial, combination therapy remains superior to monotherapy in symptomatic relief, Qmax, and quality of life [63]. However, relief of obstruction does not always prevent changes in bladder, especially when treatment is instituted too late, or was found to be ineffective.

Smooth muscle hypertrophy resulting from longstanding pBOO or a neuropathic bladder, leads eventually to an unstable bladder wall with spontaneous contractions, adding even more pressure and exaggerating the pathology progression [64]. Blockage of unwanted contractions can lead to an improved outcome after pBOO and neuropathic bladders, which is achieved

currently with the use of antimuscarinic agents [65]. Despite being the first described medication of this category to manage bladder spasms, oxybutinin remains to be the standard of care [65]. By reducing smooth muscles overactivity, intravesical pressure is lowered, and symptoms of overactive bladder are improved[66]. The use of antimuscarinic therapy is associated with several unwanted side effects which may affect patients compliance, and maybe harmful especially in the geriatric population [67]. Solitary use of antimuscarinic therapy may not be a safe option, as a weak detrusor faced by outlet obstruction makes it very susceptible to retention and subsequent intravesical pressure elevations, introducing even more injury. Antimuscarinic therapy in these circumstances should be coupled with an effective way to drain the bladder, which is being achieved currently by clean intermittent catheterization.

1.5.3.2 Clean Intermittent Catheterization

Clean intermittent catheterization (CIC) is an effective mean of mechanical bladder drainage, along with indwelling urinary catheter. CIC is basically achieved by inserting a clean catheter to drain the bladder and then removed afterwards, i.e. an in-and-out fashion, which is usually done several times a day by the patient or the caregiver. CIC has several advantages over indwelling catheters and is the preferred method of lower urinary tract drainage[68]. First described in 1972, it gained a rapid popularity over conventional methods and gradually replaced their use when indicated [69].

The use of CIC helps to avoid many of the significant complications

associated with indwelling catheters [68]. CIC use has been associated with less UTIs, lower incidence of overflow incontinence, and preservation of renal function [68]. Furthermore, it allows for better self-care and improves independence, with less mechanical obstacles to the patient (i.e. carrying drainage bags). Timed regular catheterization allows the bladder to fill several times a day, being more physiological than continuous bladder decompression, and helps avoid associated detrusor laxity and areflexia. It is generally indicated in neuropathic bladders and in patients with high intravesical pressure despite medical treatment, and in particular, if evidence of renal injury is present [68,70]. CIC can be used in adults and in pediatric patients. Nevertheless, it is less tolerable in younger patients, especially if perineal sensation is intact, due to the unpleasant sensation that accompany this method [71], which necessitates continuous follow up and support.

Although less frequent and less severe than the ones associated with indwelling catheters, complications of CIC are still encountered, including urethritis, ascending epididymo-orchitis and bacteruria [72]. Trauma and urethral bleeding occur roughly in 33% of patients on regular long-term CIC, but the incidence of urethral strictures remains low[73].

1.5.4 Lower Urinary Tract Reconstruction

When all conservative and invasive medical and surgical approaches fail, lower urinary tract reconstruction surgeries are indicated [74]. After prolonged obstruction, the bladder becomes stiff and uncompliant with significantly low capacity and high pressure. Augmentation surgeries are done as a mean to reduce

intravesical pressure and improve capacity. Augmentation cystoplasty, hence, is indicated for neurogenic and non-neurogenic end-stage bladder dysfunction [75,76]. Augmentation procedures were performed in an animal model in 1888, and in human patients one year later [74]. However, It did not become popular until the 1950s, with further approval after the use of CIC in the 1980 as a mean of drainage [74,75]. Several organs have been historically used to augment small fibrotic contracted bladders, including gastric, omentum, peritoneum and skin grafts [77]. Augmentation ileocystoplasty remains the most common procedure[74]. In this procedure, a segment of ileum is detubulerised to be patched to an opened anterior bladder wall [74]. Unfortunately, augmentation procedures are all associated with a wide burden of complications. Early surgical complications include wound infection (5 - 6.4%), bowel obstruction (3 - 5.7%) and significant bleeding (0-3%)[78]. Long term adverse sequelae include augment failure that requires revision surgery[79], metabolic disturbances [78], renal function deterioration[80], UTIs, stone formation[74,81], augment and bladder perforation[82], and carcinomas [83]. However, in search for better substitutes, a wide group of investigators are looking into autologous tissue engineered augments, aiming to reduce the fore mentioned morbidities, which we will cover in the following sections.

1.6 Current Avenues of Research

We have come to understand that the pathophysiology of the development of end-stage bladder after partial obstruction involves multiple pathways and

molecules that interact and influence the bladder on the molecular and cellular levels. Furthermore, it has been clearly established that these molecules, proteins, and genes, are expressed in a timely, chronological fashion [1]. Such findings add several considerations to our knowledge about the pathology of pBOO, and improve our understanding of each phase of bladder deterioration, helping us target each phase with appropriate therapeutic interventions.

We will discuss in brief in the sections below some of the molecular pathways that are being studied currently, and their potential significance in the pathophysiology of pBOO, as well as the potential role of mesenchymal stem cells in the prevention and therapy of pBOO.

1.6.1 Molecular Pathways

1.6.1.1 Role of Prostaglandins in BOO

Prostaglandins (PGs) are prevalent in virtually all tissues [84]. They have been linked to several biological activities in health and disease. However, an interest in the role of PGs in the pathology of bladder outlet obstruction has gained a specific interest [84]. Prostaglandins effect is mainly mediated by cyclic AMP (cAMP), which has been linked to bladder relaxation and prevention of bladder smooth muscle hypertrophy [85]. A battery of research work has been done to investigate PGs and cyclooxygenase-2 (COX-2) enzyme relation to pBOO and bladder overactivity[86-88].

PGI2, PGE2, cAMP and cGMP have been associated with the inhibition of

SMC hypertrophy in vascular grafts, and a reduction in their levels is speculated to be related to SMC hypertrophy and proliferation [89]. An experimental animal model of pBOO has demonstrated an increase in SMCs of bladder wall after 3 weeks of obstruction, with a concomitant reduction in levels of PGI2, PGE2, cAMP and cGMP [84].

COX-2 levels have been shown to be increased as a result of pBOO and bladder wall stretch in several studies[88]. However, the exact role of this enzyme is yet to be discovered. Several studies have evaluated the involvement of PGE2, its receptor EP, and EP subtypes in the pathology of BOO and overactive bladder (OAB) [86-88]. Intravesical PGE2 administration has been shown to induce bladder contraction, detrusor over activity, and reduction in bladder capacity in animals and humans [90]. In a neuropathic animal model after spinal cord injury, PGE2 level was found to increase in the bladder [88]. Furthermore, a higher level of PGE2 was detected in young patients with pBOO secondary to urethral strictures[87]. PGE2 exerts its activity through the EP receptor family of G-protein coupled receptors, and 4 subtypes have been identified [91,92]. EP1 receptor has been proposed to be related to detrusor overactivity. In a pBOO animal model, EP1 knockout mice did not show signs of overactive bladder [88]. EP3 activation has been also linked to OAB, and higher bladder capacities achieved when antagonized [93]. On the other hand, EP4 activation is suggested to suppress detrusor contractions, and selective agonist instillation was observed to decrease the frequency of spontaneous contractions in rats with BOO[86]. Manipulation of the PGs pathway may seem appealing to manage abnormal detrusor contractility secondary to pBOO,

however, aspirin instillation has been investigated in a rabbit model, but did not yield significant therapeutic effects in pBOO in terms of pathologic bladder contractility[94].

1.6.1.2 Transforming Growth Factor β

TGF-β has been involved in several cellular pathways and processes, and is being studied in different pathologic and physiologic states, including tissue injury, fibrosis, and tumor biology[95,96]. TGF-β is secreted initially as a hypoactive precursor form, which is bound to the latency-associated peptide (LAP) [97]. TGF-β can be activated in several ways, to yield the active 25 kDa homodimer form [97]. In vitro activation can be achieved by acidity (pH 4), alkali, temperature (80^{o} C) and urea treatment, while in vivo activation may involve a plasmin-mediated reaction, or proteolysis of LAP by cathepsin D or glycosidases [98]. Cells are also involved in the activation process such as with osteoclasts [99].

TGF-β has been involved extensively in cellular growth and regulation of extracellular matrix. Inhibitory effects have been observed on the growth of epithelial and endothelial cells while having stimulatory effect on mesenchyme-derived tissues exposed to low concentrations of TGF-β (at high concentrations, it inhibits growth) [100,101]. TGF-β mediated extracellular proliferation has been clearly demonstrated, as it promotes an increase in the synthesis of fibronectin and fibrillar collagens (types I, III, and V) by fibroblasts [97]. It also decreases the expression of collagenases, transin/serine protease and plasminogen activator [97]. Furthermore, it stimulates tissue inhibitors of metalloproteinases (TIMPs) further

promoting extracellular matrix proliferation [97].

TGF-β has several isoforms (TGF-β1, TGF-β2, TGF-β3) and different distribution throughout mammalian tissues [97]. Activation of TGF-β receptors leads to a sequence of intracellular events, producing activated SMAD proteins[102,103]. SMAD proteins are linked to gene expression, and observed to modulate several biological cellular functions, including fibrosis, tumor formation, and epithelial cell differentiation to myofibroblasts[104,105].

TGF-β expression has been shown to increase in response to pBOO in animal models [1,106]. Urinary levels of TGF-β has been positively correlated with the level of pBOO in animal and human studies [107]. In vitro studies of human bladder samples, treatment with TGF-β1 induced smooth muscle hypertrophy, and increased collagens type I and III deposition in the extracellular matrix [108]. In an experimental animal model of pBOO, TGF-β type II receptor knockout mice had significantly less fibrosis and smooth muscle hypertrophy compared to wild type obstructed mice[109].

1.6.1.3 Nitric Oxide

Nitric Oxide (NO) was originally described in 1987 as a vasodilator molecule [110]. Since then, our knowledge of it has been expanding, and the role of NO in different biological activities is being explored. NO has a major role in smooth muscle relaxation, and has been recognized as a neurotransmitter in the non-adrenergic, non-cholinergic neurons (NANC) [111,112]. NO has a major effect on smooth muscle relaxation. Binding of NO to guanylyl cyclase stimulates the

production of cGMP from GTP, which poses multiple biological effects, including vasodilation and smooth muscle relaxation, via activation of protein kinase A2 and G [113,114].

cGMP deactivation is mediated by numerous phosphodiestrase enzymes (PDE) which hydrolyzes cGMP to 5'-GMP. Inhibition of PDE promotes longer actions of cGMP, which has been used for the vasodilatory effect in the treatment of pulmonary hypertension, and in erectile dysfunction[115]. The role of PDE inhibitors is also being investigated in lower urinary tract pathologies, and its possible beneficiary effects on bladder smooth muscle after pBOO[116].

NO is synthesized from its precursor, L-arginine via the enzyme nitric oxide synthase (NOS), which has three different isoforms: inducable (iNOS), and non-inducable isoforms endothelial (eNOS) and neuronal (nNos) [117,118]. iNOS is involved in tissue immunity, tissue injury, inflammation and apoptosis [119]. It is suggested that iNOS over-expression may lead to the disintegration of urothelial cells and inflammation, whilst non inducible forms (eNOS and nNOS) have a down-regulatory effect on tissue fibrosis[118].

The level of expression of the different types of NOS has been studied in several animal models of pBOO. Several studies reported changes in iNOS, eNOS and nNOS levels in the obstructed bladders [116,117]. iNOS has been observed to increase in the early inflammatory phase of pBOO in an animal model, whilst eNOS and nNOS had more immunoreactivity only after 8-weeks of pBOO, which might explain their role in cellular apoptosis after chronic obstruction[118]. Furthermore, pharmocological blockage of iNOS has been observed to ameliorate

functional and fibrotic changes of pBOO, with similar findings in iNOS -/- knockout animals [117]. However, there might be some differential roles on these enzymes, as a recent study reported a significant decrease in the mRNA expression of eNOS with prolonged obstruction[120], which suggests lower capillary perfusion and hypoxia.

1.6.1.4 Gap Junctions

Gap Junctions are specialized areas on plasma membranes of cells that aid in cell-to-cell communication, and exchange of ions and molecules. [121]. Each gap junction is formed of two hemichannels, or connexons, and each connexon is composed of six connexin molecules (Cx) [122]. An interest has been raised in their potential effect on detrusor overactivity secondary to pBOO, and attempts are being done to elaborate on their role. Alterations in the expression of Cx molecules have been observed in the urothelium and suburothelial tissue in overactive bladders [123]. Several Cx molecules have been noted to increase with detrusor overactivity secondary to pBOO, including Cx43 and Cx26 [122]. The role of Cx inhibitors is speculated to be of benefit in this pathological aspect, and the inhibition of these Cx molecules might demonstrate a therapeutic role in OAB after pBOO. Kim et al. [122] looked into two Cx inhibitors, 18B-GA and oleamide, in rats with surgically induced pBOO. They observed an improvement in the cystometrographic findings after administration of these Cx inhibitors, in terms of increased inter-contractile interval, compared to obstructed rats [122].

Basic fibroblast growth factor (BFGF) has been observed to increase in

obstructed bladders, and was investigated in voiding dysfunction[124,125]. Negoro et al studied the effect of BFGF on the expression of Cx43 in vitro, and concluded that BFGF is involved in the expression of Cx43 and detrusor overactivity. Their finding was also aided by their observation of increased detrusor activity after in vivo administration of BFGF [126]. However, a lot is still to be investigated in this area to uncover the exact interactions and mechanisms involved in detrusor overactivity after pBOO.

1.6.1.5 Hypoxia-Inducible Factor 1α

HIF-1 is a heterodimeric transcription factor, from the Per-aryl hydrocarbon receptor nuclear translocator (ARNT)-Sim family [127]. It is a major modulator of gene transcription in hypoxic states [128]. It consists of α subunits (HIF-1α, HIF-2α and HIF-3α) and a β subunit (HIF-1β) [127]. It has been linked to several pathological states, including angiogenesis and tumor growth and metastasis, via regulating the expression of vascular endothelial growth factor (VEGF) [127]. HIF-1α has been observed to increase after pBOO[1,129], confirming the presence of bladder wall hypoxia. As the bladder pressure increases, hypoxia become more evident, especially in the voiding phase, which is thought to be from direct pressure against bladder wall capillaries [129]. Koritsiadis et al [129] investigated the presence of ischemia in human bladder tissue after prolonged pBOO. In this study, samples were gained from patients with prolonged BPH, stained for HIF-1α, and compared to normal human tissue. They found a significant increase of HIF-1α in the submucosal and stromal cells of the bladder after 10 years of

pBOO, with gradual decrease afterwards [129] . Galvin et al [130]studied the response of human bladder wall cells to hypoxia in vitro, and demonstrated an increase in HIF-1α and VEGF levels with time. In their experiment they observed an arrest of cellular growth and development, with an up-regulation of $p27^{kip1}$, a cell cycle inhibitor, which might contribute to the pathology of bladder decompensation with prolonged obstruction [130]. Alleviating bladder wall hypoxia after pBOO is a promising approach to prevent hypoxia-induced pathological consequences. Several HIF inhibitors are present, but are tested mainly for their effect in pathological angiogenesis in tumor formation and metastasis [127].

1.6.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are currently being studied for their several unique features and capabilities. They are thought of as multi-potent stem cells that possess several immune, regenerative, and therapeutic effects. We will go into more details on MSCs and their relation to inflammation, fibrosis and tissue repair in the following sections, starting with a brief historic background, and their markers and characteristics.

1.6.2.1 History and Background

Bone marrow mesenchymal stem cells (BM-MSCs) were originally described in 1961 by McCulloch and Tell in the stroma of bone marrow during their research on the sensitivity of mouse bone marrow to radiation. At first, these cells were referred to as spleen colony forming units (CFU-S), after being observed to form new colonies in spleens of irradiated mice. They suggested that

these cells have two main characteristics, the ability of self-renewal and replication, and the potential for differentiation into tissue-specific cells, i.e. plasticity. [131]. In the following years, Friedestein et al. demonstrated and elaborated on these two concepts on the fibroblast-like cells, and these cells were observed to form colonies in tissue culture, and were given the name of colony forming units - fibroblasts (CFU-F) [132] .

Although these cells were found originally in the bone marrow, they are now also isolated from several organs and tissues, including liver, kidney, amniotic fluid, umbilical cord, and adipose tissue [133] . Adipose derived MSCs are more abundant than bone marrow derived cells, and form more colonies in vitro[134]. The nomenclature of MSCs originated from the initial observation of their capability to differentiate in vitro into mesenchymal tissue, namely, osteocytes, chondrocytes and adipocytes[135] . Nevertheless, it is now feasible to differentiate MSCs into endoderm and ectoderm-derived cells [136] . This allowed for experiments and trials of tissue engineering and regeneration. MSCs popularity expands beyond tissue engineering, and their role as anti-inflammatory agents is being explored, alongside with their potential to repair injured tissues and organs after systemic administration. They have been observed to interfere with dendritic and T-cell functions, and to create an immunosuppressive microenvironment by cytokine secretions [137]. Furthermore, their recruitment to a specific tissue is largely enhanced in the presence of inflammatory states [6,138].

1.6.2.2 Characteristics and Cell Markers

Several characteristics are used to describe MSCs. These cells are characterized morphologically with a small cell body, and long, thin cell processes, simulating fibroblasts. They are known for their affinity to adhere to plastic, and this feature has been utilized to isolate them from bone marrow stroma despite their limited number (1 MSC in 10^4 to $5x10^7$ marrow cells) [133,139].

In regard to cell markers, MSCs do not show a specific or a unique marker, but they all express CD105, CD73, and CD90, and lack the expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR[139]. The lack of HLA-DR (MHC II) prevents recognition by host immune cells and renders these cells non-immunogenic which removes the need for immunosuppressive therapy in allogenic transplants[137].

Chemokine receptors (CCR, CXCR) are abundant on MSCs. Chamberlain et al [140] explained the expression of CCR6, CCR9, CXCR3, CXCR6 receptors on murine MSCs which are involved in the recruitment of immune cells to areas of inflammation [140]. Chemokine CCL2 has been seen to increase in inflammatory and fibrotic states, and blockage of CCL2 receptors resulted in less inflammation and fibrosis, an effect also seen in the cases of MSC systemic administration, which might give an insight to the mechanisms of recruitment to inflamed organs[6,141].

1.6.2.3 Role in inflammation and Fibrosis

MSCs have been studied for their therapeutic and reparative potential in

multiple tissue organs and diseases in animal models, with promising results. MSCs augment healing in inflamed and injured tissues, possibly by cell replacement, stimulation of original tissue regeneration, inhibition of apoptosis and fibrosis, or enhancement of angiogenesis[142,143]. MSCs were seen to exert an immunomodulatory effects as well. In vitro studies have demonstrated an inhibitory effect of MSCs on tumor necrosis factor (TNF) α, interferon (IFN) α, and T-cell inhibition through IL-10 [144].

Furthermore, MSCs have shown promising results in tissue repair and regeneration. In a bleomycin lung injury model, systemic administration of bone marrow derived MSCs has lead to a significant reduction in inflammation and collagen deposition [138]. In another model of lung injury, umbilical MSCs were seen to be recruited to areas of lung injury, but not to healthy tissue, as soon as 2 weeks after administration [145]. Furthermore, there was a significant reduction of pro-inflammatory cytokines, TGF-β, IFN-γ, and TNF-α, in this model as well [145]. These cells were not observed to differentiate into lung tissue, and despite this, their therapeutic effect was still observed, which proposes a local anti-inflammatory and repair signaling exerted by MSCs to original cells. In a murine myocardial infarct model, local injection of MSCs into myocardial scars resulted in an improvement of left ventricular function after 4 weeks [146]. However, these injected cells did not express myocardial markers until 6 months post injection, but by that time, functional improvement has abolished, which again suggests the paracrine mechanism for cellular improvement and repair rather than direct differentiation into myocardial cells [146]. Another suggestive observation of the

paracrine mechanism is that the effect of MSCs is observed even when only few cells were seen to reside in the injured tissue by immunofluorescence microscopy[6].

MSCs were observed to secrete IL-6, hepatocyte growth factor, VEGF and BFGF which all modulate cellular proliferation, inflammation and angiogenesis[147,148]. However, a mixture of both mechanisms (i.e. direct cellular differentiation and paracrine factors release) might all take place in a phasic chronological sequence to achieve the observed effects.

One experiment has been done to evaluate the role of MSCs in short-term pBOO. Woo et al investigated the effects of systemic administration of MSCs in a 4 weeks pBOO mouse model. They observed a significant improvement in bladder wall compliance, capacity, and capillary perfusion, as well as fibrosis and collagen deposition compared to the obstructed animals without MSCs administration[6]. In this model, MSCs were given after 3 days of obstruction, and bladders were harvested after 4 weeks. Nevertheless, long-term effects beyond 4 weeks has not been studied in this model, which might reveal more findings that help understand the effects of such intervention. In this study, 15 animals were injected with MSCs, but the therapeutic effects were observed only in 10 which MSCs were actually seen to be recruited to the bladder, where in the ones that did not show MSCs recruitment did not have such improvements, suggesting a local microenvironment action of these cells when they are homed to sites of inflammation.

1.6.3 Bladder Tissue Engineering

Another different, yet promising area in the avenues of research in urinary bladder is the attempts for synthetic engineered bladder substitutes. In fact, the first tissue engineered organ to be tested in clinical trials was a neobladder aimed to substitute intestine for augmentation cystoplasty [149]. The current concepts of research relies on unseeded and seeded scaffolds, depending whether cellular components are being "planted" on different types of scaffolds or not before incorporation into living tissue. More success has been achieved with the use of seeded scaffolds, as unseeded scaffolds have been faced with several obstacles. The design in Atala's work depends on seeded acellular matrices, by obtaining homologous bladder tissue and expansion in vitro, and seeding these cells on a collagen scaffold. This graft has succeeded to increase bladder capacity but not the compliance due to the use of collagen. Several other scaffold materials have been used as well in addition to collagen, including small intestine submucosa, alginate, and polymers of polyglycolic acid [150-153].

MSCs have been also used in bladder tissue engineering. Tian et al successfully differentiated MSCs into urothelial tissue in vitro [136]. In vivo trial resulted in a luminoid bladder structure with urothelium and smooth muscle layers, after incubating MSCs and embryonic bladder mesenchyme under the renal capsule [154]. Nevertheless, a more promising work comes from the use of MSCs in a polymer scaffold (POC) [155]. This scaffold has the features of short half life, and furthermore, does not interfere with cellular activities[156].

Another approach into bladder tissue engineering has been conducted

without the use of exogenous scaffolds [157,158]. In this model, the method consisted of allowing skin fibroblasts to generate an ECM in vitro in an ascorbic acid environment, as previously described in a skin model [159] followed by seeding with urothelial cells on top of fibroblasts tissue layers. The same researchers improved a previously described method of dynamic pressure cycles during graft development and formation, to enhance the graft's histological and functional characteristics[158,160].

1.7 Conclusion and Formulation of Research Question

The ongoing exploration of the pathological mechanisms of pBOO and the therapeutic attempts promise a huge change in the way that pBOO was classically looked at. The presence of a valid animal model of pBOO, which is reproducible and constant, has further accelerated the results in this field.

Different pathways are being examined, either on the molecular, cellular and translational aspects, seeking the effective employment of these collective findings into patient care. Knowing the exact pathological findings in each phase of pBOO that leads to an end-stage bladder will allow us to effectively target the pathological mechanisms involved. From the initial inflammatory, hypertrophied compensated phase, to the fibrosed dysfunctional bladder, interventions should be tailored to be phase-specific to effectively translate the bench work into clinical practice. Furthermore, our understanding of the initiating events will aid in the prophylaxis and anticipation of the deleterious events before taking place.

Mass spectrometry-based protein identification is a powerful tool to identify

and quantify proteins in different biological systems, and has been utilized in many pathological conditions to better understand the changes in protein expression in disease states. We will investigate the proteomic profile of bladder tissue after pBOO, aiming to identify major changes in protein expressions associated with this pathology, and possibly identifying new pathways that might serve as novel therapeutic targets.

MSCs have shown a great potential in the anti-inflammatory aspects, as well as with the healing and regenerative processes. We will explore further into their potential role in pBOO. We aim at preventing the pathological changes associated with pBOO, by systemic administration of MSCs early on the disease processes. We speculate that this approach will prevent the disease progression and provide protection to bladder's anatomical composition, and more importantly, its function. To further examine MSCs capabilities in our model, we will look into their effect on the common systemic and local inflammatory and hypoxic markers that are thought to exacerbate the pathology and result in end-stage bladder disease.

We hypothesize that early administration of MSCs will prevent the deleterious changes via preventing the inflammatory cascade leading to bladder decompensation. Animals will be subjected to pBOO surgically. Bladder function will be assessed with urodynamic studies. MSCs will be injected via tail vain, and bladders will be studied at several time intervals, assessing changes in a chronological manner.

These experiments, and their results, will add to current knowledge of

the pathology of pBOO, which will further expedite our steps towards the uncovering of the exact mechanisms of bladder dysfunction. Provided with a better understanding, and through relentless efforts, we will reach the cure, and by doing so we serve our noble goal, improving our patients' health and well-being.

1.8 References

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Chapter 2

Early Changes in Proteomic Profile of Urinary Bladder After Partial Bladder Outlet Obstruction

2.1 Introduction

pBOO is a commonly seen problem in adult and pediatric urology, and a significant cause of morbidity and mortality in these patients. As the obstruction ensues, different mechanisms and pathways are activated, with the potential end result of bladder fibrosis [1]. Multiple factors have been all linked to this pathology, including inflammation, hypoxia, and remodeling of ECM components [1,2]. However, what happens after acute obstruction, and the exact mapping of molecular changes over the time course of pBOO are yet to be discovered.

Proteomics is a collective term used to describe a wide array of protein studies. The term was first described in 1997 by Peter James, succeeding genomics, the study of genes[3]. Different methods and techniques are being used to identify global protein changes in disease conditions, aiming to achieve better understanding and identification of possible therapeutic targets. Mass spectrometry-based analysis is a powerful tool to identify and quantify proteins, providing a window to the proteome of different biological systems.

Herein, we explored the changes on the proteomic level of urinary bladder tissues in the first 14 days post pBOO, aiming to identify major changes in proteins and gene expressions involved in the initiation of pathological cascades, and relating these changes to the mechanisms of injury and the pathways activated.

2.2 Methods

To conduct this experiment, full approval from the University of Alberta Animal Care and Use Committee was obtained. Three un-obstructed animals were used as a reference to normal tissue, and surgical induction of pBOO was performed as previously described[4] for 3, 7 and 14 days with 3 animals per time point. Main outcomes were the measurement of bladder weight, urodynamic studies, and bladder tissue proteomic analysis. Real-Time Polymerase Chain Reaction (RT-PCR) was performed to determine gene transcription trends of proteins identified via mass spectrometry in this study.

2.2.1 Surgical Induction of pBOO:

Isoflourane anesthesia was used for induction and maintenance throughout the procedure. After confirmation of depth of anesthesia with toe pinch, and stabilization of respiratory and heart rate, animals were placed in supine position, shaved, and iodine solution was applied to abdominal and pelvic area. A lower midline skin incision was made, access to peritoneal cavity was achieved after dissecting the abdominal wall layer by layer. Urinary bladder was identified, and fine dissection was performed for proper exposure and identification of urethra, ureters, and adjacent organs. An 18-gauge angiocatheter was introduced into the bladder through bladder dome, and advanced into the urethra to serve as a urethral stent for calibration and prevention of complete obstruction. A 2-0 silk tie suture was passed below ureters bilaterally, placed around the urethra, and ligated gently with the angiocatheter in place, creating an external physical partial obstruction to bladder outlet *(fig 2.1)*. The angiocatheter was then removed, and bladder dome

closed with a 5-0 vicryl suture in a water-tight fashion. Abdominal wall, fascia, and skin were closed using a 3-0 vicryl continouos suture. Isoflourane was then discontinued and animals kept on oxygen per nasal cone until recovery.

2.2.2 Measurements of Bladder Weight & Urodynamics:

Under isoflourane anesthesia, an 18-gauge angiocatheter was introduced into the bladder through a superior cystostomy and connected to a water infusion pump at 0.1 ml/minute. Continuous pressure monitoring and readings recorded (cm H_2O) using a low-pressure transducer. The study was terminated when visible urinary leakage was observed from the urethral meatus. Bladder capacity, leak point pressure, and maximum pressure were recorded. Bladder wall compliance was calculated to reflect change of pressure with volume infusion (ml / cm H_2O). Animals were then killed humanely, bladders were excised and wet weight recorded. A segment of bladder tissue was then fixed in 4% paraformaldehyde and Shandon Cryomatrix (Thermo Scientific, UT). Paraffin blocks sectioned and stained with hematoxylyn & eosin. The remaining tissue was snap frozen in liquid nitrogen for subsequent protein extraction and analysis.

2.2.3 Protein Extraction from Bladder Tissue:

Equal samples of bladder tissue were obtained from each animal at sacrifice (200 mg bladder tissue). Tissue was submerged in liquid nitrogen and homogenized while frozen in a Micro-Dismembrator (B. Braun Biotech Inc., Allentown, PA). Tissue powder was suspended in a cell lysis buffer. Solution was kept on ice for 30 minutes, vortexed several times, and then centrifuged. Supernatant obtained and protein concentration measurement was performed

using the Bradford Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), to yield a final concentration of 2 mg/ml for each sample. Bovine serum albumin was used as a standard. Lysate was then used immediately or snap frozen and stored until further analysis.

2.2.4 Gel Electrophoresis and Staining:

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 12% BisTris Criterion precast gels (Bio-Rad, Hercules, CA, USA) were used. Molecular weight ladder was loaded in first gel lane and 40 μL of each sample was loaded with loading buffer. Gel was submerged in SDS-PAGE running buffer and run at 150V for 10 minutes, followed by 200V until dye visualized at the bottom of the gel. Staining performed overnight using Coomassie Blue protein stain.

2.2.5 Liquid Chromatography-Mass Spectrometry Analysis:

Each gel lane was cut into ten regions, and after in-gel tryptic digestion, analysis was performed using liquid chromatography-mass spectrometry (LC-MS/MS) in the Institute of Biomolecular Design (IBD) at the University of Alberta. Briefly, the excised gel bands were de-stained twice in 100mM ammonium bicarbonate (AmBic)/acetonitrile (ACN) (50:50). The samples were then reduced (10mM β-mercaptoethanol 100mM AmBic) and alkylated (55mM iodoacetamide in 100mM AmBic). After dehydration of the gel, trypsin (6 ng/ul) was added to just cover the gel pieces and the digestion was allowed to proceed overnight (~16 hrs.) at room temperature. Tryptic peptides were first extracted from the gel using 97% water/2% ACN/1% formic acid

followed by a second extraction using a 1:1 mixture of extraction buffer and acetonitrile.

Fractions containing tryptic peptides dissolved in aqueous 25% v/v ACN and 1% v/v formic acid were resolved and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Scientific) coupled to the LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100μm inner diameter (300Å, 5μm, New Objective). Peptide mixtures were injected onto the column at a flow rate of 3000 nL/min and resolved at 500 nL/min using 60 min linear ACN gradients from 0 to 45% v/v aqueous ACN in 0.2% v/v formic acid. The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 60 000 and m/z range of 400–2000. The ten most intense multiply charged ions were sequentially fragmented by using collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap; after two fragmentations all precursors selected for dissociation were dynamically excluded for 60 s. Data was processed using Proteome Discoverer 1.3 (Thermo Scientific) and the non-redundant reviewed Rattus norvegicus protein database from Uniprot was searched using SEQUEST (Thermo Scientific). Search parameters included a precursor mass tolerance of 10ppm and a fragment mass tolerance of 0.8 Da. Peptides were searched with carbamidomethyl cysteine as a static modification

and oxidized methionine as a dynamic modification. For quantification of proteins between samples, spectral counting was used[5,6].

2.2.6 Quantitative Real Time Polymerase Chain Reaction (RT-PCR):

RNA extraction was performed from snap frozen bladder tissue after homogenization and suspension in Trizol solution, using RNeasy spin columns (Qiagen, Mississauga, ON, Canada). After DNAse digestion for 60 minutes, random primers were used to synthesize first cDNA strand (Sigma, Oakville, ON, Canada). RT-PCR was then performed (Power SYBR Green PCR Master Mix, ABI, Foster City, CA). 25 μL used containing 0.2 μM of genes of proteins identified using upstream and downstream primers, and 1 μL of first strand product. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene was used as control. Amplifications were done using an ABI 7300 real-time system (Applied Biosystems, Foster City, CA). For standardization of amplification curves, HPRT amplification was used. Table 2.1 outlines forward and reverse primers sequences of genes used in this study.

2.2.7 Statistical Analysis:

Proteins present in all samples were used for statistical analysis. Data reported here as mean fold change from normal control ± standard error around the mean of peptide spectral counts for each protein. Analysis of variance (ANOVA) was performed for statistical analysis for all presented data (STATA software Version 12.1). A value of $p \leq 0.05$ was used to determine statistical significance.

2.3 Results

2.3.1 Animal Health:

All animals remained healthy throughout the duration of experiment, with no mortalities. There was no significant difference in animals weight pre-procedural and at time of sacrifice.

2.3.2 Urodynamics and Bladder Weight:

pBOO induced an increase in bladder weight compared to unobstructed group, observed significantly in the 3 days group (303.3±24.0 mg vs. 200.7±4.6 mg, p-value 0.01), and in the 14 days group (535.0±82.5 mg vs. 200.7±4.6 mg, p-value 0.02) (fig 2.2). Bladder capacity showed an initial drop from baseline in 3- and 7-day groups, but increased in 14-day group compared to normal bladders (2.2±0.3 vs. 0.9±0.2 ml, p-value 0.02) (fig 2.3). Leak point pressure remained relatively unchanged initially, with a trend towards increasing in the 14-day obstruction group but not statistically significant (fig 2.4). Bladder compliance followed the pattern of bladder capacity, with an initial reduction followed by an increase in the 14-day group, however not significant in all groups (fig 2.5).

2.3.3 Proteomic Analysis:

Stained gels after resolving protein solutions showed homogenous outcomes in all lanes (fig 2.6). An average of 613 proteins per sample were identified initially, and after isolating proteins repeatedly detected in all samples, 140 proteins were subjected to statistical testing. Table 2.2 outlines proteins with significant change from un-obstructed bladder tissue, and their fold-change from

baseline in all experimental groups. Marker of endoplasmic reticulum stress, 78-kDa glucose-regulated protein (GRP78), was found to increase after pBOO being statistically significant in the 14-day group (fold change 3.7±0.3, p-value 0.01). Ras homolog transforming protein member A (RhoA) showed a 1.9±0.2 fold-change after 14 days of obstruction (p-value 0.044), with an increase in RhoA-GDP dissociation inhibitor level (1.95±0.1 fold, p-value 0.037). Cytoskeleton molecules, actin and tubulin-α/β, showed a statistically significant increase after 14 days of obstruction (table 2.2). Small leucine-rich proteoglycans (SLRPs) decorin and lumican, decreased after 14 days of pBOO (fold change 0.35±0.04 and 0.34±0.03, p-value 0.048 and 0.002, respectively).

2.3.4 RT-PCR:

GRP78 mRNA expression showed a significant reduction in the 7-day obstruction group compared to 0-day group (0.56±0.12 fold, p-value 0.039), and then increased after 14-days obstruction (mean fold change 2.57±0.61, p-value 0.064) (figure 2.7). RhoA mRNA decreased initially in the 3-day group (0.27±0.04 fold, p-value 0.03) then increased significantly after 14 days of pBOO (1.93±0.15, p-value 0.04) (figure 2.8). Down-regulation of decorin mRNA was observed in all experimental groups, being statistically significant in the 3-day group (fold change 0.20±0.11, p-value 0.02) (figure 2.9). Lumican mRNA level decreased in all groups, however, did not reach statistical significance (figure 2.10). In keeping with our previous work, TGFβ1 & HIF1α mRNA levels increased significantly after 14 days of pBOO (3.40±0.30 fold for TGFβ and 4.40±1.00 fold for HIF1α, p-values <0.01 and 0.03, respectively).

2.4 Discussion

What happens from pBOO to bladder fibrosis and end-stage bladder remains to be elusive. Multiple factors have been seen to play a role in this pathology, including mechanical strain, bladder wall hypoxia, and inflammation[1]. Several factors and pathways are known to interplay throughout the time course of pBOO, including TGFβ, CTGF, HIF1α, and ECM proteoglycans [1,2]. However, the exact mechanisms and interactions are yet to be discovered.

Mass spectrometry protein identification is a superior tool to identify proteins and changes in their abundance, and has been utilized to understand several pathological conditions [7,8]. We previously demonstrated that several changes on the functional and molecular level have already occurred by 4 weeks after pBOO[1]. However, the response happens early and likely multifactorial. Proteomics offer us an insight into a wide spectrum of proteins, and allows us to examine the changes in their expression and to detect novel pathways and potential intervention targets. In this current work, we describe the changes in the proteome of urinary bladder in a temporal fashion after 3, 7 and 14 days of pBOO. The majority of identified proteins can be grouped into being related to changes in cellular structure, stress, inflammation, hypoxia and oxidative stress. While some of the proteins detected in this study correlate with our previous work, such as with decorin down-regulation [1,2], we identified new proteins described for the first time in association with pBOO, which might reveal to be of possible clinical benefit.

2.4.1 GRP78 and Endoplasmic Reticulum Stress:

GRP78 is a member of 70kDa heat shock proteins, and one of the most abundant molecular chaperons in the endoplasmic reticulum (ER) that is involved in proper protein folding [1,2,9].

During states of stress, unfolded and misfolded proteins accumulate in the ER inducing endoplasmic reticulum stress (ERS), which activates the quality control process known as the unfolded protein response (UPR), characterized by an increase in chaperons involved in protein homeostasis including heat shock proteins[3,9]. In general, heat shock proteins exert a protective effect on cells in stressful conditions, such as hypoxia, metabolic stress, inflammatory cytokine exposure, and apoptosis-inducing environments, and serve as a biomarker of cellular stress and disease [4,10]. Activation of UPR results in either proper folding of proteins, and if not feasible, degradation and triggering of apoptotic sequences[1,9,1,2,11]. Our findings confirm the presence of ERS after 14 days of pBOO, evident by the increase in GRP78 protein level *(figure 2.7)* and heat shock cognate 70 protein level *(table 2.2)*.

In normal conditions, GRP78 is stable and bound to UPR activators: activating transcription factor 6 (ATF6), translation initiation factor 2 kinase (PERK), and inositol-requiring enzyme 1 (IRE1) [7,8,12]. The presence of unfolded proteins dissociates GRP78 from these molecules, and increases transcription of UPR target genes, including GRP78 [13]. GRP78 binds to exposed hydrophobic areas on unfolded proteins and restores proper protein form [13,14]. ERS and UPR activation is seen in several pathologies and fibrotic conditions, including heart

failure, chronic kidney disease, cystic fibrosis, renal and interstitial lung fibrosis[12,13,15,16].

Maintaining low levels of unfolded proteins and reducing endoplasmic reticulum stress is essential in maintaining the normal physiology of a cell, and preventing cellular apoptosis. In an animal study of liver diseases, GRP78 knockout mice had exacerbated pathological sequelae and mortality [17]. 4-phenylbutyrate (4-PBA), a molecular chaperon that was shown to stabilize protein configuration and improve ER protein folding [18], provided neuroprotective effects in mice with hypoxic brain injury [19]. In the urinary bladder, GRP78 was noted to increase after 24 hours of complete urethral ligation in animals, and treatment with 4-PBA resulted in lower ERS response in-vitro, and less apoptosis in-vivo [20]. In this previous model, applying mechanical strain to bladder tissue in-vitro did not show evidence of ERS, however, ERS was present after exposure to low oxygen conditions suggesting the role of bladder wall hypoxia rather than mechanical pressure in the induction of ERS [20].

If this stress continues, cellular apoptosis is the end result of continuous accumulation of misfolded proteins in the cell and ERS activation. This has been demonstrated in an animal model of chronic kidney disease, where the presence of ERS was associated with increased proximal renal tubular cellular apoptosis [21]. Instituting proper therapy and hence, reducing cellular stress, results in lower ERS activation and cellular apoptosis. This has been described in a rat model of myocardial infarction, where treatment with atorvastatin resulted in lower GRP78 and caspase-12 levels [22]. Treatment with candesartan alleviated some of the

pathological sequelae and ERS response in kidneys in a model of ureteric obstruction [23]. These findings suggest the role of ERS as a mean of monitoring disease progression, cellular stress, and response to treatment.

2.4.2 RhoA/RhoA Kinase pathway:

RhoA is a small GTPase that belongs to the Ras superfamily. It has been found in our study to increase significantly in protein and mRNA expressions after 14 days post pBOO. Activation of RhoA from its inactive form RhoA-GDP, results in many downstream effects on the cellular and molecular level, which contribute to the pathology of pBOO. The active form, RhoA-GTP, exerts its effects through 2 main effectors: RhoA Kinase (ROCK), and Diaphanous-related formin (Dia), which results in increased smooth muscle contractility, cytoskeletal modulation, and formation of stress fibers [24]. It has been previously shown that RhoA/ROCK pathway is involved in urinary bladder smooth muscle contractility in humans and animals [25-27].

Hypoxia and mechanical stress, two of the main factors in pBOO, have been shown to activate RhoA/ROCK [28,29]. RhoA, through its downstream effector, ROCK, increases Ca^{++} sensitivity in bladder smooth muscles, and inhibits myosin light-chain (MLC) phosphatase activity. This results in an increase in MLC phosphorylation and increased smooth muscle contractility[26,30,31]. Several experimental studies found RhoA/ROCK to increase following pBOO [32,33], and administration of Y27632, a ROCK inhibitor, resulted in lower contractility of obstructed bladder smooth muscles [32,34]. Collectively,

RhoA/ROCK pathway activation after pBOO seems to be a major responsible pathway for detrusor overactivity after pBOO.

Moreover, the activation of RhoA/ROCK seems to augment the pathology after pBOO by modulating nitric oxide pathway. In vascular endothelium, activation of RhoA/ROCK resulted in reduction of eNOS gene expression [35], thus, reducing perfusion and augmenting hypoxia and fibrosis [36]. In spontaneous hypertensive rats, which are prone to develop an overactive bladder, treatment with verdanafil resulted in less activation of ROCK, and increased cGMP levels with improved bladder outcomes[37]. This confirms the interaction between NOS and ROCK pathways after pBOO.

RhoA/ROCK activation might play a role in bladder smooth muscle hypertrophy as well. In vascular smooth muscle cells, activation of RhoA/ROCK resulted in vascular SMC hypertrophy [35]. However, mechanical strain, in addition to RhoA/ROCK activation, activated extracellular signal-regulated kinase (ERK) 1/2 and c-Jun NH2-terminal kinase (JNK) pathways, resulting in vascular SMCs hypertrophy, indicating that multiple pathways are involved in producing bladder SMCs hypertrophy [38-40].

The increase in bladder contractility after pBOO, required to overcome the increased resistance to voiding, might result in an exacerbation of the downstream effects on the bladder wall. This might explain in part the positive outcomes of controlling bladder wall contractility with the anticholinergic oxybutynin, which is the current proven treatment of pBOO [41].

TGFβ has been documented to increase after pBOO and thought to be responsible for many of its pathological changes [1]. TGFβ and RhoA/ROCK pathways seem to interact on many levels. TGFβ is thought to activate Rho/ROCK pathway in a SMAD dependent and independent manner [42]. It has been found to activate RhoA/ROCK pathway in neural crest cells, vascular endothelial cells, and rat pulmonary arterial smooth muscle cells [43]. The interaction between RhoA/ROCK and TGFβ /SMAD pathway is evident as well in actin modulation in fibroblasts [44-47]. Moreover, administration of TGFβ to synovial-derived mesenchymal stem cells resulted in the activation of RhoA/ROCK pathway, which induced cytoskeletal reorganization [48]. Together, TGFβ and RhoA/ROCK pathways interaction seem to play a central role in the outcomes after pBOO.

2.4.3 Small Leucine-Rich Proteoglycans:

SLRPs are ECM proteoglycans that are involved in collagen fibrillogenesis [2]. Altered expression has been demonstrated in several pathological and fibrotic conditions [49]. Our laboratory has previously shown that decorin is down-regulated 4 weeks after pBOO [2]. Decorin binds to collagen fibrils, making them thinner and more regular [50], and this reduction promotes abnormal collagen deposition and fibrosis. Here, we detected a significant reduction in decorin protein level after 2 weeks of pBOO, which promotes a pro-fibrotic environment *(fig 2.9)*.

Another member of SLRPs, lumican, was noted to decrease after 14 days of obstruction as well. Lumican is one of the major proteoglycans in the ECM of

cornea, dermis, and muscle connective tissue [51]. As with other SLRPs, the main function of lumican is the regulation of ECM collagen, contributing mainly to corneal transparency [52]. Lumican-null mice demonstrated delayed wound healing, increased recruitment of inflammatory macrophages, and increased TGFβ level [53]. The homogenous effect of pBOO observed in this study on detected SLRPs could be explained by the increase of TGFβ level. TGFβ was found to have an inverse relationship with decorin in fibrotic diseases[54,55], and its activation resulted in reduction in lumican level in many studies, both in-vivo and in-vitro [56-58]. However, the reduction of lumican level might be an early response to TGFβ, as its level has been noted to be elevated in fibrotic conditions such as hepatic fibrosis and post burn hypertrophic scarring, suggesting its role in scar remodelling[49,59]. TGFβ-induced downregulation of lumican was prevented by blocking RhoA/ROCK pathway in corneal stromal keratocytes [60], suggesting the importance of both pathways in regulating SLRPs.

Significant increases in TGFβ and HIF1α mRNA expressions were observed after 2 weeks of pBOO in this current study, confirming our previous findings [1]. These proteins were not detected by mass spectrometry, which highlights the limitations of this approach. Protein identification relies mainly on identification of peptides in a sample and matching them to computer-based databases. Several factors have been reported to interfere with the process of proteins identification, including extremes of molecular weight and protein length (very high or very low), peptide characteristics (e.g. hydrophobicity), and the database selection [61]. These factors might attribute to the inability to detect some

of the proteins that have been previously associated with pBOO. However, despite these limitations, mass spectrometry protein identification remains to be a very reliable technique in proteomics, and is being utilized extensively in the modern era of global protein studies.

2.5 Conclusion

Multiple pathways are activated in the early phase after pBOO. Bladder wall hypoxia and inflammation results in an increase in GRP78 and HSc70, denoting accumulation of unfolded proteins and endoplasmic reticulum stress response activation, promoting a pro-apoptotic environment. Mechanical strain, in addition to hypoxia, results in the activation of RhoA/ROCK pathway, with its downstream effects of increased muscle contractility and hypertrophy, stress fibers formation, and interfering with nitric oxide metabolism adding more hypoxic injury and promoting fibrosis. Inflammation and TGFβ activation is evident by the changes noted in decorin and lumican, favoring abnormal collagen deposition. TGFβ and RhoA/ROCK pathways interact and seem to play a central role in this pathology. Therapeutic targeting of all initiating events after pBOO, i.e mechanical strain, bladder wall hypoxia and inflammation, seems to be the appropriate approach to ameliorate the pathological sequelae and progression to bladder fibrosis. Figure 2.11 summarizes the changes in protein expression detected in this study, and their possible inducers, downstream effects, and interactions after pBOO.

2.6 Tables

Table 2.1 List of PCR primers sequences.

Gene	Forward Sequence	Reverse Sequence
GRP78	5'-CCAGCTTACTTCAATGATGCAC-3'	5'-CTTCTCTCCCTCTCTCTTATCC-3'
RhoA	5'-AGGACCAGTTCCCAGAGGTT-3'	5'-ACTATCAGGGCTGTCGATGG-3'
Decorin	5'-ACACCAACATAACTGCTATTCC-3'	5'-AGACTGCCATTTTCCACAAC-3'
Lumican	5'-CACCAGAATGTAACTGTCCC-3'	5'-TCAGCTTAGAGAAGACCTTTCC-3'
TGFβ1	5'-GAGGTGACCTGGGCACCAT-3'	5'-GGCCATGAGGAGCAGGAA-3'
HIF1α	5'-TGCTTGGTGCTGATTTGTGA-3'	5'-GGTCAGATGATCAGAGTCCA-3'

Table 2.2 Protein identification and quantification results, and their fold change from normal unobstructed bladder tissue.

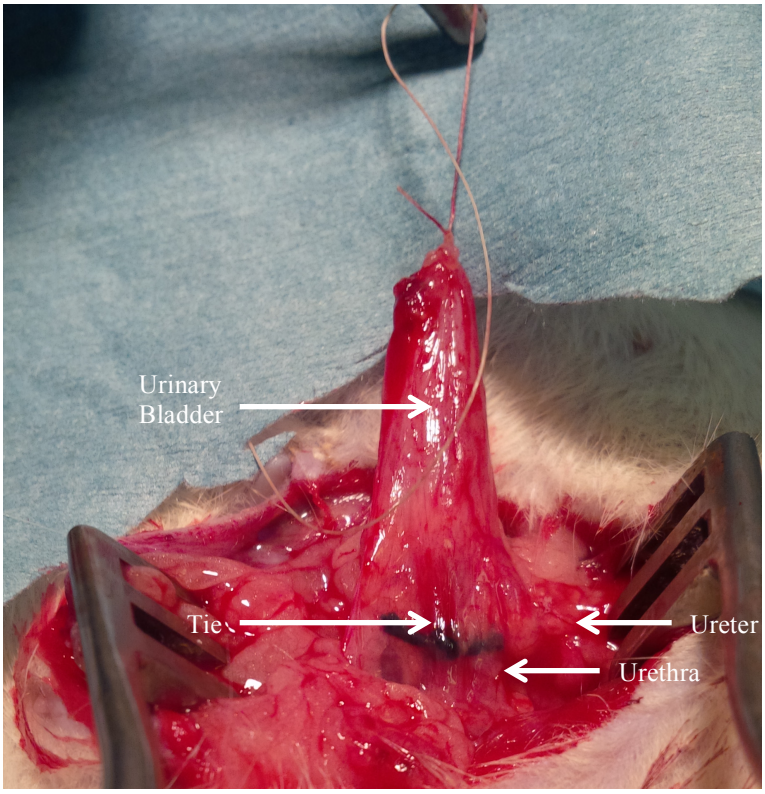
Accession	Gene	Protein	Ratio (O/N)[1]		
			3 Days	7 Days	14 Days
Up-regulated					
P06761	Hspa5	78 kDa glucose-regulated protein	2.04	2.15	3.66*
P69897	Tubb5	Tubulin β-5 chain	2	2.1	3.01*
P45592	Cfl1	Cofilin-1	0.55	0.16	2.65*
P20761	Igh-1a	Ig γ-2B chain C region	1.82	1.01	2.63*
Q6P9V9	Tuba1b	Tubulin α-1B chain	1.75	1.73	2.49*
P10111	Ppia	Peptidyl-prolyl cis-trans isomerase A	0.68	0.3	2.34*
P63018	Hspa8	Heat shock cognate 71 kDa protein	0.94	1.13	2.16*
Q07936	Anxa2	Annexin A2	1.49	0.79	2.16*
O88989	Mdh1	Malate Dehydrogenase, Cytoplasmic	1.04	0.61	2.14*
P09006	Serpina3n	Serine protease inhibitor A3N	4.93*	1.22	2.02
Q5XI73	Arhgdia	Rho GDP-dissociation inhibitor 1	0.87	0.88	1.95*
P61589	Rhoa	RhoA transforming protein	0.8	1.3	1.9*
P60711	Actb	Actin, cytoplasmic 1	0.94	0.41	1.7*
Q62930	C9	Complement component C9	2.17*	0.98	0.91
Q6P734	Serping1	Plasma protease C1 inhibitor	2.74*	2.18*	0.89
Down-regulated					
P47875	Csrp1	Cysteine and glycine-rich protein 1	0.34	0.21*	1.09
P31232	Tagln	Transgelin	0.50	0.16*	1.06
Q01129	Dcn	Decorin	0.59	0.59	0.35*
P51886	Lum	Lumican	0.75	0.58	0.34*

[1] **Represent ratio of obstructed to normal unobstructed groups**

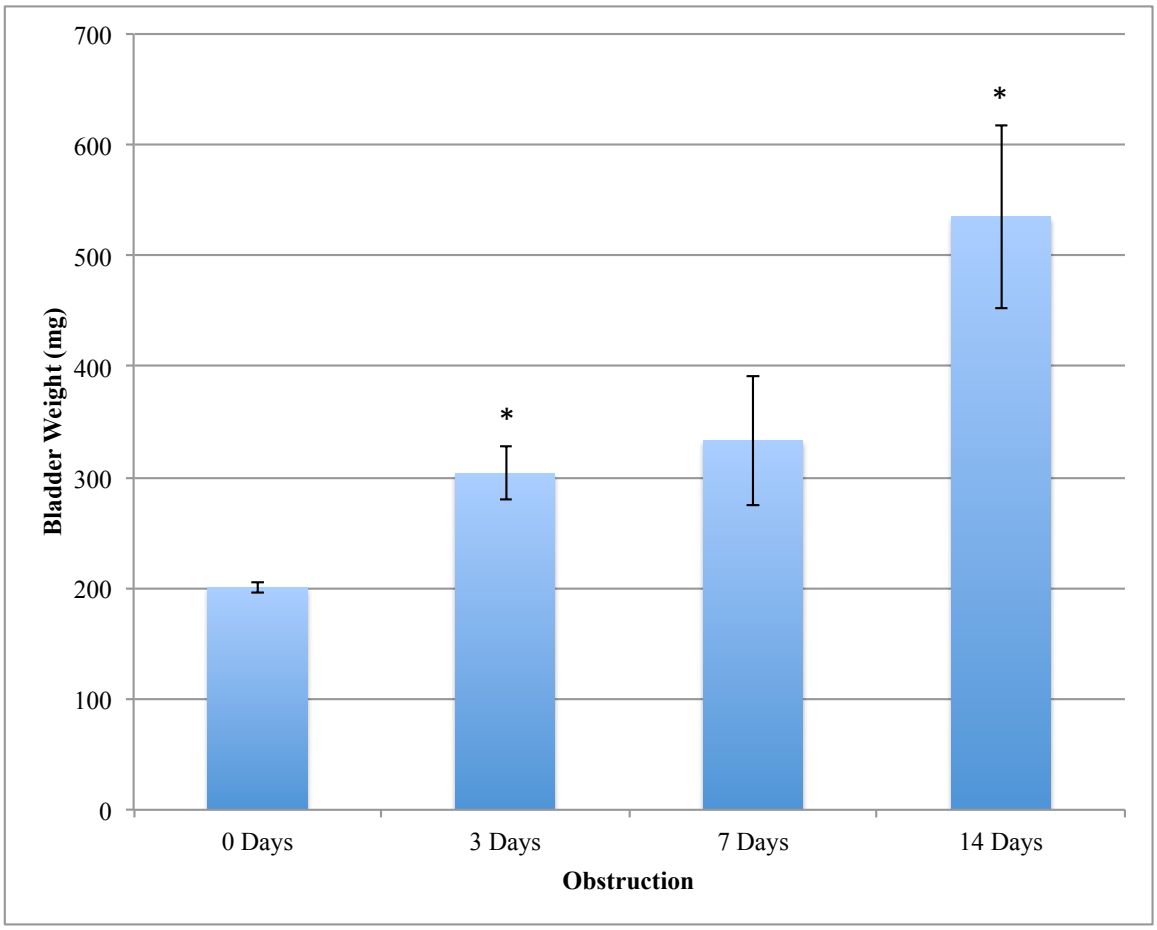
*** *p-value* < 0.05 vs. normal unobstructed group**

2.7 Figures

Figure 2.1 Surgical induction of pBOO.

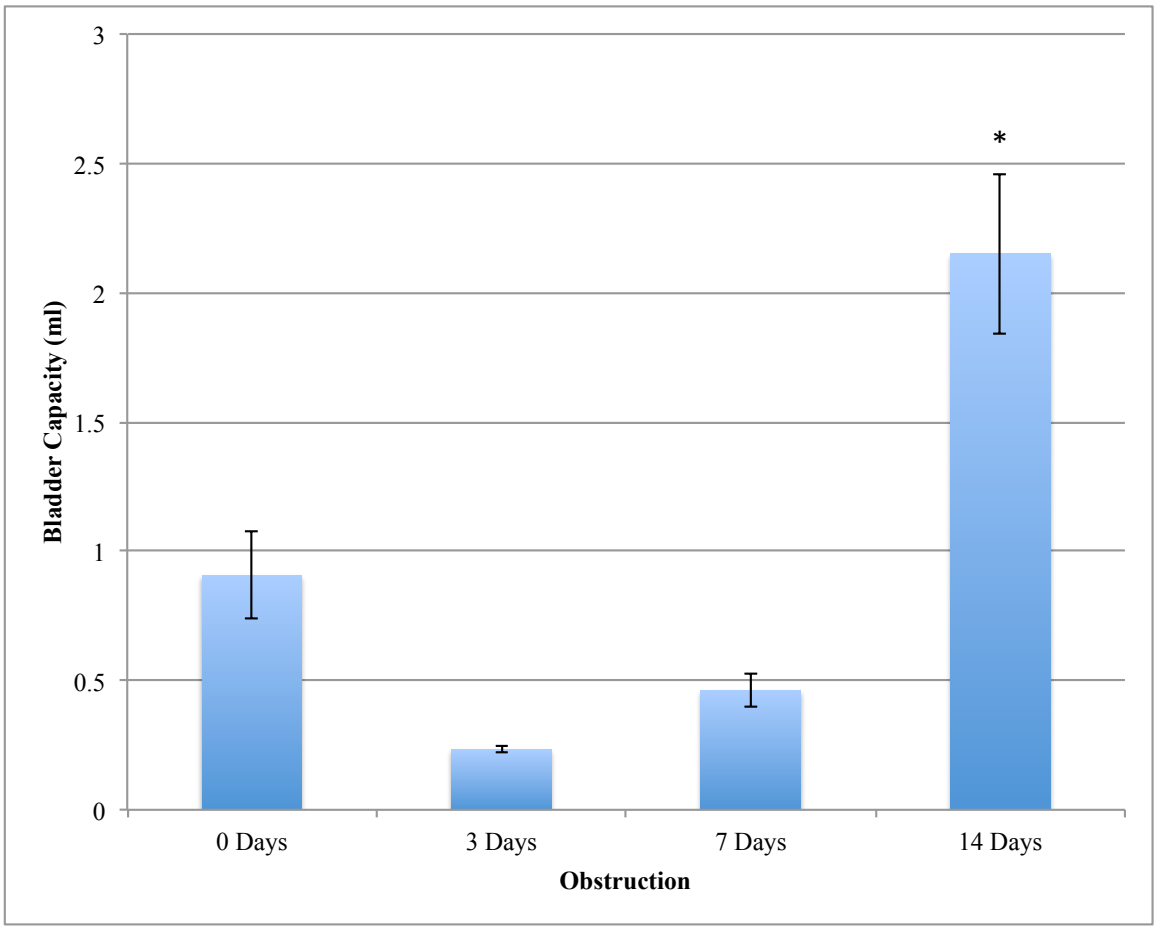


Figures 2.2 Bladder weight changes after pBOO.



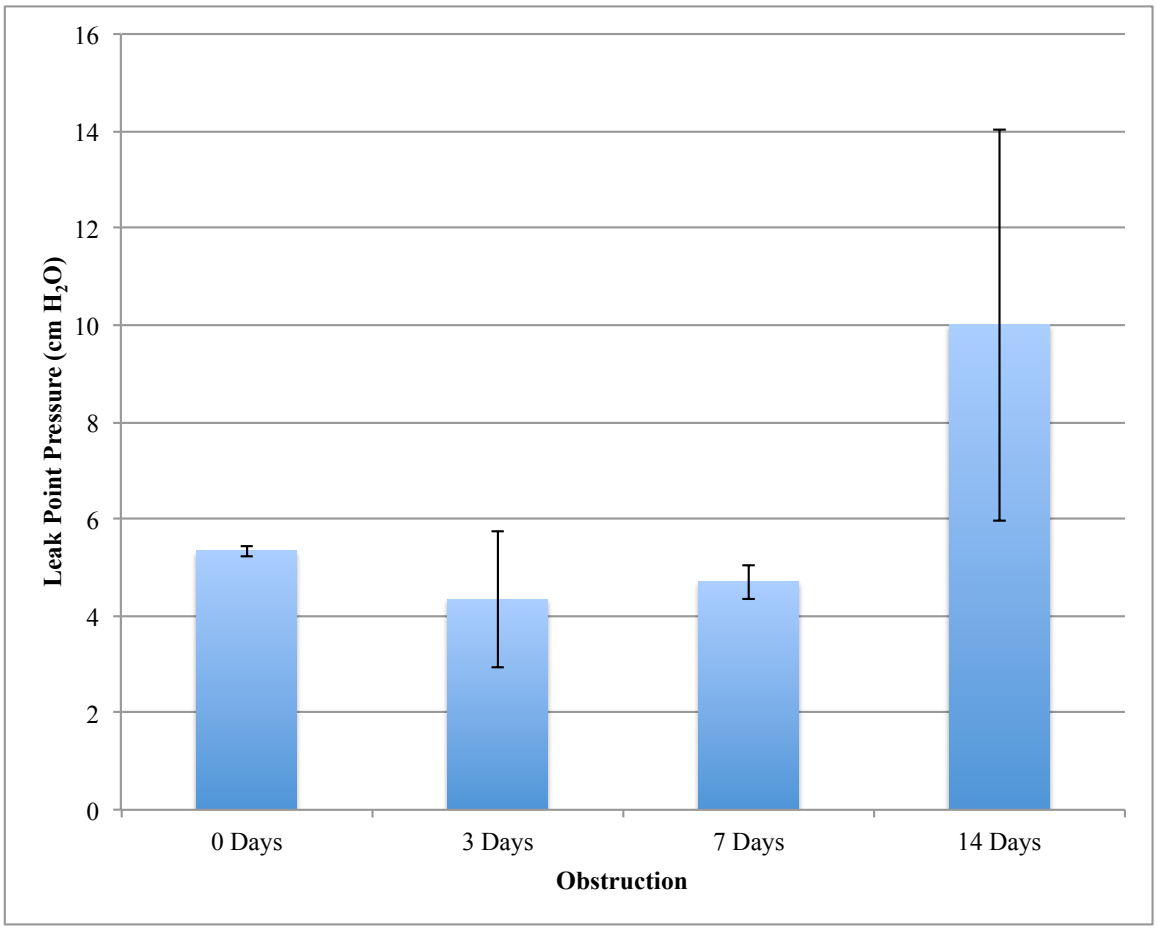
** p-value < 0.05 vs. unobstructed.*
Error bars represent standard error around the mean.

Figure 2.3 Bladder capacity changes after pBOO.



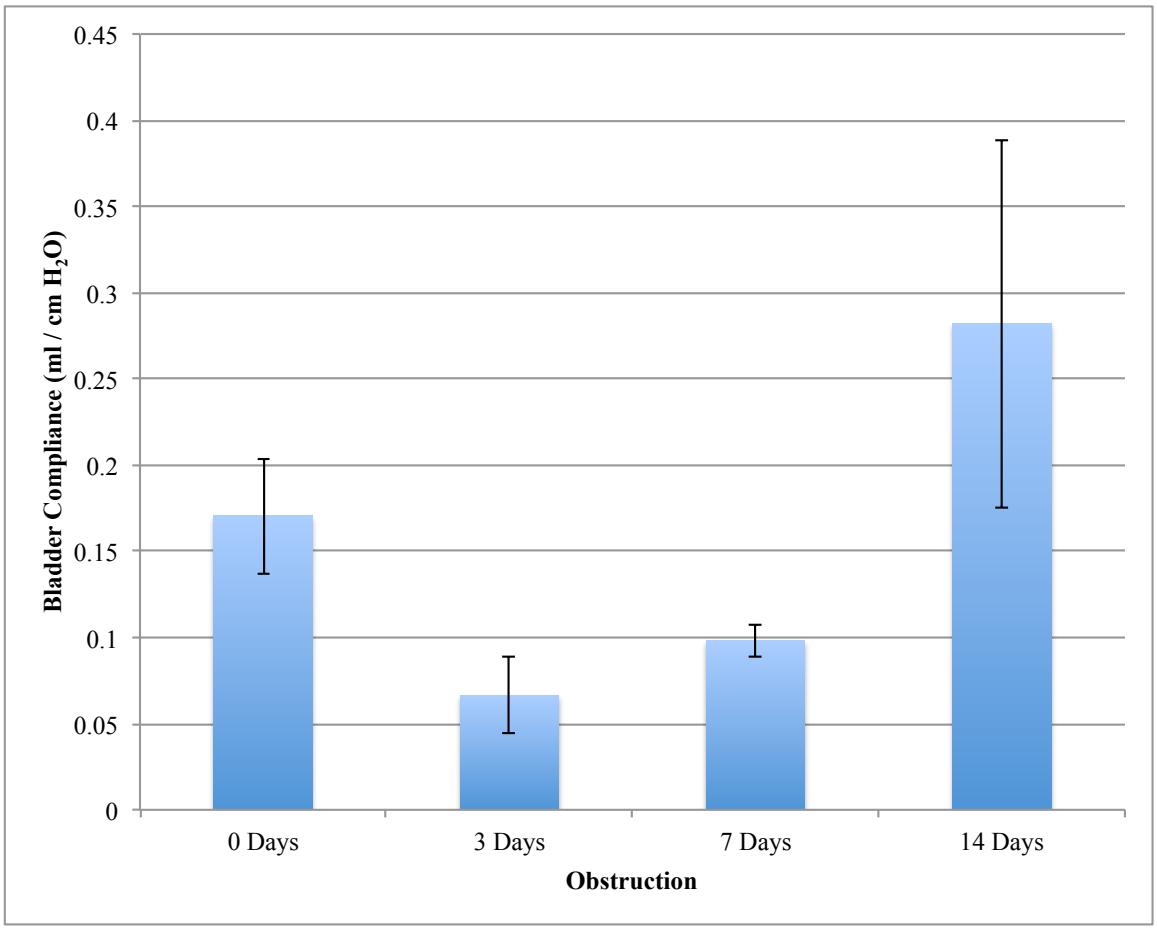
** p-value < 0.05 vs. unobstructed.*
Error bars represent standard error around the mean.

Figure 2.4 Leak point pressure measurements after pBOO.



Error bars represent standard error around the mean.

Figure 2.5 Calculated bladder wall compliance changes after pBOO.



Error bars represent standard error around the mean.

Figure 2.6 Image of Coomassie Blue Stained Gel.

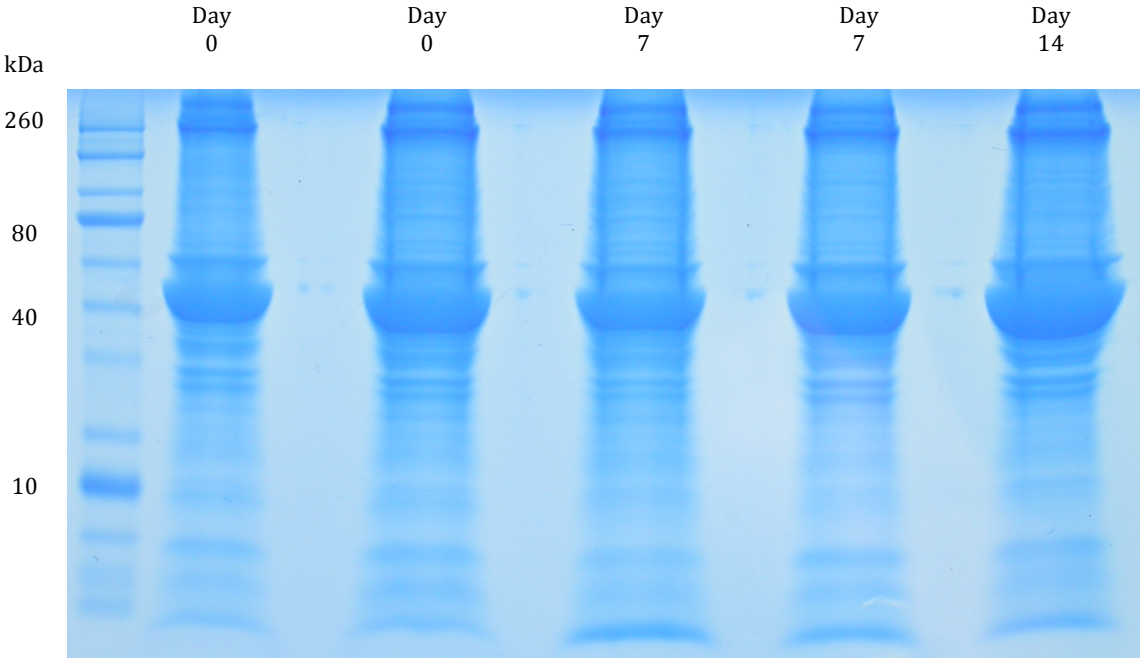
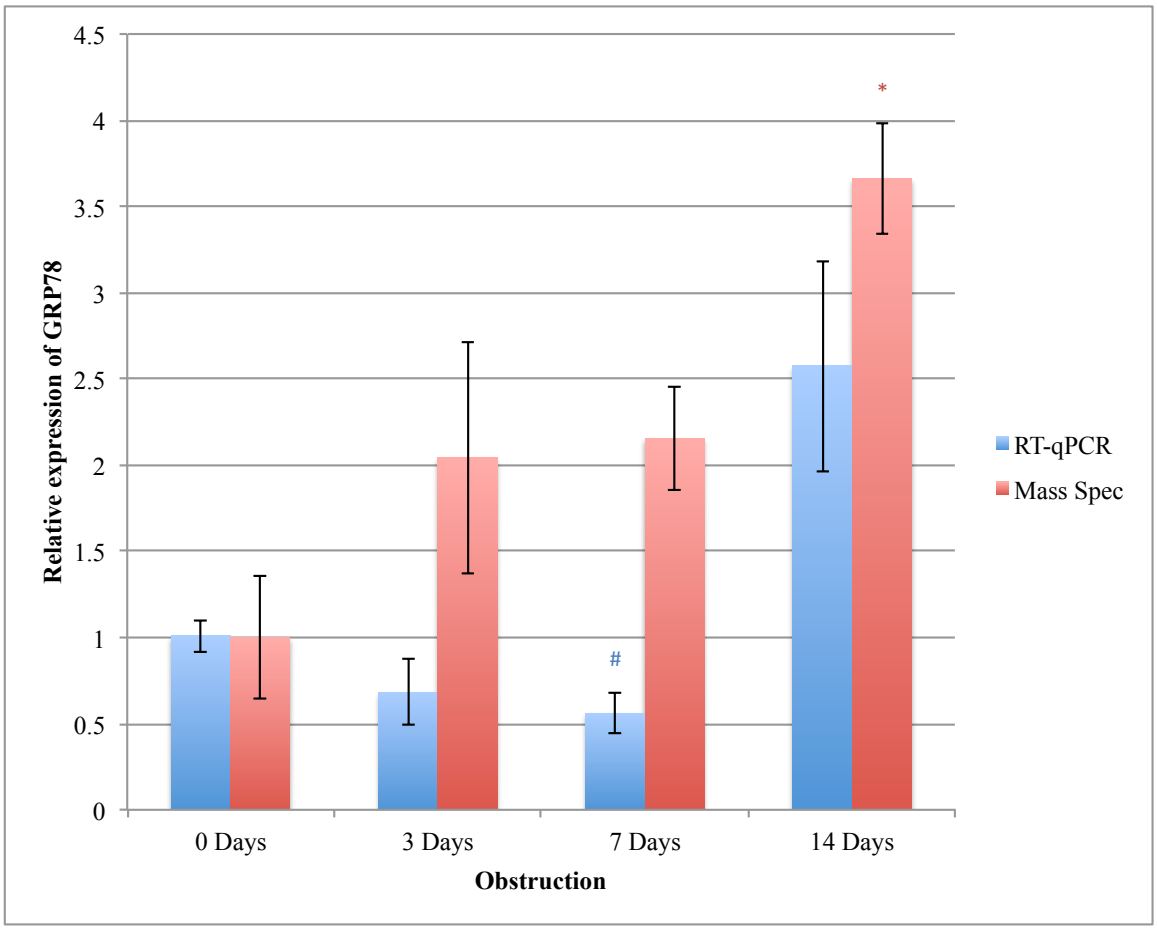
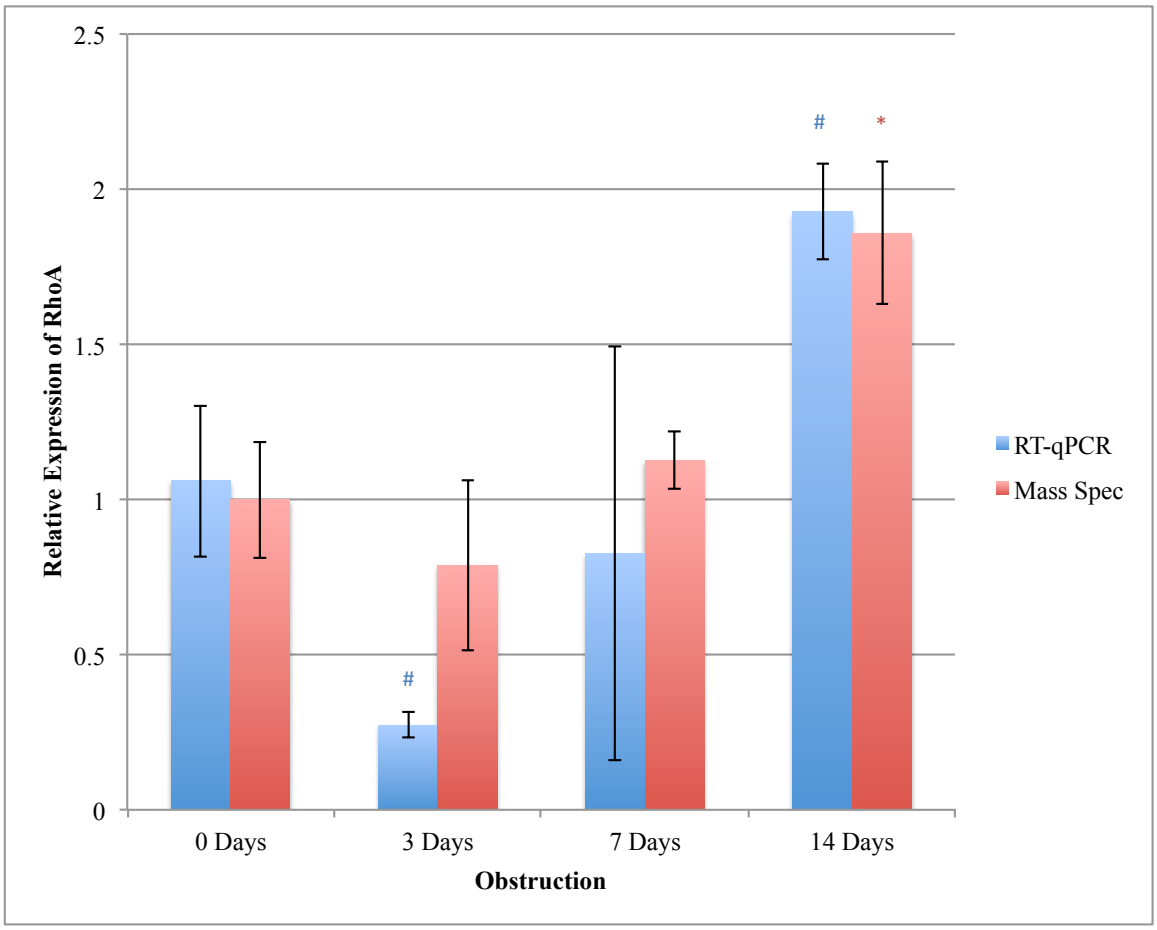


Figure 2.7 Protein and mRNA expression of ER stress marker GRP78.



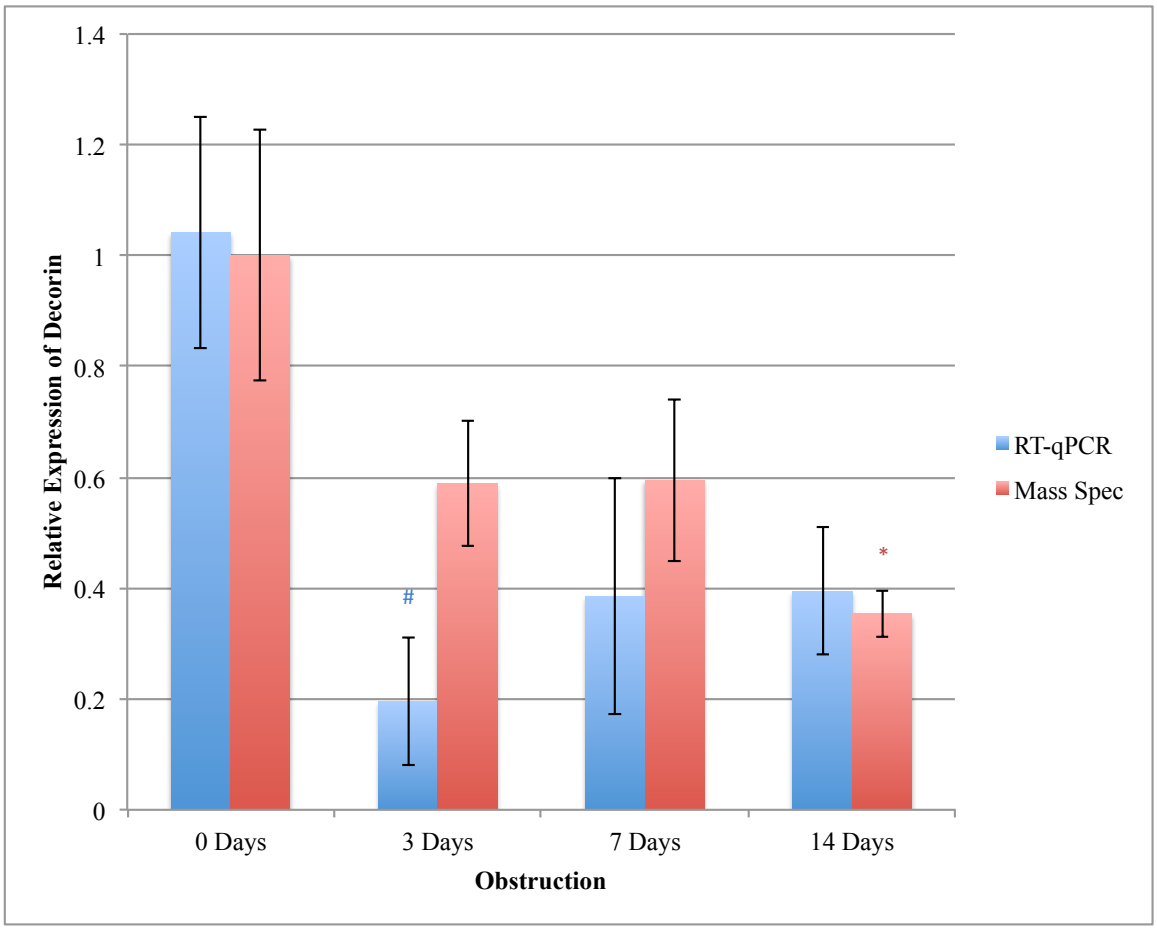
** p-value < 0.05 vs. unobstructed for Mass Spectrometry data.*
 # p-value < 0.05 vs. unobstructed for RT-qPCR data.
Error bars represent standard error around the mean.

Figure 2.8 Protein and mRNA expression of RhoA.



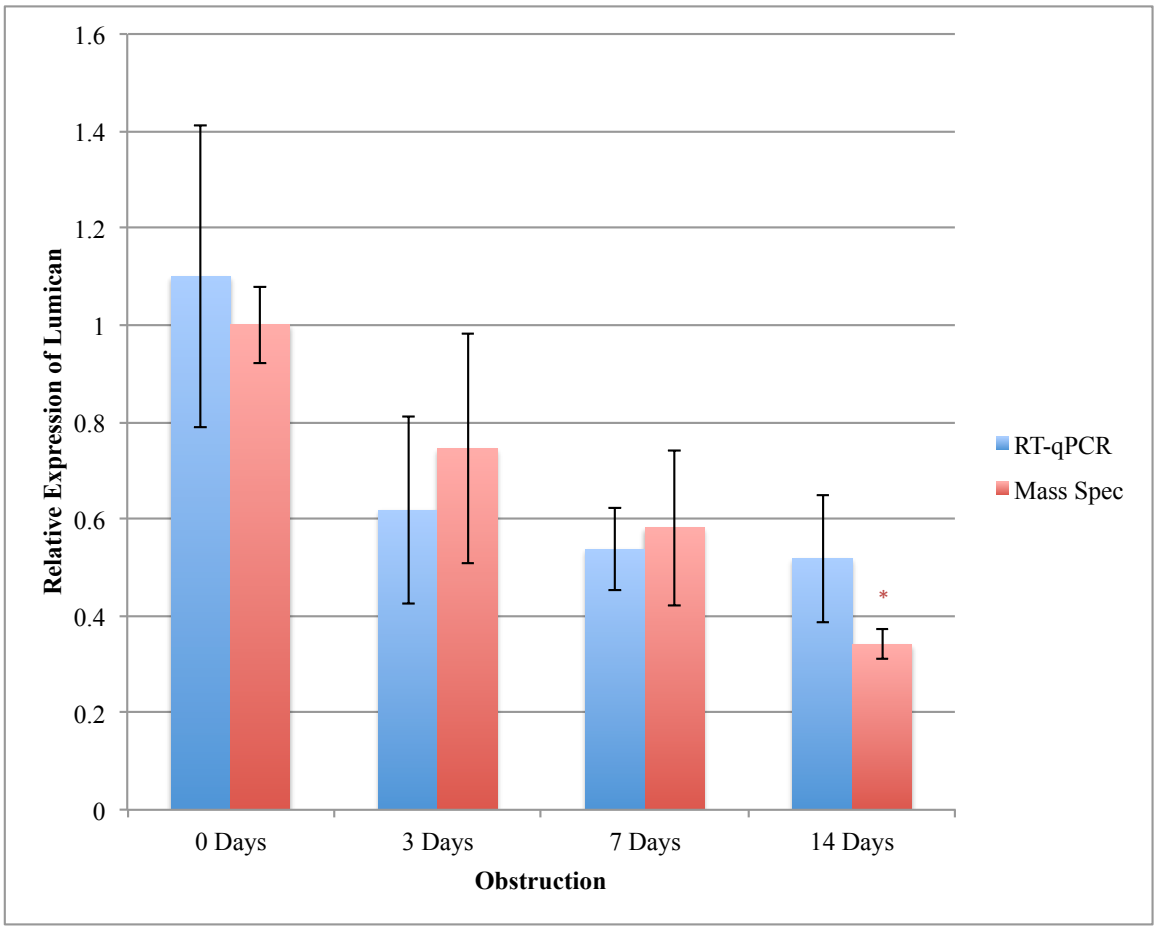
** p-value < 0.05 vs. unobstructed for Mass Spectrometry data.*
 # p-value < 0.05 vs. unobstructed for RT-qPCR data.
Error bars represent standard error around the mean.

Figure 2.9 Protein and mRNA expression of decorin.



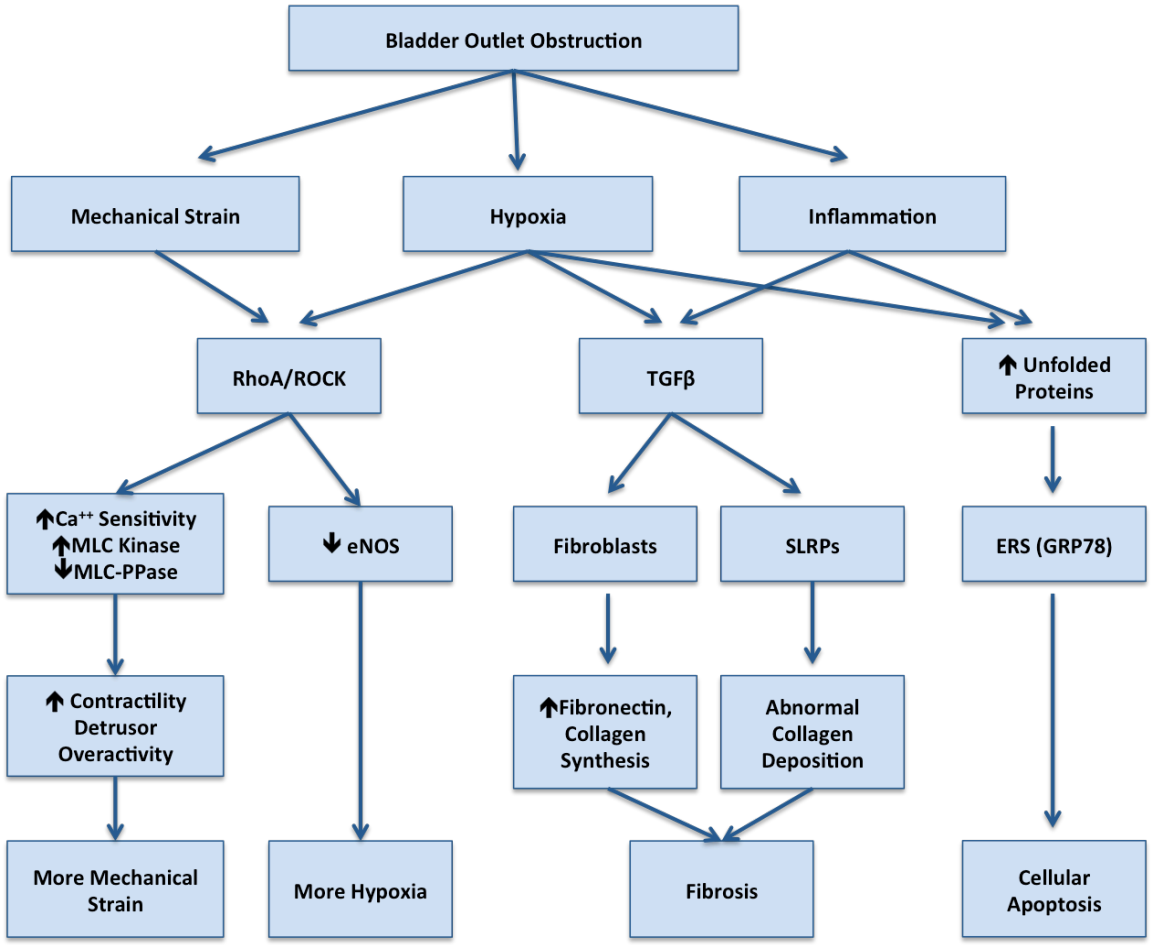
** p-value < 0.05 vs. unobstructed for Mass Spectrometry data.*
 # p-value < 0.05 vs. unobstructed for RT-qPCR data.
Error bars represent standard error around the mean.

Figure 2.10 Protein and mRNA expression of lumican.



** p-value < 0.05 vs. unobstructed for Mass Spectrometry data.*
Error bars represent standard error around the mean.

Figure 2.11 Summery of detected proteins and their possible effects & interactions reported in the literature.



2.8 Endnotes

A version of this chapter has been submitted for publication.

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Chapter 3
The Role of Mesenchymal Stem Cells in Partial Bladder Outlet Obstruction

3.1 Introduction

pBOO, defined as an increase in pressure required for voiding, is a ubiquitous problem in urology. The etiologies of pBOO affect a wide range of age groups, from BPH in adult men to posterior urethral valves in the pediatric population. High intravesical pressure induces a sequence of inflammatory changes initially, which if left untreated, is followed by SMC hypertrophy, fibrosis and end-stage bladder development[1].

Mechanical strain, bladder wall hypoxia, oxidative stress, and inflammation have been all implemented in the pathogenesis of pBOO[1,2]. However, targeting bladder SMCs contractility with the anti-cholinergic oxybutynin remains the only proven therapy of pBOO[3]. Several mediators and pathways are induced in response to pBOO, and thought to modulate its pathological outcomes, including TGFβ, RhoA, HIF1α, and ERS[1,4,5].

MSCs are currently being investigated for their therapeutic and reparative potentials in animal models and clinical trials[6-8]. They are seen to be recruited to injured and inflamed tissues and exert therapeutic immunomodulatory actions[9]. These cells were demonstrated to exert an inhibitory effect on many inflammatory markers, including TGFβ, IFN α/γ, and TNFα[9,10]. Furthermore, they were observed to secrete several growth factors that are involved in tissue regeneration and angiogenesis[11,12]. In this current study, we hypothesized that the administration of MSCs will result in decreased inflammatory response and

hypoxic state in the early phase after pBOO. We induced pBOO surgically and MSCs were administered intravenously. Outcomes were measured in terms of bladder weight, urodynamics, and RT-PCR studies of inflammatory, hypoxic, and ERS markers, in addition to ECM proteoglycans.

3.2 Methods

Full approval from the University of Alberta Animal Care and Use Committee was obtained prior to conducting this study. A total of 15 female Sprague-Dawley rats (weight > 200 gm) were used divided into 5 groups. Three (n=3) animals were used as normal unobstructed group, and pBOO was induced surgically for 7 and 14 days, with (treatment group) and without (control group) intravenous administration of green fluorescent protein (GFP) producing-MSCs. Immunofluorescent microscopy studies of bladder tissues were performed to detect the presence of MSCs. Functional and molecular outcomes were measured in terms of changes in bladder weight, urodynamics, and RT-PCR for inflammatory markers TGFβ and RhoA, hypoxic marker HIF1α, ERS marker GRP78, and ECM proteoglycans decorin and lumican.

3.2.1 Surgical Induction of pBOO:

Under isoflourane anesthesia, animals were subjected to surgical induction of pBOO as previously described[13]. Lower midline abdominal incision was made, accessing the peritoneal cavity and exposing urinary bladder and surrounding tissue. An 18-guage angiocatheter was introduced through a bladder dome cystostomy and advanced into the urethra for calibration and avoidance of complete urethral ligation. A 2-0 silk suture was then passed inferior to vesicoureteric junction, and ligated gently around the urethra to produce partial obstruction. Closure of bladder dome was then performed using a 5-0 vicryl suture after removal of the angiocatheter. Abdominal wall and skin were closed thereafter and animal was allowed to recover.

3.2.2 Measurements of Bladder Weight & Urodynamics:

At time of sacrifice, bladders were excised and wet weight recorded. For urodynamic studies, 18-guage angiocatheter was introduced into the bladder, and was then connected to water infusion pump (0.1 ml/minute), and a low-pressure transducer (cm/H_2O). Pressure readings were recorded during filling and at time of urinary leakage per urethra (leak point pressure). Bladder capacity was then recorded. Bladder wall compliance was calculated to represent intravesical pressure changes with volume infused (ml/cm H_2O).

3.2.3 Mesenchymal Stem Cells:

Sprague-Dawley rats mesenchymal stem cells, which have been transfected with a lentiviral construct encoding a GFP expression motif, were obtained commercially (Cyagen Biosciences Inc, Sunnyvale, CA, product RASMX-01101). They were acquired in passage 3, and passaged up to passage 8 using company's recommended growth medium and protocol (GUXMX-90011). A dose of $5x10^6$ cells suspended in 0.5 ml saline was administered intravenously (tail vein) at time of surgical induction of pBOO to treatment group animals.

3.2.4 Detection of MSCs in Bladder Tissue:

A section of bladder tissue was obtained from all animals and fixed in 4% paraformaldehyde solution. Unstained paraffin-block slides were obtained, deparaffinized and prepared with xylene and alcohol, washed with PBS, and then mounted using 4',6-diamidino-2-phenylindole (DAPI) containing mounting-solution (ProLong Gold Antifade mounting medium, Invitrogen, ON, Canada). Images were captured using Carl Ziess fluorescence microscope using DAPI and

FITC fluorescence filters (Carl Ziess Microimaging Inc., Thornwood, NY). Control group bladder tissues were used as a negative control.

3.2.5 Expression of Target Genes:

Snap frozen bladder tissues were used after homogenization with a Micro-Dismembrator (B.Braun Biotech Inc., Allentown, PA). Tissue powder was then suspended in Trizole solution, and total RNA was extracted using RNeasy spin columns (Qiagen, Mississauga, ON, Canada). DNAse digestion for 60 minutes was performed, and then random primers were used to synthesize first cDNA strand (Sigma, Oakville, ON, Canada). Real-time RT-PCR was then conducted (Power SYBR Green PCR Master Mix, ABI, Foster City, CA). 1 μL of first strand product was used, along with 25 μL containing 0.2 μM of target genes with upstream and downstream primers. For control, HPRT gene was used. An ABI 7300 real-time system (Applied Biosystems, Foster City, CA) was used for amplifications. HPRT amplification was used for standardization of amplification curves. *Table2.1* outlines forward and reverse primers sequences of genes used in this study.

3.2.6 Statistical Analysis:

Data are presented as mean ± standard error around the mean. ANOVA was performed for all presented data (STATA software Version 12.1). A value of $p \leq 0.05$ was used to determine statistical significance.

3.3 Results

3.3.1 Fluorescence Microscopy Imaging:

GFP-positive cells were detected in all treatment groups after 7 and 14 days of pBOO. Figure 3.1 represents two panels taken from two different animals from the treatment group after 14 days of obstruction.

3.3.2 Bladder Weight and Urodynamics:

pBOO induced an increase in mean bladder weight in all groups, being statistically significant from unobstructed animals after 14 days (535.0±82.5 vs. 200.7±4.6 mg, p-value 0.016) (figure 3.2). No statistically significant difference observed between control and treatment group in all time points.

Bladder capacity increased significantly in the control group after 14 days compared to unobstructed group (2.15±0.30 vs. 0.90±0.17 ml, p-value 0.02). Treatment with MSCs resulted in significantly lower bladder capacity than controls after 14 days, resembling normal unobstructed bladder capacity (0.91±0.29 vs. 2.15±0.3 ml, p-value 0.04) (figure 3.3). No statistically significant changes observed in leak point pressure and bladder wall compliance between control and treatment group, and compared to unobstructed bladders at all time points (figures 3.4 & 3.5).

3.3.3 RT-PCR:

Obstruction for 14 days induced a statistically significant increase in TGFβ in group compared to unobstructed group (mean fold change 3.40±0.30, p-value <0.01). Treatment with MSCs resulted in lower TGFβ level after 14 days

compared to control, with the difference being marginally significant (2.10±0.38 vs. 3.40±0.30, p-value 0.059) (figure 3.6).

HIF1α mRNA expression was found to be significantly lower in the 7 days control group compared to unobstructed (0.15±0.03 fold, p-value 0.02), and significantly higher in the 14 days control compared to unobstructed (4.39±0.98 fold, p-value 0.03). Treatment group had significantly lower HIF1α mRNA expression compared to untreated group (0.37±0.18 vs. 4.39±0.98 fold, p-value 0.02) (figure 3.7).

RhoA transforming protein mRNA was found to increase in the 14 days control group (1.92±0.16 fold, p-value 0.04). Treatment group had lower mRNA expression of RhoA compared to control (0.45±0.23 vs. 1.92±0.16 fold, p-value 0.01) (figure 3.8). Endoplasmic reticulum chaperon, GRP78, was significantly lower in the treatment group compared to control after 14 days (0.83±0.12 vs. 2.57±0.61, p-value 0.049) (figure 3.9).

Mean mRNA expression of lumican was generally lower in obstructed groups compared to unobstructed; however, not statistically significant (figure 3.10). pBOO induced a reduction in decorin mRNA levels from unobstructed, being marginally significant in the 14 days control group (0.40±0.10 fold, p-value 0.053) (figure 3.11). Treatment with MSCs did not result in a significant difference between treatment and control groups for decorin and lumican mRNA expression in all time points (figure 3.10 & 3.11).

3.4 Discussion

Treatment with mesenchymal stem cells has demonstrated promising results in many organs and pathological conditions, and much enthusiasm is present in the literature regarding their beneficial roles. They are thought to possess a wide range of immunomodulatory effects, to promote tissue healing and regeneration, prevention of apoptosis, in addition to angiogenesis enhancement[14,15]. They have been also shown to exert an inhibitory effect on many inflammatory markers, including TNFα, IFNα/γ, TGFβ, in addition to secreting anti-inflammatory cytokines, including IL-10[9,10].

MSCs are recruited to injured and inflamed organs with positive outcomes in many studies[9,16,17]. In a model of lung injury, umbilical MSCs were found in lung tissue, and resulted in significant reduction in TGFβ, IFNγ and TNFα[9]. Furthermore, in a model of murine myocardial infarction, local injection of MSCs resulted in improved left ventricular function[17]. In the urinary bladder, MSCs administration resulted in better bladder wall compliance and hypoxic state after 4 weeks in a mouse model of pBOO[16]. These cells are known to exhibit several chemokine receptors (CCRs, CXCRs) which are involved in immune cells recruitment to sites of inflammation, including CCR6, CCR9, CXCR3, CXCR6[18]. Presence of such receptors on MSCs is probably what enhances their recruitment to inflamed areas. In this current experiment, injected MSCs were found in bladder tissue in all treatment group samples (figure 3.1).

TGFβ is a protein that is involved extensively in cellular growth and ECM proliferation, and has been previously documented to increase after pBOO[1,19]. It is

known to promote fibronectin and collagen fibrils production by fibroblasts, with a stimulatory effect over TIMPs, promoting collagen deposition and fibrosis[20]. In the urinary bladder, it was seen that treating human bladders with TGFβ in vitro resulted in smooth muscle hypertrophy and collagen deposition[21], while TGFβ type 2 receptor knockout mice had less SMC hypertrophy and fibrosis after pBOO[22]. Here in this study, TGFβ mRNA expression was observed to be significantly elevated in response to pBOO after 14 days. Treatment with MSCs resulted in a lower magnitude of increase, which despite not reaching statistical significance (p-value 0.059) suggests a potential beneficial role on TGFβ levels after pBOO that could be detected with increased sample size.

Bladder wall hypoxia is a documented problem after pBOO, which is thought to be responsible for many of its pathological sequelae[1,16]. It is evident in this current study with the increase in HIF1α level after 14 days of pBOO. Treatment with MSCs was successful in reducing HIF1α level, indicating less bladder wall hypoxia (0.37±0.18 vs. 4.4±0.98 fold, p-value 0.015). This observation could be explained by that MSCs are known to secrete VEGF and BFGF, which are involved in inducing angiogenesis, and hence, providing better perfusion to injured organs[11,12].

Mechanical strain, in addition to hypoxia, are known to activate RhoA/ROCK pathway in many studies[23,24]. RhoA level was observed to be significantly increased after 2 weeks of pBOO in control animals in this current work. RhoA activation is known to increase bladder contractility through Ca^{++} sensitization adding further mechanical strain and stretch to bladder wall after

pBOO[25-27]. Furthermore, it has been observed to interfere with eNOS in vascular endothelium, adding even more hypoxia[28]. Our treated animals exhibited significantly lower RhoA mRNA expression compared to control group after 14 days of pBOO (0.45±0.2 vs. 1.9±0.15 fold, p-value <0.01), and hence, better control of bladder wall contractility. Interfering with RhoA pathway might result in more eNOS availability, reducing hypoxic strain on bladder wall, adding more beneficial outcomes in terms of bladder wall hypoxia.

Proteomic analysis of bladder tissue after pBOO in our previous experiment showed the presence of endoplasmic reticulum stress response after 2 weeks of pBOO, evident by the increase in GRP78 protein level. GRP78 is a molecular chaperon in the ER that is involved in proper protein folding[29]. Cellular stress is known to induce an accumulation of unfolded and misfolded proteins in the lumen of endoplasmic reticulum, and if repair is not feasible, cells were observed to proceed to apoptosis[29,30]. In this experiment, GRP78 mRNA was observed to increase after 14 days of pBOO, and treatment with MSCs resulted in a significantly lower GRP78 mRNA expression (0.83±0.12 vs. 2.57±0.6, p-value 0.049). The reduction in GRP78 seen after treatment confirms MSCs role in reducing cellular stress, and their inhibitory effect on apoptosis seen in other studies[14,15].

The negative outcomes in mRNA expressions of decorin and lumican seen in this study both in treatment and control group might be related to duration of obstruction. We have demonstrated previously that pBOO induced downregulation of decorin mRNA; however this was seen after 4 weeks of

pBOO[31]. ECM remodeling seems to be a later response to inflammation in the bladder wall, and further time points are needed to examine whether MSCs have a role or not in this aspect of the pathology.

3.5 Conclusion

pBOO resulted in a significant activation of many inflammatory and hypoxic pathways. TGFβ activation is seen which leads to ECM proliferation and collagen deposition. Stimulation of RhoA/ROCK pathway is evident, increasing bladder smooth muscles contractility and possibly interfering with nitric oxide metabolism adding more hypoxic injury. HIF1α is elevated after pBOO confirming hypoxia as a pathological factor. ERS and cellular apoptosis are induced by pBOO as well. Treatment with MSCs resulted in lower mRNA expression of hypoxic, inflammatory, and cellular stress markers after pBOO. Figure 3.12 summarizes changes in mRNA expression of major studied genes in unobstructed, 14 days control and 14 days treatment groups. These improvements are reflected on bladder function, where treated animals had normal bladder capacity up to 14 days. More time points are needed to examine the role of treatment with MSCs on remaining aspects of bladder function, mainly intravesical pressure and bladder wall compliance.

3.6 Figures

Figure 3.1 Fluorescence microscopy images showing GFP-expressing MSCs in bladder tissue of 14 days treatment group.

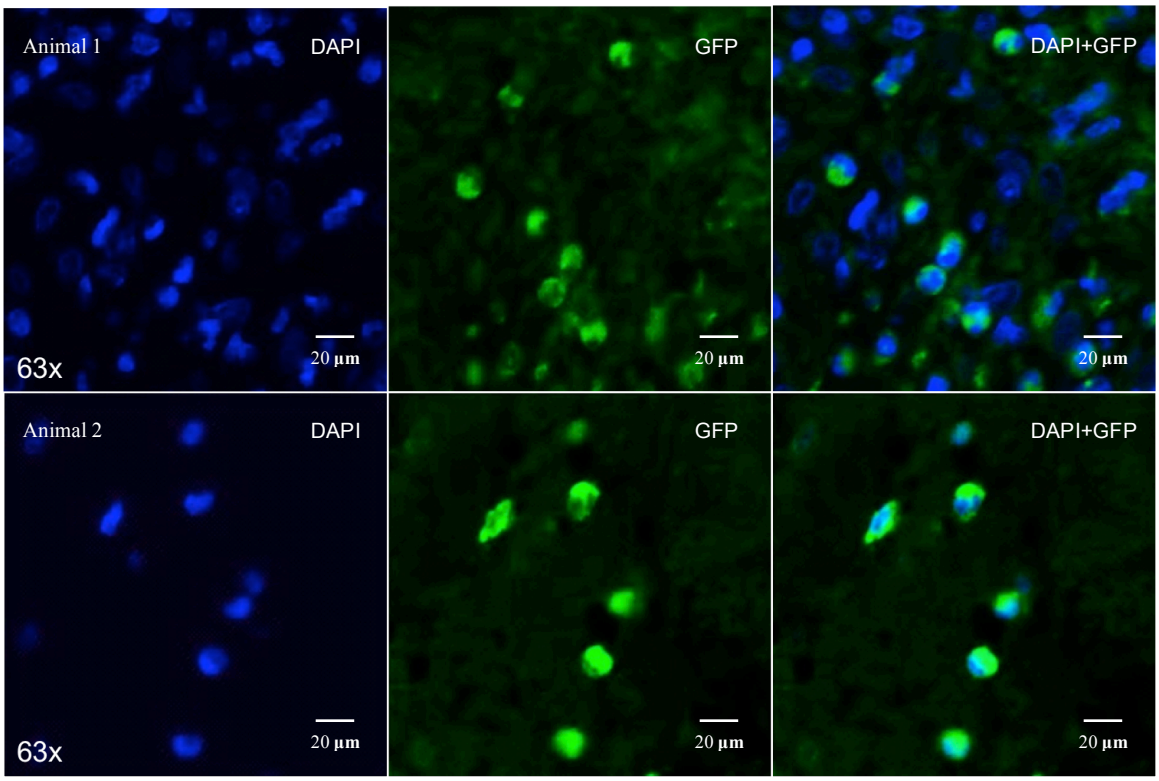
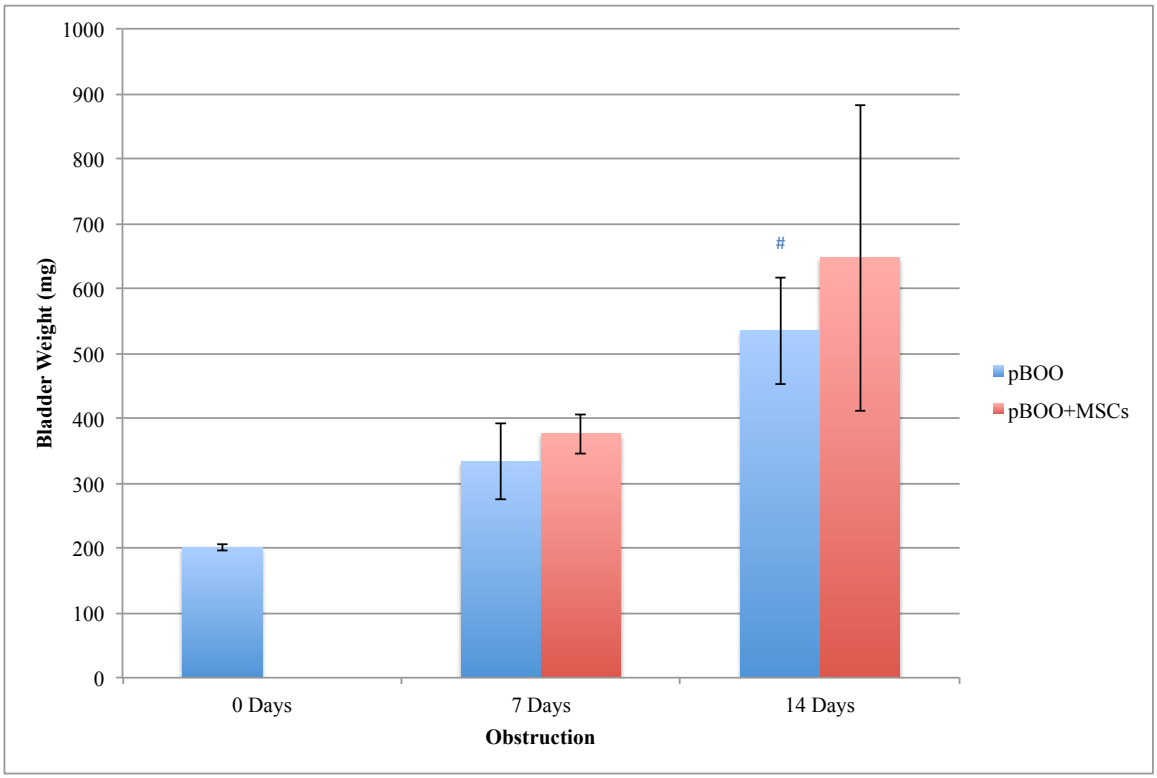
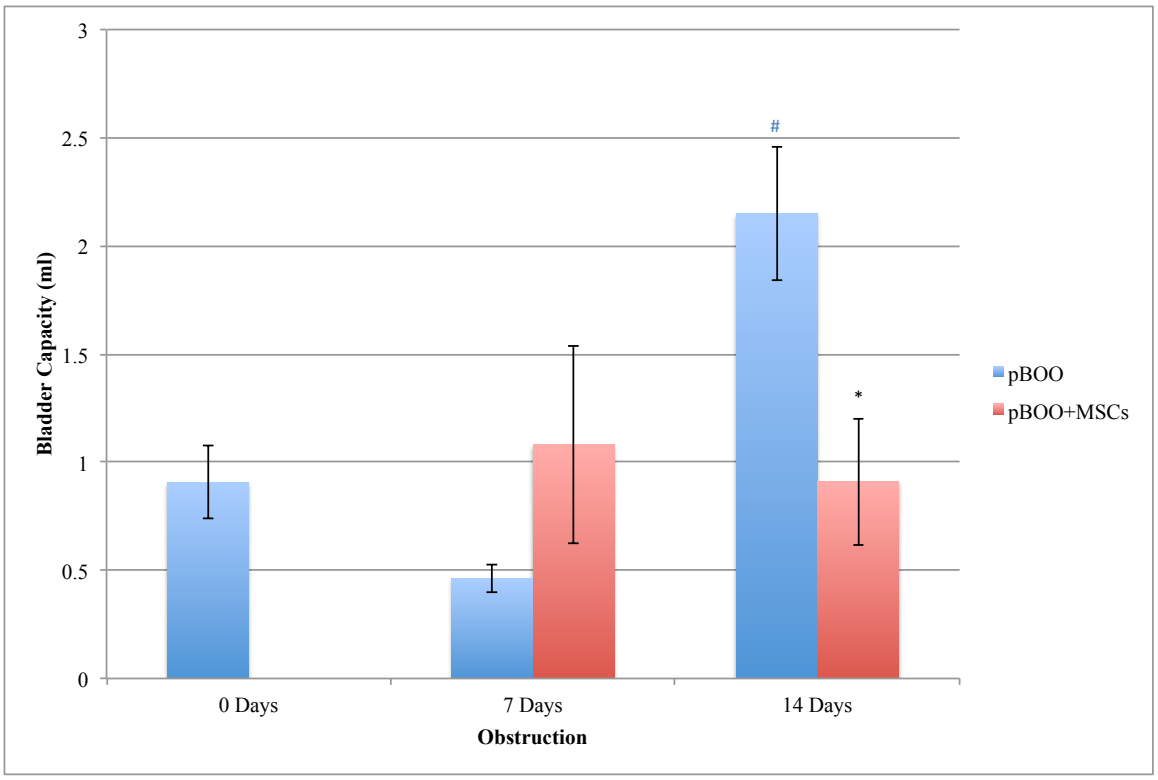


Figure 3.2 Bladder weight changes over the time course of the experiment in control and treatment groups.



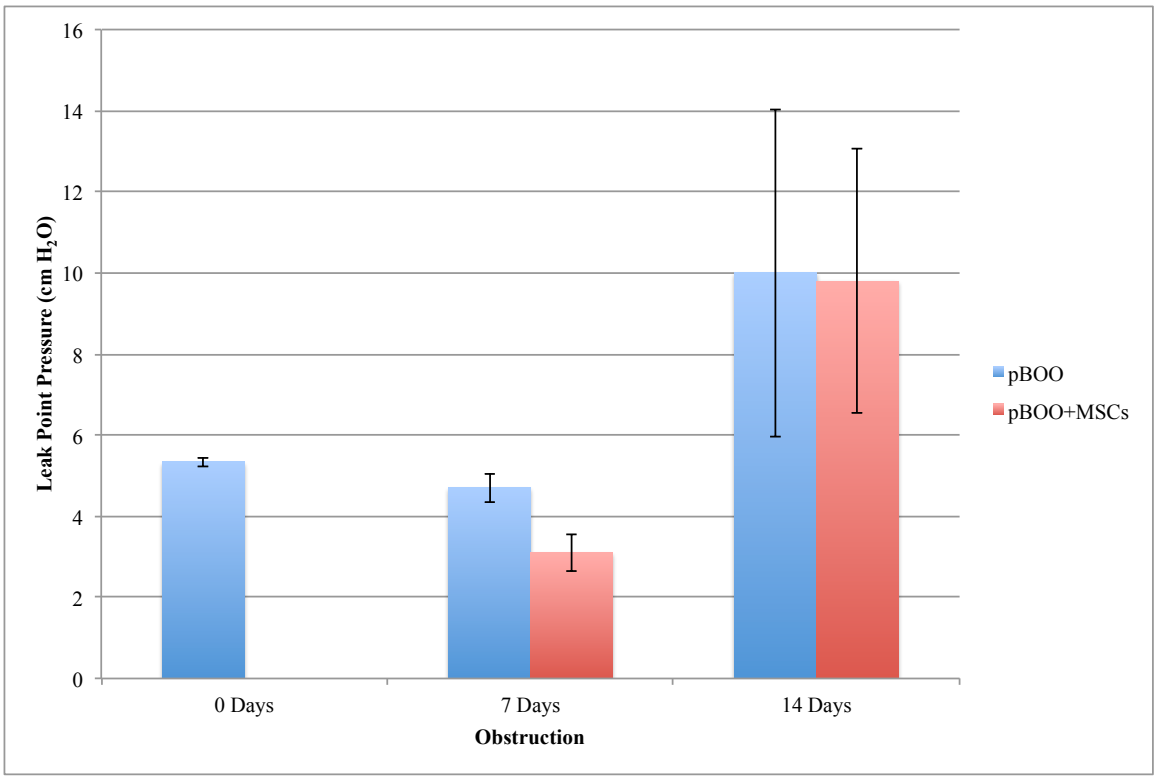
 # p-value < 0.05 vs. unobstructed.
Error bars represent standard error around the mean.

Figure 3.3 Bladder capacity changes over the time course of the experiment in control and treatment groups.



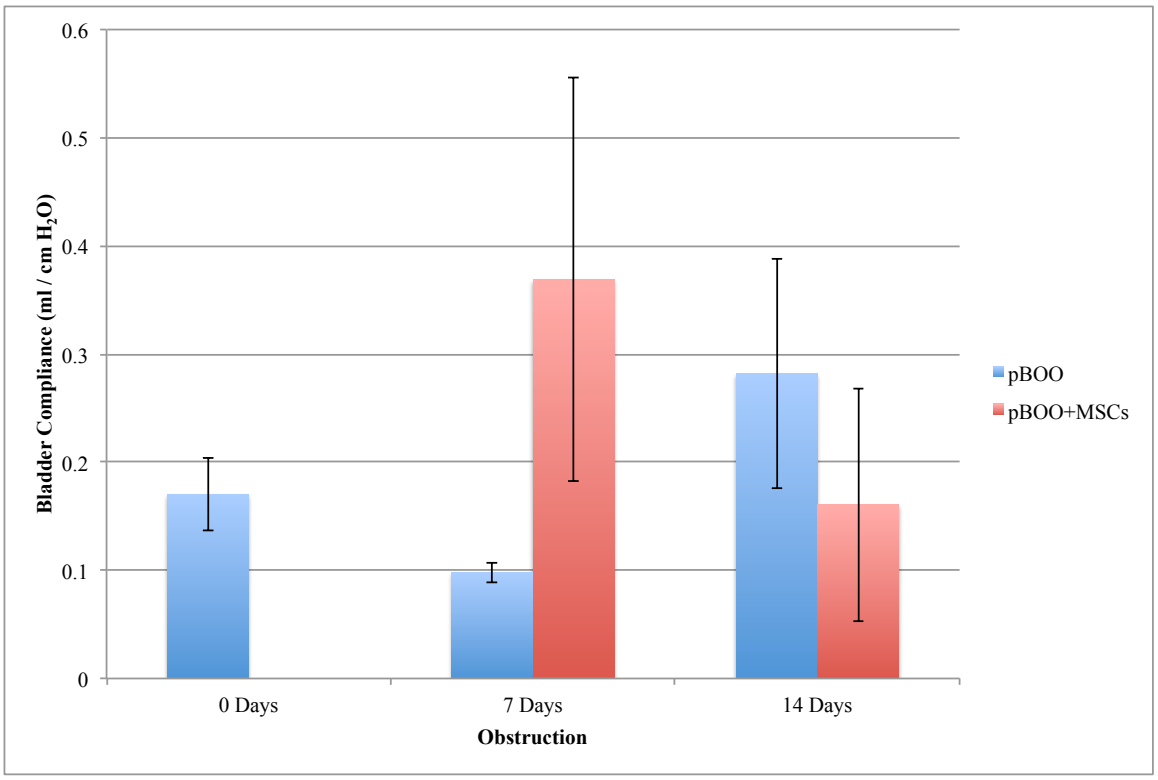
 # p-value < 0.05 vs. unobstructed.
** p-value < 0.05 in treatment vs. control groups.*
Error bars represent standard error around the mean

Figure 3.4 Leak point pressure changes over the time course of the experiment in control and treatment groups.



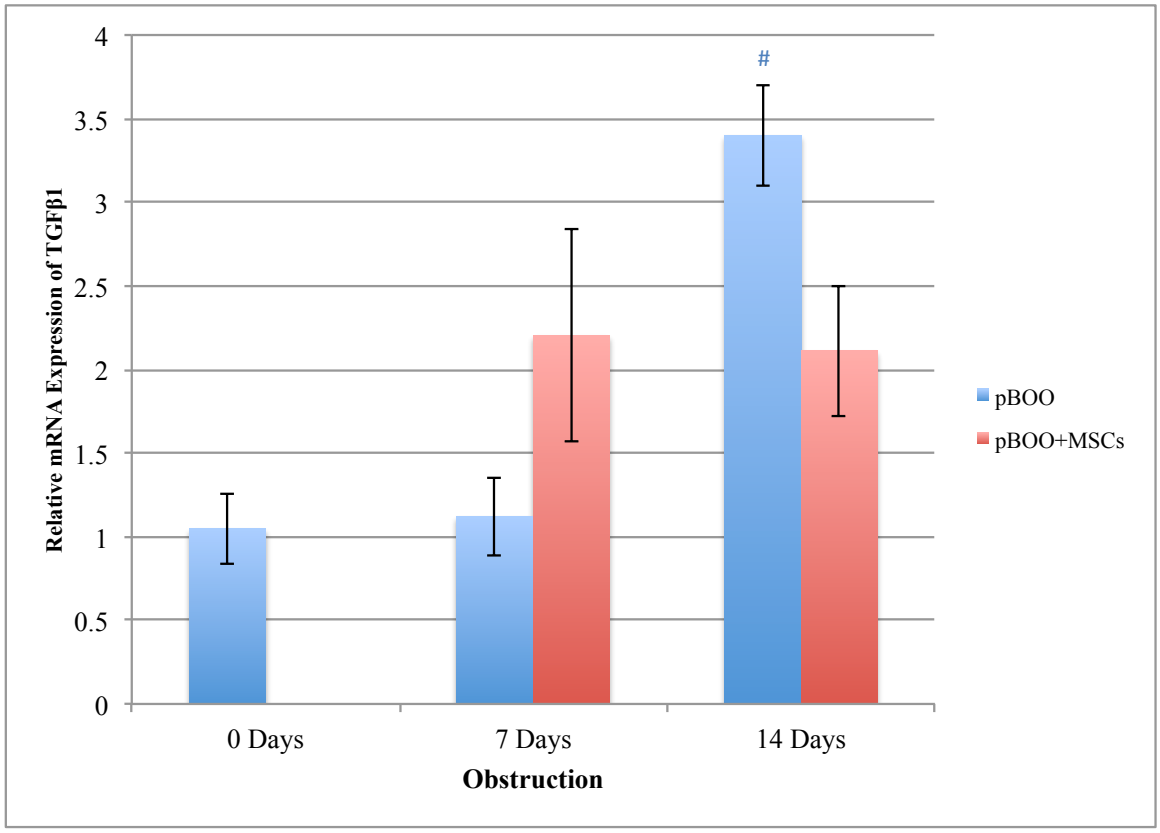
Error bars represent standard error around the mean

Figure 3.5 Bladder compliance changes over the time course of the experiment in control and treatment groups.



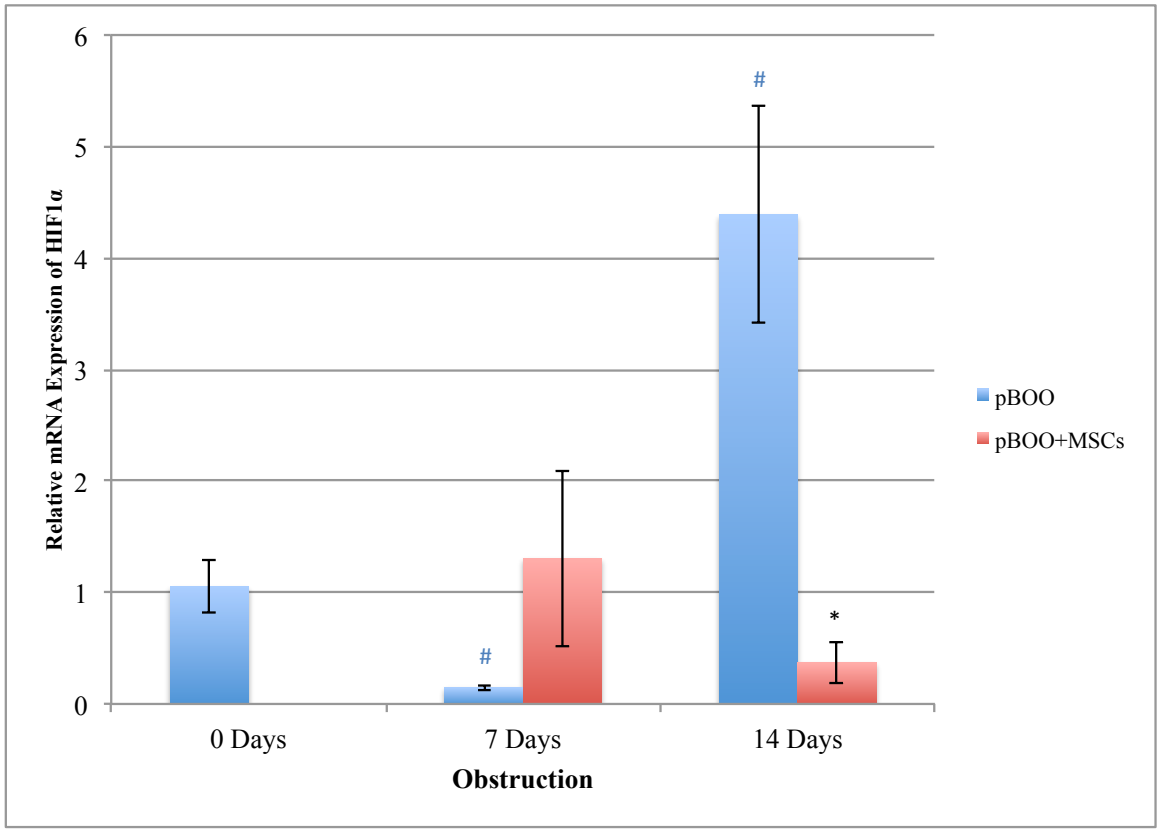
Error bars represent standard error around the mean

Figure 3.6 TGFβ1 mRNA expression in control and treatment groups.



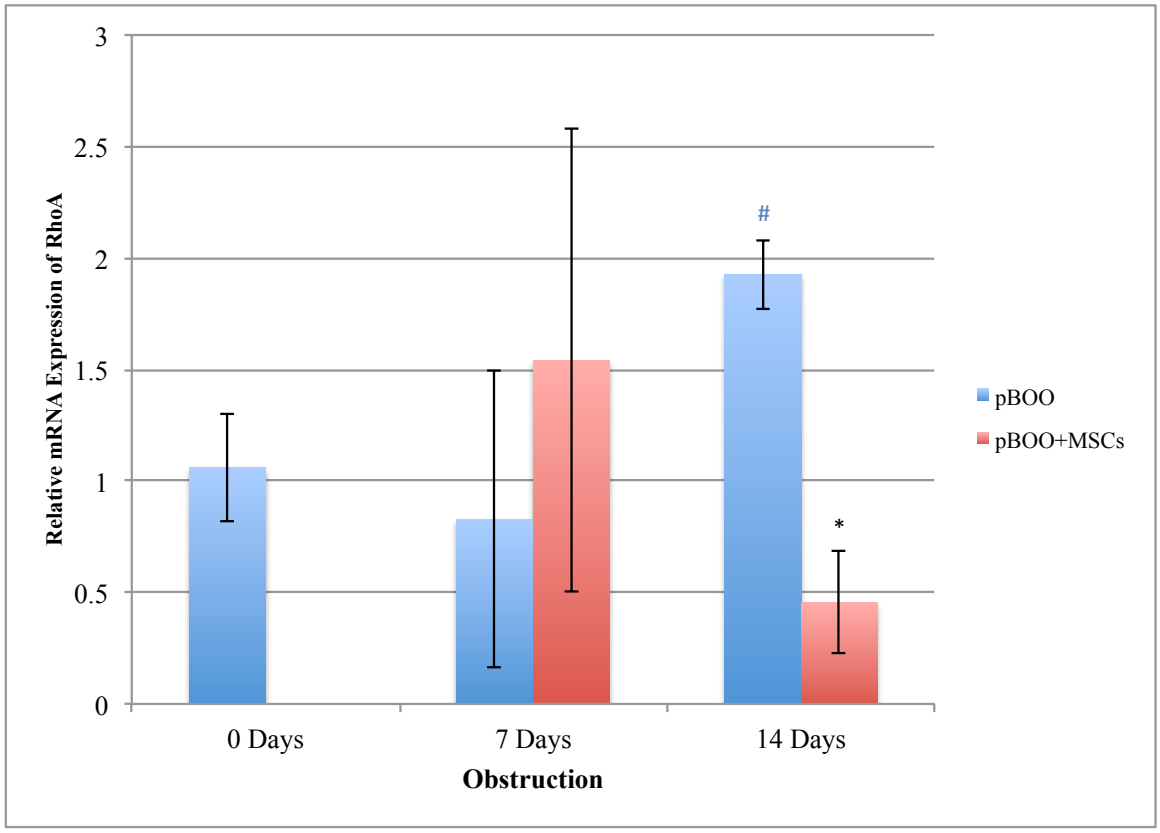
 # p-value < 0.05 vs. unobstructed.
Error bars represent standard error around the mean

Figure 3.7 HIF1α mRNA expression in control and treatment groups.



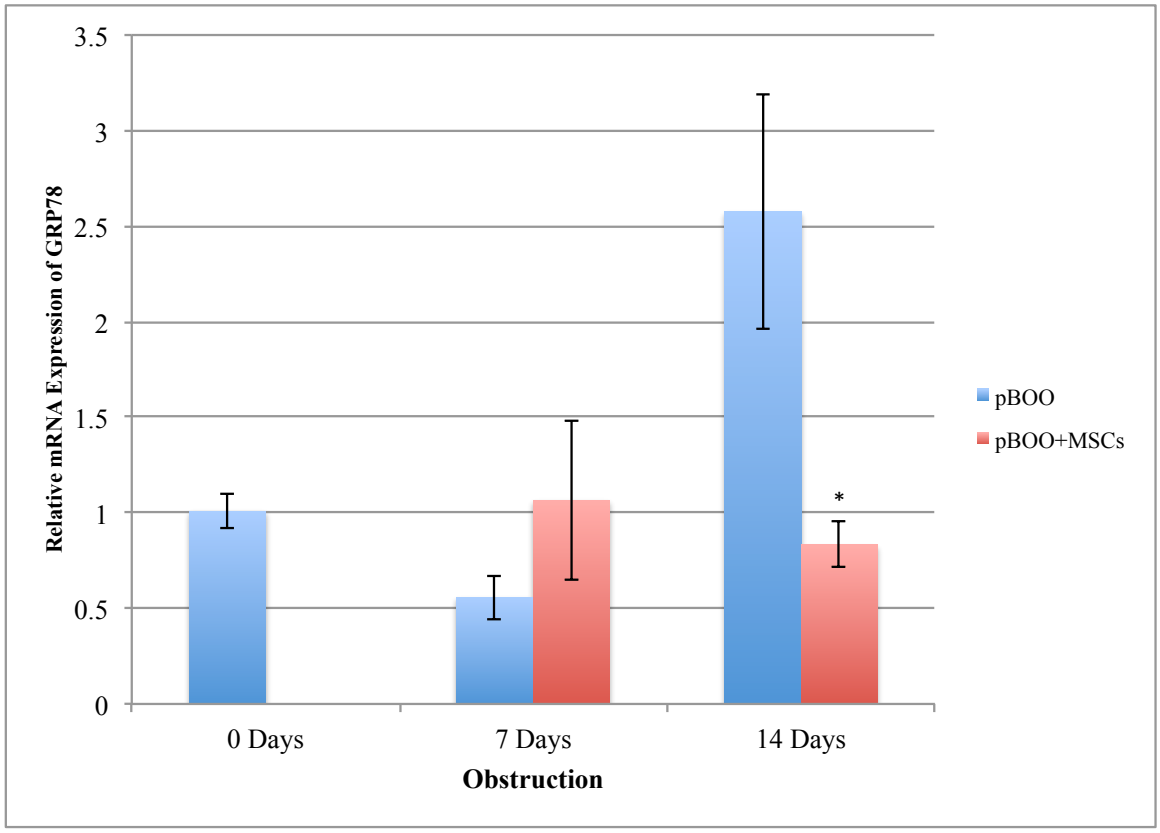
 # p-value < 0.05 vs. unobstructed.
** p-value < 0.05 in treatment vs. control groups.*
Error bars represent standard error around the mean

Figure 3.8 RhoA mRNA expression in control and treatment groups.



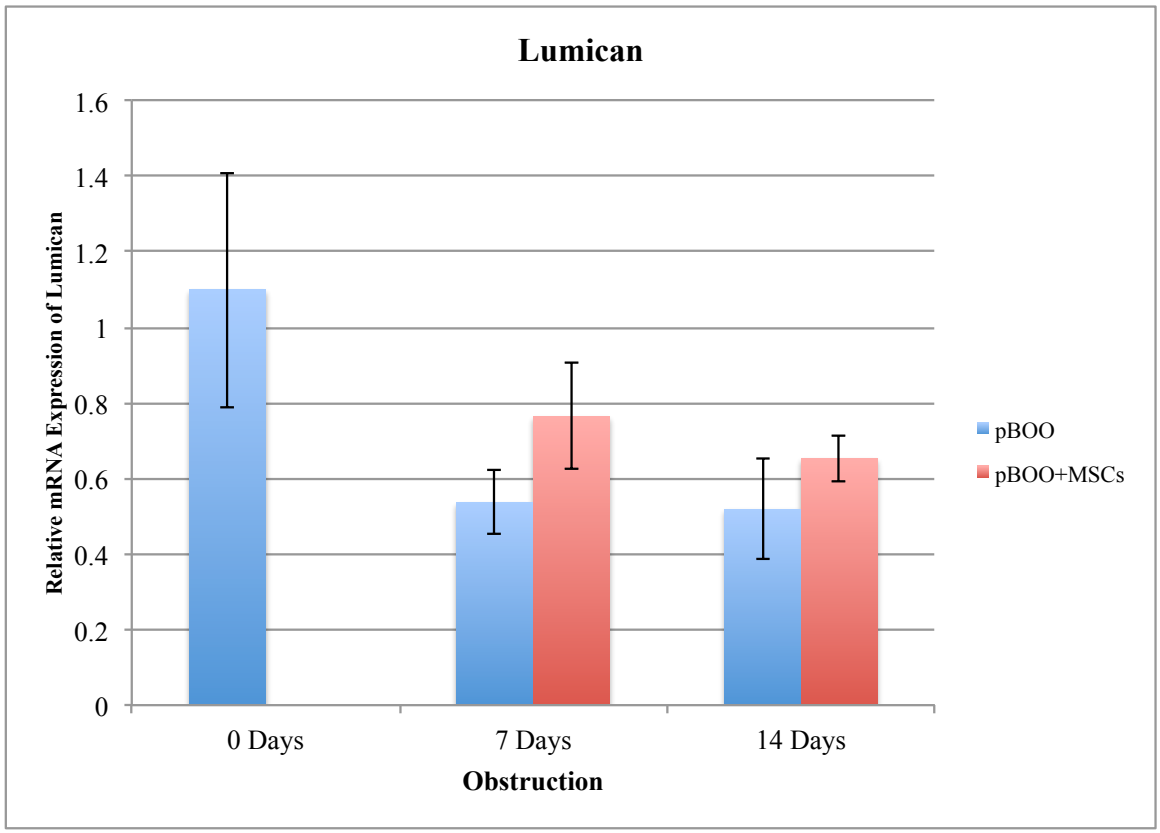
 # p-value < 0.05 vs. unobstructed.
** p-value < 0.05 in treatment vs. control groups.*
Error bars represent standard error around the mean

Figure 3.9 Marker of ER stress (GRP78) mRNA expression in control and treatment groups.



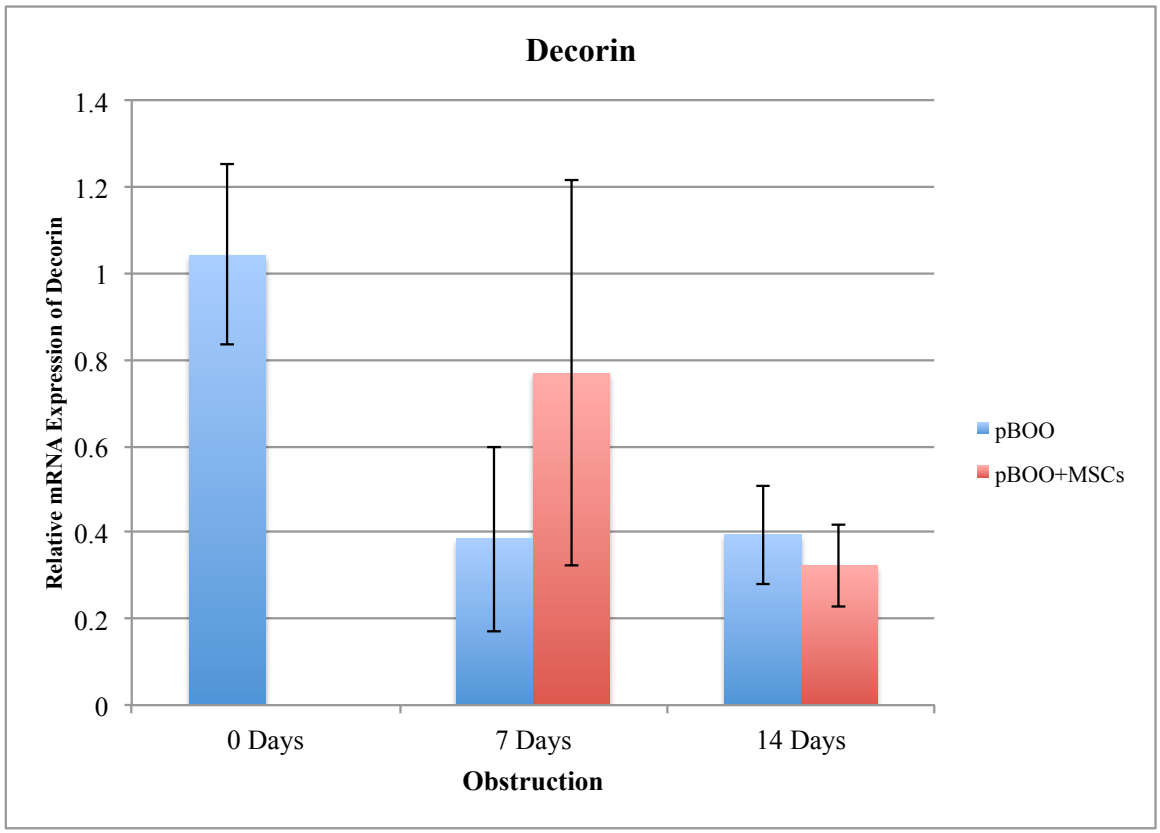
** p-value < 0.05 in treatment vs. control groups.*
Error bars represent standard error around the mean

Figure 3.10 Lumican mRNA expression in control and treatment groups.



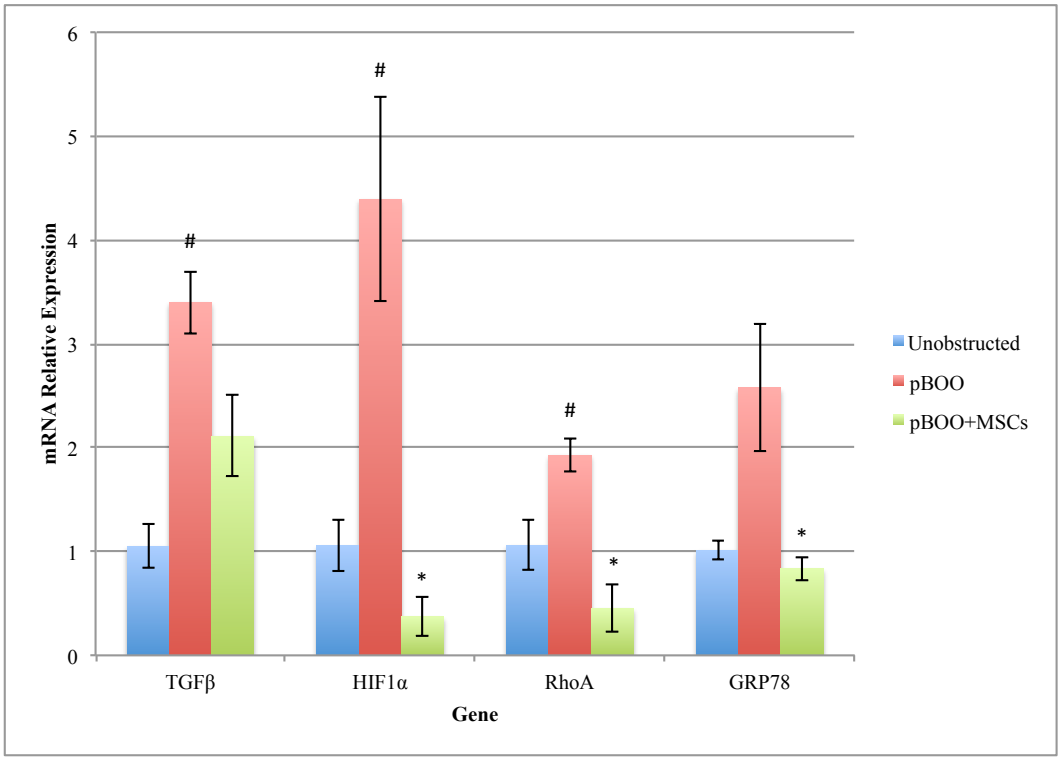
Error bars represent standard error around the mean

Figure 3.11 Decorin mRNA expression in control and treatment groups.



Error bars represent standard error around the mean

Figure 3.12 Summary of mRNA expression of some examined genes in unobstructed & after 14 days of pBOO in control and treatment groups.



 # p-value < 0.05 vs. unobstructed.
** p-value < 0.05 in treatment vs. control groups.*
Error bars represent standard error around the mean

3.7 Endnotes

A version of this chapter has been submitted for publication.

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Chapter 4

Conclusion and Future Directions

pBOO remains to be an important problem in modern urological practice. The presence of obstruction forces the bladder to generate more pressure for proper voiding, leading to a sequence of bladder wall inflammation, hypoxia, and eventually fibrosis with its deleterious outcomes on patients' lives.

Here in this current work, we tried to understand the early changes in bladder wall protein composites, to identify early changes that might be a "trigger" to later pathological consequences on the molecular and functional level. Utilizing mass spectrometry based analysis we described the early changes in the proteome of urinary bladder after pBOO. Some results came to support our previous work, such as with decorin reduction, and we described some new proteins and pathways that are activated after pBOO, which added to our understanding of the pathology.

Our proteomic analysis demonstrated a significant role for GRP78 and ERS, RhoA, TGFβ, and SLRPs in the pathology of pBOO. This shows the interplay of mechanical stretch, hypoxia, and inflammation in inducing the pathological changes observed.

ERS after pBOO, evident by the increase of GRP78, results in a sequence of initial corrective measures that help in protein homeostasis and proper protein folding; however, if this stress persists, this pathway triggers intracellular apoptotic signals, leading to the loss of cellular population [1 2]. Cellular loss has been observed in a previous work in our laboratory, where after prolonged

duration of obstruction, cells are replaced by collagen, denoting the "fibrotic phase" of pBOO[3].

Bladder wall hypoxia is thought to be a major mechanism of injury after pBOO [3]. Here we confirmed the presence of hypoxia in the early phase after pBOO by detecting high levels of HIF1α.

Transforming protein RhoA was seen to increase as well after pBOO. This increase activates a sequence of cellular pathways and mechanisms, and thought to result in increased bladder smooth muscle contractility (i.e. bladder instability) adding more pressure inside the bladder wall [4-6]. Furthermore, RhoA is though to interfere with eNOS, leading to reduction in bladder wall perfusion and augmentation of hypoxia [7].

TGFβ is seen to increase after pBOO confirming our previous findings [3]. Its activation results in an increase in ECM proliferation and collagen deposition, promoting bladder fibrosis [8]. It also interferes with ECM proteoglycans, promoting irregular collagen fibrils organization [9].

The second experiment in this thesis involved the administration of MSCs after pBOO. This resulted in several important physiological and biomedical benefits. MSCs are known to exert many immunomodulatory and therapeutic effects, and have been studied in many animal models of injury with promising positive results [10,11]. In our model of pBOO, treated animals exhibited lower ERS marker expression, which might results in a decreased rate of cellular apoptosis. Less RhoA levels were detected, suggesting better control of muscle contractility. Angiogenesis induced by MSCs is a possible mechanism for the reduced bladder

wall hypoxia observed with lower HIF1α. Despite not reaching statistical significance, the reduction in TGFβ mean mRNA expression in treated animals suggests a positive outcome, and might need higher sample size to detect. These changes on the molecular level were translated on bladder function. Despite the limited effect on urodynamics seen, which is most likely due the short duration of the study, we observed maintenance of bladder capacity around normal levels in treated group, compared to significant increase in the control group after 14 days of pBOO.

Further research is needed to delineate more changes on bladder functionality. We can look to the presented work here as a "proof of concept" that MSCs can actually halt the pathological changes after pBOO. Longer duration of follow up is needed to detect further benefits of MSCs treatment on the progression to bladder fibrosis.

Linking our proteomic findings to the genomics and transcriptomics of pBOO will be a valuable addition to our knowledge, and a future direction to pursue. This would help properly understand the whole spectrum of changes that pBOO induces on cells and their regulation of transcription to translation.

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