

# Mesenchymal stem cells ameliorate partial bladder outlet obstruction-induced epithelial-mesenchymal transition type II independent of mast cell recruitment and degranulation

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**Cite as:** Kadam R, Wiafe B, Metcalfe PD. Mesenchymal stem cells ameliorate partial bladder outlet obstruction-induced epithelial-mesenchymal transition type II independent of mast cell recruitment and degranulation. *Can Urol Assoc J* 2021;15(1):E29-35. <http://dx.doi.org/10.5489/auaj.6501>

Published online July 17, 2020

## Abstract

**Introduction:** Partial bladder outlet obstruction (pBOO) results in increased urinary storage pressure and significant morbidity. Increased pressure results in a sequence of programmed events: an initial inflammatory phase, smooth muscle hypertrophy, and fibrosis. Although epithelial-mesenchymal transition (EMT) and mast cell accumulation play intermediary roles in some fibrotic conditions, their role in pBOO has not yet been elucidated. Mesenchymal stem cell (MSC) therapy is emerging as a promising treatment for several conditions. It potently inhibits bladder deterioration after pBOO; however, its mechanism of action is insufficiently understood. Thus, we hypothesize that EMT type II pathway plays a significant role in pBOO, aided by the recruitment and activation of mast cells, and these are potently inhibited by MSCs.

**Methods:** pBOO was surgically induced in female Sprague-Dawley rats and simultaneously treated with MSCs. Treatment effect was determined after two or four weeks and compared to untreated controls. Immunohistochemistry was used to measure markers characteristic of EMT (vimentin, collagenase, and collagen). Whole and degranulated mast cell counts were also performed.

**Results:** pBOO resulted in an increased expression of collagenase, vimentin, and collagen. Mast cell recruitment increased proportionately to the length of bladder obstruction. MSC treatment significantly mitigated the EMT type II response, but mast cell recruitment and degranulation were unaffected.

**Conclusions:** Our results demonstrate the involvement of EMT type II in the pathophysiology of pBOO and confirm its mitigation with MSC treatment independent of mast cells response. The observations provide insight into the mechanism of action and have therapeutic ramifications.

## Introduction

Partial bladder outlet obstruction (pBOO) is a ubiquitous condition in urology, with etiology ranging from prostatic hyperplasia in elderly males to posterior valves in the fetus. Several studies have promoted the idea that following pBOO, the bladder detrusor muscle generates a high-pressure state, causing programmed progressive deterioration of the bladder with accompanying biochemical and molecular changes. An initial inflammatory phase gives rise to bladder smooth muscle hypertrophy, followed by compensatory angiogenesis, infiltration of the extracellular space with collagen, and resultant decompensatory bladder fibrosis.<sup>1,2</sup> However, despite this widespread recognition, current treatment modalities for pBOO remain either crude or inadequate. Neither clean intermittent catheterization nor oral anticholinergics target the physiological pathways to inhibit the progression of this pathology. This gap necessitates the use of a bimodal therapy that attenuates the pathological processes underlying pBOO, and at the same time, can promote regeneration of healthy bladder tissue.

In recent years, mesenchymal stem/stromal cells (MSCs) have propelled to the forefront as a promising treatment for inflammatory and fibrotic diseases. MSCs are multipotent cells, most commonly isolated for clinical use from adults from the bone marrow and adipose tissue.<sup>3</sup> MSCs possess a lack of immunogenicity and can modulate immune responses, making them efficacious in the treatment of rheumatoid arthritis and graft vs. host disease.<sup>4,5</sup> Alongside these properties, the paracrine effects of MSCs have been shown to attenuate fibrosis in the liver, lungs, and kidneys.<sup>6-8</sup> Concerning pBOO, we have previously demonstrated the anti-inflammatory and anti-fibrotic effects of MSC treatment in-vitro using hypoxic bladder smooth muscle cells, and in-vivo using acute and long-term pBOO rat models.<sup>9-11</sup> Since MSC therapy for pBOO holds great promise, it necessitates a fuller understanding of the underlying mechanisms that drive pBOO and the effect that MSCs have on them.

Type 2 epithelial to mesenchymal transition (EMT type II) is a cellular process that can generate aberrant myofibroblasts whose fibrotic activity has adverse effects on organ function.<sup>12</sup> The process is a double-edged sword that is initially beneficial in wound healing; however, in cases of prolonged inflammation, it results in excessive extracellular matrix deposition and fibrosis.<sup>13</sup> During EMT type II, cells lose their typical epithelial architecture, apical-basal polarity and instead gain migratory abilities, a spindle-shaped mesenchymal phenotype, and express vimentin — a type III intermediate filament.<sup>12,14</sup> Transforming growth factor-beta (TGF- $\beta$ ), a pleiotropic profibrotic factor, is critical to this process, and acts via downstream phosphorylation of mothers against decapentaplegic homolog 2/3 (SMAD2/3), leading to eventual myofibroblast differentiation.<sup>15,16</sup> Collagenases can degrade extracellular matrix components<sup>17</sup> and promote pericellular collagenolysis to facilitate cell migration and invasion.<sup>18,19</sup>

Mast cells are multifunctional immune cells that originate in the bone marrow as naive progenitor cells, are recruited via chemotactic inflammatory cytokines, and mature upon penetrating tissue.<sup>20</sup> The hallmark of their activation involves degranulation and secretion of cytokines such as TGF- $\beta$ 1 and chymase, which promote fibrosis.<sup>21,22</sup> Since MSCs have an immunomodulatory effect, they have the potential to alter mast cell accumulation and degranulation and inhibit their deleterious effects.

The role of EMT type II, within the context of pBOO, has not yet been elucidated. Thus, we hypothesize that the EMT type II pathway is a primary pathway through which pBOO results in fibrosis, and that mast cells play a prominent role in this process. MSCs achieve their therapeutic, anti-fibrotic effects by attenuating these processes.

## Methods

### Animal and study design

All protocols were approved by the University of Alberta Ethics Committee for the Care and Use of Experimental Animals. Twenty healthy adult female Sprague Dawley rats (approximately 250–300 g) were randomly assigned to five groups as follows: 1) obstructed for two weeks (pBOO-2weeks); 2) obstructed for four weeks (pBOO-4weeks); 3) obstructed with MSC therapy for two weeks (pBOO+MSCs-2weeks); 4) obstructed with MSC therapy for four weeks (pBOO+MSCs-4weeks); and 5) unobstructed controls (n=4 in each group). The animals resided two per cage with free access to food and water.

### Induction of pBOO

Rats in pBOO groups underwent urethral ligation under isoflurane anesthesia to create partial bladder outlet obstruction

as described earlier.<sup>2,11</sup> In sum, the bladder was exposed, and an 18-gauge angiocatheter was inserted into the urethra. A 2-0 silk suture was then tied around the urethra, and the catheter was removed. All operated rats were provided with cotrimoxazole-water and maintained on meloxicam (1 mg/kg) and buprenorphine (0.02 mg/kg) to manage pain.

### MSC treatment

Immediately after pBOO, the therapy groups were given a tail vein injection of Sprague Dawley bone marrow-derived MSCs commercially obtained from Cyagen Biosciences Inc, (Sunnyvale, CA, U.S.) in 2015. The cells obtained were characterized by the supplier to be positive for CD90, CD44, and CD29, and negative for CD34, CD45, and CD11b/c by flow cytometry analysis. They also tested negative for bacteria, fungi, and mycoplasma. The MSCs were cultured to expand in alpha-minimal essential medium ( $\alpha$ -MEM) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (290  $\mu$ g/ml), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (100 mM), sodium pyruvate (1 mM), penicillin-streptomycin (100 U/ml) (Life Technologies, Burlington, Canada), and 5 ng/ml fibroblast growth factor-2 (FGF-2) (Neuromics, Edina, MN, U.S.). MSCs between passages two and eight were used for this experiment. One million stem cells in the log phase of growth per 0.5 ml PBS were injected per animal. At the experimental endpoint, bladder tissue was harvested and part fixed in 4% paraformaldehyde for immunohistochemistry studies.

### Antibodies

Mouse monoclonal antibodies against collagenase (DSHB H18G8), and vimentin (DSHB 3CB2) deposited by Werb Z and De La Rosa EJ, respectively, were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. A secondary horseradish peroxidase-conjugated antibody rabbit anti-mouse (abcam6728) was purchased from the indicated supplier.

### Immunohistochemistry studies

Formalin-fixed bladder tissues were embedded in paraffin blocks and sectioned into 5  $\mu$ m thickness. Then, the sections were deparaffinized by one-hour incubation at 65°C, followed by two changes of xylene and rehydration in decreasing concentrations of alcohol and water. Samples underwent heat-mediated antigen retrieval in sodium citrate solution, followed by blocking of endogenous peroxidase activity in a solution of methanol and hydrogen peroxide. A 5% BSA solution was used to block the tissues for an hour and a half. Overnight incubation with a primary antibody concentra-

tion of 1:100 vimentin and 1:45 collagenase in 2% BSA was performed. The following day, washes were followed by incubation in 1:200 secondary antibody in TBST for an hour and a half. Following subsequent washes, the immunoreaction was visualized with 3'3'-diaminobenzidine (DAB Sigma D8001). Tissues were counterstained with Mayer's hematoxylin (Sigma MHS32), dehydrated in 100% ethanol, cleared in xylene, and mounted for microscopic visualization. Microscopic images were taken using an Eclipse Ti-S microscope (Nikon Canada, Mississauga, Canada).

### Toluidine blue tissue-staining and mast cell degranulation count

Toluidine blue stock solution was prepared from powder (Sigma 198161) diluted in 70% ethanol. At the time of the experiment, a working solution was prepared by combining the stock solution with 1% NaCl with a pH of 2.3. Following deparaffinization at 65°C, xylene clearance and rehydration, as indicated by steps outlined earlier, slides were stained in toluidine blue working solution for 2–3 minutes. Next, slides were washed in distilled water, dehydrated in 100% ethanol, cleared in xylene, and mounted. Microscopic images were taken using an Eclipse Ti-S microscope (Nikon Canada, Mississauga, Canada). Images at a higher magnification were taken using a Zeiss Axio Imager Microscope with 20x 1.3 NA Plan Apocromat lens and captured with Infinity3-3UR camera with Infinity analyze and capture software for windows.

A mast cell degranulation count was performed with an Eclipse Ti-S light microscope by two independent observers. Per tissue, mast cells in 100 high-power fields were counted. Whole non-degranulated mast cells that had an intact, uniform, dark violet-indigo color with little or no space visible inside them were counted per field. The number of degranulated mast cells and their extracellular granules that exhibited a lighter purple color were also noted.

### Trichrome staining

Deparaffinized bladder tissues were incubated in Bouin's solution, washed, and stained using Gomori's stain for 15 minutes. Differentiation was performed in 0.5% acetic acid for two minutes and washed sections were dehydrated, cleared in xylene, and mounted. Microscopic images were captured using Eclipse Ti-S microscope.

## Results

### EMT type II response

Vimentin, a marker of the pervasiveness of EMT, increased with obstruction. The protein was immunolocalized to the detrusor after two and four weeks of obstruction. However,

MSC treatment led to a significant decrease in its expression (Fig. 1A). Similarly, collagenase expression increased with obstruction time and was prevalent in the lamina propria, as well as the detrusor. MSC treatment attenuated the expression of these proteins at the same time points (Fig. 1B).

### Mast cell response

Mast cell accumulation in the bladder tissue increased with obstruction but remained unchanged with MSC treatment. Even though the mast cells were broadly distributed throughout the layers of the bladder, there appeared to be more in the suburothelial layer and around blood vessels (Fig. 2). Increase in the number of mast cells was directly proportional to the obstruction time, and degranulated mast cells increased in accordance. Nonetheless, treatment with MSCs neither affected the total mast cell count nor the proportion of degranulation (Fig. 3).

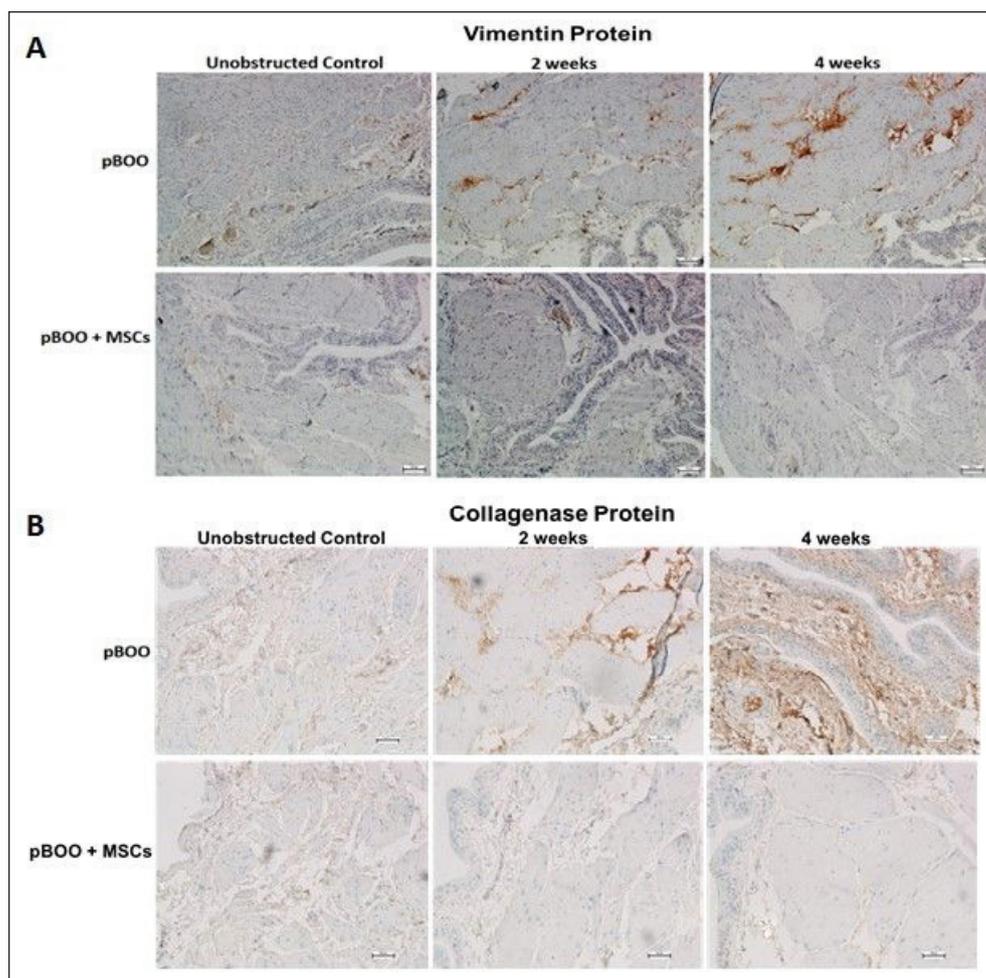
### Collagen accumulation response

As revealed by Trichrome staining, pBOO groups displayed an increase in collagen deposition in the lamina propria and intra-detrusor spaces. This collagen infiltration of the bladder smooth muscles was heightened in four-week obstructed bladders, resulting in the observed distorted histology. However, MSC injection resulted in the inhibition of connective tissue infiltration at the same time points, and the treated groups displayed normal bladder histology (Fig. 4).

## Discussion

EMT type II is a critical process in the physiology of inflammation; however, when unchecked, it can result in pathological fibrosis. We have previously shown that pBOO results in fibrosis and MSCs can inhibit this. In this investigation, we wished to more pointedly explore the role of EMT and mast cells in pBOO. We demonstrated the expression of EMT markers and their inhibition by MSC treatment. This study reveals that activation of the EMT type II program does play a role in achieving the fibrotic pathophysiology of pBOO, thus supporting our hypothesis.

Our previous investigation using the same animal population demonstrated that obstruction upregulates the gene expression of TGF- $\beta$ 1 – a master-inducer capable of sustaining the entire EMT process.<sup>11</sup> Additionally, our lab previously confirmed the transcriptional upregulation of SMAD2/3 alongside increased protein expression of phosphorylated SMAD2/3 in bladder urothelium after pBOO.<sup>11</sup> The TGF- $\beta$ 1-SMAD2/3 pathway is closely tied to myofibroblast differentiation<sup>16</sup> and EMT.<sup>15</sup> Specifically, the phosphorylated SMAD2/3 combine with SMAD4 to form a heterotrimer complex, which translocates to the nucleus to regulate



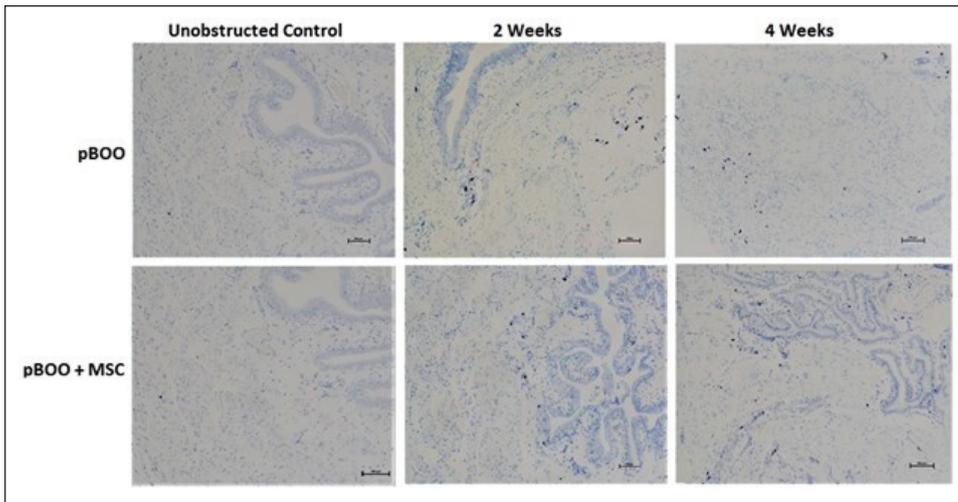
**Fig. 1.** Increased vimentin and collagenase expression and activity in partial bladder outlet obstruction (pBOO) was inhibited by mesenchymal stem cells (MSCs). **(A)** Vimentin protein expression progressively increased with pBOO duration and decreased significantly with MSC treatment to normal expression. **(B)** Collagenase protein levels, also identified in brown staining, increased significantly in the lamina propria and detrusor after pBOO; however, collagenase levels were downregulated in MSC groups. Representative micrographs were captured at 10x magnification, and scale bars represent 100  $\mu$ m.

gene expression and generate myofibroblasts.<sup>23</sup> Given the well-established relationship between EMT and myofibroblast activation, the pBOO-induced EMT in the current study may result in the concomitant increase in myofibroblasts accumulation leading to fibrosis. Collagenases play an important role in cell invasion and migration through pericellular collagenolysis.<sup>18,19</sup> One characteristic feature of pBOO is the increased deposition of collagen. In this study, we also found a copious amount of collagen deposition in the lamina propria and detrusor with increased obstruction time. Results of the current study show the activation of EMT type II sustains the pathway of this disease. Thus, the concurrent increase in protein expression of vimentin, collagenase, and collagen confirms the contributive role of EMT type II in pBOO-induced bladder fibrosis via the TGF- $\beta$ 1-SMAD2/3 pathway.

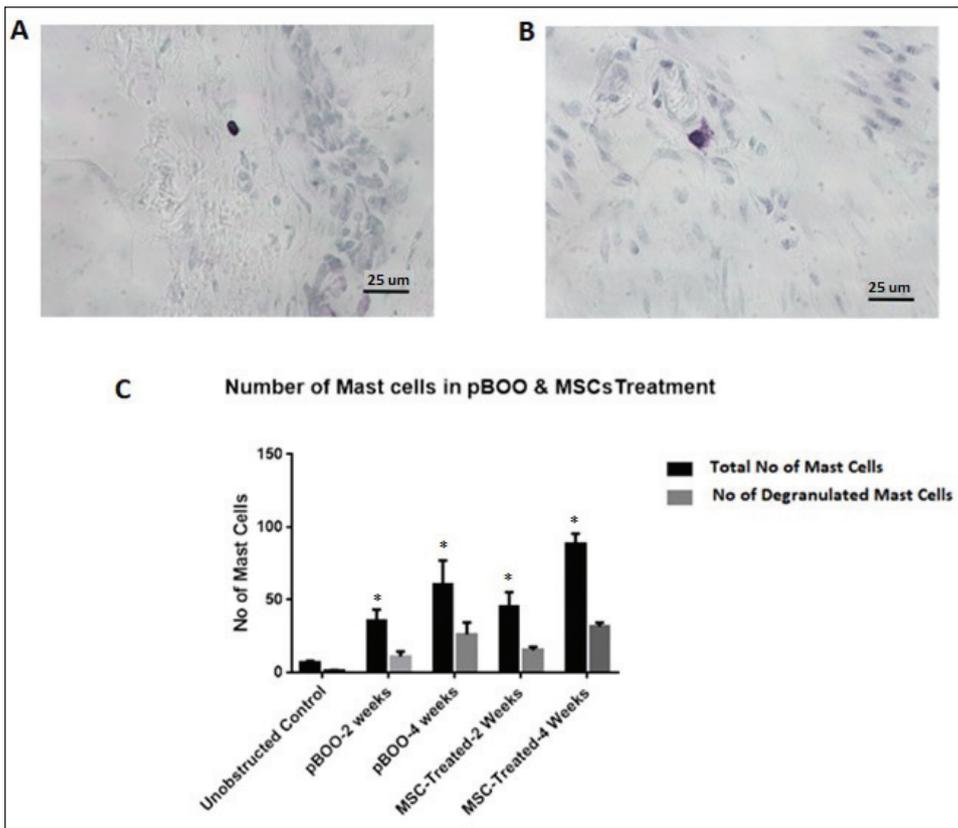
In keeping with current findings, EMT type II results in a simultaneous increase in mast cell accumulation and associated degranulation. A study conducted by Michishita et al<sup>24</sup> also found an upregulation of mast cells when examining pBOO in a rat model at one week of obstruction. The inflammatory bladder disease — bladder pain syndrome/interstitial cystitis (BPS/IC) — is the most common cause of mast cell recruitment.<sup>25</sup> Previous studies have implicated mast cells in fibrosis since they are capable of secretion and chymase-mediated activation of TGF- $\beta$ 1 and, hence, the induction of the pathophysiology of fibrotic diseases,<sup>21,22</sup> as noted with renal fibrosis after ureteric obstruction.<sup>26</sup> Thus, in this study, mast cells serve as a twofold marker for inflammation, as well as the consequent EMT process. The increased mast cell count with obstruction demonstrates increased inflammation of the bladder after pBOO. In our previous investigation, we demonstrated that the gene transcription of TNF- $\alpha$  increased in obstructed bladders.<sup>11</sup> TNF- $\alpha$  is a potent chemoattractant for mast cells and could, hence, be the mechanism underlying the observed mast cell accumulation in the current

study.<sup>20</sup> We were also able to demonstrate that pBOO results in the associated degranulation of mast cells. Therefore, the inflammatory process is integral to mast cell recruitment and activation.

MSCs inhibit EMT type II without affecting mast cell accumulation and degranulation. Since our previous work demonstrated that MSCs downregulate TGF- $\beta$ 1 and SMAD2/3, and since the EMT type II process canonically occurs via the signaling of stated cytokines, it is likely that their inhibition is the primary cause behind the ensuing decrease in myofibroblasts, collagenase, and collagen expression. Regarding the unaffected mast cell accumulation and degranulation, a few explanations are possible. Firstly, MSCs may likely exert their effects by interacting with other sources of pro-fibrotic mediators, such as the macrophage and T-helper cells.<sup>27</sup> The effect of MSCs on these additional components of the immune



**Fig. 2.** Mast cell accumulation increased with obstruction and was unaffected by mesenchymal stem cells (MSCs). Mast seen as dark blue cells, were broadly distributed throughout the bladder layers. Representative micrographs were captured at 10x magnification, and scale bars represent 100  $\mu$ m. pBOO: partial bladder outlet obstruction.

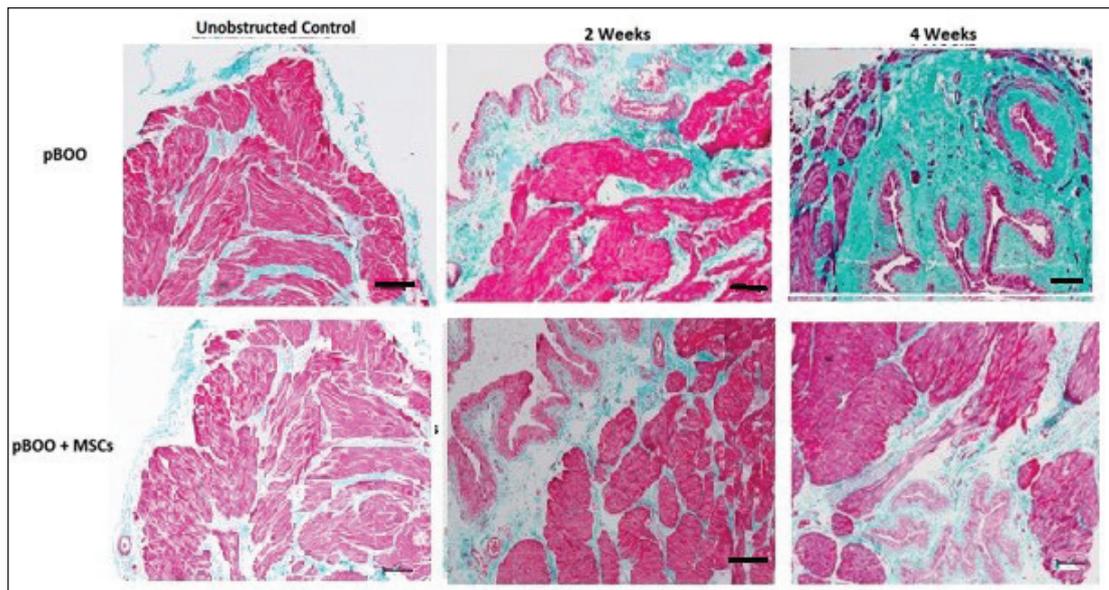


**Fig. 3.** Mast cell count increased with obstruction time, and degranulated mast cells increased correspondingly. Treatment with mesenchymal stem cells (MSCs) did not affect the total number of mast cell recruitment, nor the rate of degranulation. **(A)** A representative, non-degranulated mast cell displays a dark violet uniform color and a non-vacant, intact shape. **(B)** A degranulated mast cell is light purple in color and has dot-like extracellular granules surrounding the deficient cell. **(C)** The total number of mast cells increased with obstruction with concordant increase in degranulation. \*Statistical difference between unobstructed controls and other experimental groups. Both types of mast cells were not significantly affected by MSCs. Micrographs were captured at 40x magnification, and scale bars represent 25  $\mu$ m. pBOO: partial bladder outlet obstruction.

system deserves further investigation. Secondly, the effect of MSCs on mast cells could be a function of time, better observed after a more extended period than the four weeks researched in this study. Lastly, mast cell mediators initially play a role in wound healing, a process that goes awry with chronic inflammation and leads to fibrosis. Thus, perhaps this mechanism is conserved by MSCs due to its initially advantageous protective purpose, and MSCs instead downregulate cytokines like TGF- $\beta$ 1 and SMAD2/3 that are more proximally related to fibrosis. Despite having no significant effect on mast cells, MSC treatment has global benefit, as our previous work has shown a predominant decrease in inflammation, smooth muscle cell hypertrophy, fibrosis, and most importantly, improvement in urodynamic parameters.<sup>10,11</sup>

We wish to acknowledge a few of the limitations of this study. This manuscript is designed as a descriptive, qualitative assessment of the role of EMT in bladder fibrosis. We were unable to perform quantitative (RT-PCR, ELISA) experiments because of limited quantities of tissue available. However, we feel confident that our previous studies have robustly confirmed the degree of inflammation and fibrosis.<sup>2,9-11</sup> Therefore, our descriptive exploration of EMT as a mechanism towards this is justified as a means of describing the pathophysiology and one mechanism by which MSC exert their protective effect.

Our model is designed to resemble clinical conditions, such as posterior urethral valves, neurogenic bladders with detrusor sphincter dyssynergia, and prostatic hypertrophy. We have delineated many biochemical pathways and means of inhibition, however, acknowledge multiple shortcomings. The acute severity of obstruction and timing of therapeutic administration is less



**Fig. 4.** Collagen expression and infiltration increased in the detrusor and lamina propria layers with obstruction time. However, mesenchymal stem cell (MSC) treatment lead to an inhibition of collagen accumulation at the same time points. Bladder collagen stained blue whereas muscles and blood vessels are stained red. Scale bar represents 100  $\mu$ m. pBOO: partial bladder outlet obstruction.

relevant to clinical practice. No human receives treatment the minute an acute, nearly complete obstruction occurs. However, we have been using MSC treatment at the time of surgical induction of pBOO, as this has given us the most efficacious means of mitigating the inflammatory response and preventing detrusor deterioration. Because our injections at the time of pBOO induction have been so effective, we have not felt the need to repeat them but instead, have begun working on experiments to confirm the efficacy of treatment after pBOO and once bladder changes have occurred. We will give the MSC two and four weeks after pBOO and determine its effectiveness at repairing damaged tissue instead of preventing the damage.

## Conclusions

Our finding that pBOO-induced fibrosis occurs via the EMT type II pathway reinforces its similarities with other fibrotic pathologies. We suspect that pBOO fibrosis is liable to similar mechanisms that affect such diseases as cirrhosis, renal fibrosis, and pulmonary fibrosis, wherein EMT type II pathway is demonstrably involved. We look forward to future studies, to determine the efficacy of MSC treatment in a delayed fashion, with a pre-established EMT pathway, to determine if MSCs can reverse this pathophysiology.

**Competing interests:** The authors report no competing personal or financial interests related to this work.

This paper has been peer-reviewed.

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