Original Paper

The Antimuscarinic Agent Tolterodine Regulates Bladder Extracellular Matrix in Partial Bladder Outlet Obstruction in Rats

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Key Words

Muscarinic receptor • Extracellular matrix • Bladder outlet obstruction • Metalloprotease

Abstract

Background/Aims: Antimuscarinic agents can delay the progression of bladder dysfunction caused by bladder outlet obstruction (BOO). To date, the relationship between muscarinic receptor activity and the bladder extracellular matrix (ECM) remains unclear. Thus, an animal model of partial BOO (PBOO) in female rats was established to explore the variation in bladder wall ECM proteins under PBOO conditions with antimuscarinic agent administration. **Methods:** Rats were randomly divided into three groups: sham, PBOO, and PBOO plus tolterodine. Picrosirius red staining was used to examine the smooth muscle and collagen content of bladder samples. Gene microarray and RT-PCR were performed to survey the expression of ECM proteins, receptors, and metabolism regulators in the rat bladder. Positive results were further evaluated by immunohistochemistry. **Results:** Picrosirius red staining showed that smooth muscle volume significantly increased in the PBOO and PBOO plus tolterodine groups ($p < 0.05$), while collagen significantly increased in the PBOO group ($p < 0.05$) but not in the PBOO plus tolterodine group. Gene microarray and RT-PCR revealed that none of the collagen subtypes exhibited significant changes after PBOO establishment and tolterodine administration. However, matrix metalloproteinases (MMPs) increased significantly in the PBOO plus tolterodine group ($p < 0.05$). Additionally, PBOO inhibited the expression of non-collagen ECM proteins in the rat bladder wall, while tolterodine induced the expression of non-collagen ECM proteins and ECM receptors. **Conclusions:** Tolterodine decreased the volume of collagen in PBOO rat bladder wall, possibly via MMPs, and regulated the expression of ECM proteins and receptors.

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Introduction

Bladder outlet obstruction (BOO) can result in structural and functional changes in the detrusor muscle of the bladder [1–3]. In the early stages of BOO, the bladder wall thickens to compensate for higher outlet resistance. However, over time, the detrusor muscle becomes decompensated and then lower urinary tract symptoms (LUTS) appear, ultimately progressing to urinary retention with overflow incontinence [3, 4]. Studies have shown that hypertrophy can be found in both smooth muscle cells and the extracellular matrix (ECM) of the bladder wall in BOO [5], where the ECM hypertrophy is mainly due to collagen accumulation, and the more collagen accumulates, the worse the LUTS are [6, 7]. The main reason for collagen accumulation in the bladder wall in BOO is inhibited collagen catabolism rather than increased synthesis; this process is controlled by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [8, 9]. An effective method for terminating or even reversing the morphological changes in the bladder wall is to relieve BOO as early as possible [3, 4]. However, even if the obstruction is relieved, the existing collagen accumulation in the bladder wall and that of other non-collagen proteins is difficult to resolve completely, and in some patients, LUTS persist [10, 11]. Therefore, a specific treatment to effectively activate collagen catabolism would be helpful for improving LUTS caused by long-standing BOO.

Acetylcholine, which activates muscarinic receptors on detrusor myocytes, is the main contractile transmitter of the bladder wall. A muscarinic antagonist can inhibit activation of muscarinic receptors, leading to decreased sensitivity of C-fibers and reduced involuntary contraction of the detrusor muscle at the urinary storage stage [12]. Thus, the bladder can achieve greater capacity with decreased urgency and frequency. Consequently, an antimuscarinic agent is effective for relieving LUTS caused by an overactive bladder. An antimuscarinic agent can also effectively improve LUTS caused by long-standing BOO because these symptoms are also caused by hypersensitive bladder filling and involuntary detrusor muscle contraction [3, 4]. Hence, an antimuscarinic agent does not just relieve the short-term symptoms. A study on bronchial smooth muscle showed that prolonged action of an antimuscarinic agent remodeled the airway wall ECM, delayed smooth muscle hyperplasia, and changed the adhesive interaction between myocytes and ECM, ultimately improving the functional status of the airway smooth muscle [13]. The question is whether the prolonged effects of antimuscarinic agents can be obtained in the bladder, and whether the inhibition of muscarinic receptors can result in remodeling of the ECM and decomposition of collagen, consequently relieving LUTS caused by long-term BOO.

To address this, we established an animal model of partial BOO (PBOO) in Sprague–Dawley rats. The antimuscarinic agent tolterodine, was administered to the rats to examine differences in collagen accumulation and metabolism. Furthermore, expression of other non-collagen proteins and receptors in the bladder wall was examined to elucidate whether an antimuscarinic agent can affect the functional status of the bladder wall ECM as a therapeutic agent.

Materials and Methods

Animals

Female Sprague–Dawley rats (220 to 250 g) were used. The rats had free access to water and food and were maintained in a temperature-controlled room under a 12-h light/dark cycle. The rats were randomly subdivided into three groups: sham, PBOO, and PBOO plus tolterodine (0.36 mg/kg), with five rats in each group. Tolterodine, a commonly used antimuscarinic agent, was dissolved in distilled water and administered by gavage. The sham and PBOO groups received the same volume of distilled water. Treatment was initiated on the first day after surgery and continued once daily for 3 weeks. All animal experiments were carried out in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and approved by the West China Hospital Committee on Animal Care.

PBOO animal model

Rats were anesthetized by intraperitoneal injection of 0.9% chloral hydrate (3 mL/kg). Then, a suprapubic midline incision was made to expose the bladder and proximal urethra. An urethra catheter (outer diameter: 1 mm) was passed into the urethra and was then ligated with 4-0 silk around the proximal urethra to simulate PBOO. The catheter was then removed and the abdominal incision was closed. The sham group was subjected to the same procedure except for the urethra ligation. All the rats received 150 mg/kg body weight ampicillin intramuscularly after the procedure.

Microarray analysis

Microarray analysis (service provided by Kangchen Biotech, Shanghai, China) was performed to evaluate the bladder gene expression in the PBOO model and tolterodine-treated group using a Whole Rat Genome Oligo Microarray (Agilent Technologies, Santa Clara, CA).

Briefly, total RNA samples were isolated from rat bladder wall fragments using TRIzol Reagent (Life Technologies, Carlsbad, CA). Sample labeling and array hybridization were performed according to the One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies). In total, 100 µL of the hybridized sample was dispensed into the gasket slide, which was then assembled with the gene expression microarray slide. Slides were incubated for 17 h at 65°C in an Agilent Hybridization Oven and scanned by the Agilent DNA Microarray Scanner (part number G2505C).

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies). Differentially expressed genes with statistically significant differences between groups were identified and shown in Microsoft Excel documents.

Reverse transcription polymerase chain reaction

RT² Profiler PCR Array (Agilent Technologies) was used to measure rat bladder ECM and adhesion molecule mRNA expression. Total RNA was isolated from fragments of rat bladder wall using TRIzol Reagent. RNA was eluted in 20 µL nuclease-free water and stored at –70°C until use. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Reverse transcription and amplification were performed using 2× SuperArray PCR master mix (Agilent Technologies). PCR conditions were 95°C for 10 min and 40 amplification cycles (95°C for 15 s, 60°C for 1 min and optical read), followed by 95°C for 10 s and melting from 65°C to 95°C at 0.5°C per 5 s. Data analysis was carried out using the comparative cycle threshold method with Bio-Rad iQ5 software.

Histological analysis

Bladder specimens were fixed in 4% paraformaldehyde for 24 h, then embedded in paraffin, cut into 4-µm sections, and mounted on microscope slides. The specimens were stained with picosirius red to examine the smooth muscle and collagen content of the tissue. Images from each slide were captured using a digital camera (Olympus Corporation, Tokyo, Japan) in five randomly chosen areas at 100× magnification. The areas of collagen (red) and muscle tissue (yellow) were quantified using Image-Pro Plus (Media Cybernetics Inc., Rockville, MD). The integrated optical density (IOD) of smooth muscle or collagen was calculated and compared between groups.

Immunohistochemistry

For immunohistochemistry, fibronectin, integrin α4, integrin β2, MMP7, and TIMP1 were detected using the following primary antibodies from Abcam (Cambridge, UK): rabbit anti-fibronectin (ab2413), rabbit anti-MMP7 (ab189277), rabbit anti-TIMP1 (ab61224), rabbit anti-integrin α4 (ab202969), and rabbit anti-integrin β2 (anti-CD18, ab131044). The slides were stained according to the streptavidin-biotin-peroxidase complex (SABC) method. Ten randomly chosen representative areas at 400× magnification from each section were captured and analyzed using Image-Pro Plus software. Immunohistochemical scores (IHS) were calculated and compared between groups.

Statistical analysis

Results are presented as the mean ± standard deviation. Results between groups were compared by paired *t* test or analysis of variance. A value of *p* < 0.05 and fold-change > 1.5 in microarray analysis was considered a difference requiring verification. A value of *p* < 0.05 in the PCR and image analyses was considered significant.

Results

Collagen deposition in the PBOO rat bladder wall is relieved by tolterodine treatment

In the picrosirius red staining test, bladder wall thickness increased significantly after 3 weeks of PBOO. Furthermore, muscle area IOD increased by $121.4\% \pm 55.7\%$ (203.8 ± 55.5 vs. 92.1 ± 34.0 , $p = 0.009$) and collagen increased by $64.4\% \pm 25.4\%$ (403.1 ± 63.7 vs. 245.2 ± 88.6 , $p = 0.046$), compared with the sham group. When rats were treated with tolterodine for 3 weeks together with PBOO, the detrusor muscle IOD was significantly higher than that of the sham group (269.6 ± 43.7 vs. 92.1 ± 34.0 , $p < 0.001$), but there was no significant difference in the IOD compared with the PBOO group (269.6 ± 43.7 vs. 203.8 ± 55.5 , $p = 0.1$). However, 3 weeks of tolterodine treatment together with PBOO reduced the rat bladder collagen IOD to the sham group level (244.0 ± 59.9 vs. 245.2 ± 88.6 , $p = 0.858$), which was significantly lower than that of the PBOO group (244.0 ± 59.9 vs. 403.1 ± 63.7 , $p = 0.023$; Fig. 1). These results indicate that tolterodine either inhibited collagen synthesis or activated collagen catabolism in the PBOO bladder wall to reduce collagen accumulation.

Reduction in collagen volume is mediated by MMPs in the PBOO model rat with tolterodine treatment

Forty-six collagen gene transcripts were screened using an Agilent whole-genome microarray, of which 24 transcripts had potential expression differences between any two groups ($p < 0.05$, fold change ≥ 2). These were further verified by PCR, but no significant difference was observed ($p > 0.05$; Fig. 2), suggesting that the accumulation of collagen in the PBOO rat bladder and the reduction in collagen volume in the PBOO plus tolterodine treatment rat bladder were not associated with collagen expression.

Fifty-three collagen metabolic regulator gene transcripts were screened by microarray, of which 22 transcripts had potential expression differences, and of these, 10 were further confirmed by PCR ($p < 0.05$; Fig. 3A and B). The results showed that antimuscarinic agent treatment of PBOO rats promoted an obvious increase in expression of MMPs (*Adamts1*, 2.04-fold, $p = 0.047$; *Mmp10*, 3.55-fold, $p = 0.004$; *Mmp8*, 5.56-fold, $p = 0.013$; *Mmp9*, 5.96-fold, $p = 0.046$), and in *Timp1* (3.92-fold, $p = 0.045$). Compared with the PBOO plus tolterodine group and the sham group, there was also a significant increase in *Adamts8* (19.47-fold, $p = 0.006$), *Mmp7* (457.24-fold, $p < 0.001$), and *Mmp13* (3.40-fold, $p = 0.025$) expression. These results suggest that tolterodine promoted the gradual decomposition of accumulated collagen in the PBOO rat bladder by regulating the expression of MMPs and TIMPs.

This increased expression, as well as the distribution, of MMP7 and TIMP1 in each group was further examined by

Fig. 1. Rat bladder paraffin sections stained with picrosirius red. Red-stained areas in the muscular layer represent collagen fibers, and yellow stained areas represent myofibers. Each sample slide was captured in five randomly chosen areas at 100 $\times$ magnification and analyzed using Image-Pro Plus. Smooth muscle or collagen IOD was calculated and is shown in the histogram. Tolterodine can either inhibit collagen synthesis or activate collagen catabolism in the PBOO bladder wall to relieve collagen accumulation. * $p < 0.05$ compared with the sham group. Scale bar is 400 μ m.

immunohistochemistry. MMP7 and TIMP1 were mainly distributed in the ECM (Fig. 3C). Compared with the sham group, the MMP7 IHS in the PBOO group was 1.22-fold higher ($p = 0.038$) and that of TIMP1 was 1.54-fold higher ($p = 0.086$); the MMP7 IHS in the PBOO plus tolterodine group was 2.06-fold higher ($p = 0.036$) and that of TIMP1 was 2.62-fold higher ($p = 0.013$; Fig. 3D). These results support the abovementioned changes in gene expression.

Tolterodine promotes expression of non-collagen ECM proteins and ECM receptors

Gene expression of non-collagen ECM proteins in the PBOO rat bladder with tolterodine treatment significantly changed compared with the PBOO

Fig. 2. Collagen gene expression of the rat bladder wall upon PBOO modeling and antimuscarinic agent treatment evaluated by rat genome-wide microarray analysis (A) and RT-PCR (B). Accumulation of collagen in the PBOO rat bladder and reduction in collagen volume in the PBOO plus tolterodine treatment rat bladder are not associated with collagen expression.

Fig. 3. Expression of matrix metalloproteinases and their inhibitors in rat bladder wall upon PBOO modeling and antimuscarinic agent treatment evaluated by rat genome-wide microarray analysis (A), RT-PCR (B) and immunohistochemistry (C). Tissue samples were stained with-MMP7 and -TIMP1 antibodies using the SABC method. Each slide was captured in 10 randomly chosen areas at 400× magnification and analyzed using Image-Pro Plus. The IHS was calculated and is shown in the histogram (D). Tolterodine can promote gradual decomposition of accumulated collagen in PBOO rat bladder by regulating the expression of MMPs and TIMPs. * $p < 0.05$ compared with the sham group. Scale bar is 100 μ m.

Fig. 4. Expression of cell adhesion molecules in rat bladder wall upon PBOO and antimuscarinic agent treatment evaluated by rat genome-wide microarray analysis (A), RT-PCR (B), and immunohistochemistry study (C). Tissue samples were stained with an anti-fibronectin antibody using the SABC method. The IHS was calculated and is shown in the histogram (D). * $p < 0.05$ compared with the sham group. Scale bar is 100 μm .

Fig. 5. Expression of ECM receptors in rat bladder wall upon PB00 and antimuscarinic agent treatment evaluated by rat genome-wide microarray analysis (A), RT-PCR (B), and immunohistochemistry study (C). Tissue samples were stained with anti-integrin $\alpha 4$ and -integrin $\beta 2$ antibodies using the SABC method. The IHS was calculated and is shown in the histogram (D). * $p < 0.05$ compared with the sham group. Scale bar is 100 μm .

group. Forty ECM non-collagen transcripts were screened by microarray, and 25 of these showed potential differences between any two groups ($p < 0.05$, fold change ≥ 2). Of these 25, 13 ECM transcripts were further confirmed by PCR ($p < 0.05$; Fig. 4A and B). PBOO mainly downregulated the expression of non-collagen ECM mRNAs (*Hapln1*, -9.00-fold, $p = 0.038$; *Lama1*, -2.60-fold, $p = 0.039$; *Postn*, -2.17-fold, $p = 0.002$; *Thbs2*, -2.11-fold, $p = 0.004$; *Tnc*, -2.60-fold, $p = 0.006$; *Vtn*, -8.30-fold, $p = 0.002$), and the antimuscarinic agent upregulated the expression of non-collagen ECM mRNAs (*Fn1*, 2.48-fold, $p < 0.001$; *Postn*, 2.39-fold, $p = 0.013$; *Spp1*, 7.67-fold, $p = 0.015$; *Vcan*, 4.04-fold, $p = 0.010$).

Tolterodine treatment promoted expression of ECM receptor genes. Thirty-nine transcripts with potential differences were screened from 87 ECM receptor gene transcripts by microarray; of these 11 ECM receptors were confirmed to have significant expression

changes after further PCR validation ($p < 0.05$; Fig. 5A and B). The results showed that the expression changes in ECM receptor genes in the bladder wall were not significant in the PBOO group; however, tolterodine treatment increased the expression of *Itgb4* 3.08-fold ($p = 0.034$), *Itgb2* 2.28-fold ($p = 0.032$), and *Sell* 7.32-fold ($p = 0.042$).

The increased expression and distribution of fibronectin (*Fn1*), integrin $\alpha 4$ (*Itga4*), and integrin $\beta 2$ (*Itgb2*) in tissue slices from each group was examined by immunohistochemistry. Fibronectin was mainly distributed in the ECM (Fig. 4C). Compared with the sham group, the fibronectin IHS in the PBOO group did not significantly change ($p = 0.165$); however, in the PBOO plus tolterodine group it was increased 1.88-fold ($p = 0.049$; Fig. 4D). integrin $\alpha 4$ and integrin $\beta 2$ were mainly distributed in the plasma membrane (Fig. 5C). Compared with the sham group, the integrin $\alpha 4$ ($p = 0.118$) and integrin $\beta 2$ ($p = 0.055$) IHS in the PBOO group showed no significant change. However, the integrin $\alpha 4$ IHS was increased in the PBOO plus tolterodine group 3.00-fold ($p = 0.003$) and integrin $\beta 2$ was increased 3.99-fold ($p = 0.001$; Fig. 5D). These results support the abovementioned changes in gene expression.

Discussion

The rat PBOO model is a common pathological model for research on bladder function. This animal model has a defined modeling scheme that can be easily implemented, with significant differences between the experimental group and the control group. The rat PBOO model was initially established by Mattiasson and colleagues [14]. After several attempts, the 1-mm-catheter-guided partial ligation of the female rat urethra, which does not totally close the urethra and improves bladder outlet resistance, was adopted. Shakirova and colleagues [15] have reported that shortly after establishing a PBOO model, the weight of the detrusor muscle may increase rapidly; the weight of the bladder increased from 80 mg to 480 mg after 6 weeks of obstruction. The increase in detrusor muscle weight is mainly due to smooth muscle cell hypertrophy in the bladder, while there is relatively less hyperplasia. The cross-sectional area of smooth muscle cells after 6 weeks of obstruction may increase 10-fold [16]. At this point, a urodynamic test may result in elevated maximal micturition pressure, increased residual urine, increased frequency of detrusor muscle contractility, and reduced bladder wall compliance [17]. Relieving the BOO state is the most effective means of curbing the development of this condition. However, if BOO persists for a long time, the resultant structural changes and bladder wall dysfunction may not be completely alleviated [10, 11].

Bladder wall dysfunction is associated with metabolic disorders of bladder wall ECM [18]. BOO causes strain injury to the bladder wall, leading to rupture of collagen fibers, which gradually break down into short-chain collagens that are deposited in the intercellular space [19]. Because of the sustained BOO, the newly synthesized collagen fibers will gradually decompose into short-chain collagens, leading to gradual aggravation of collagen deposition; short-chain collagens lack good elasticity, and along with the increase in collagen deposition, the ECM gradually loses its elasticity, resulting in decreased bladder wall constriction and compliance [6, 7, 19]. Several clinical and pathological studies have pointed out that the ECM/cell ratio is an important indicator of bladder function [18, 20, 21]. The higher the ratio, the poorer the bladder compliance and the more severe the patient's LUTS. Therefore, in addition to the early relief of the BOO state, identifying a therapeutic strategy for promoting collagen decomposition will help relieve the strain injury to the bladder wall caused by BOO.

Acetylcholine muscarinic M_2 and M_3 receptors are the main cause of bladder detrusor muscle contraction; thus, blocking the effect of acetylcholine on muscarinic receptors can relax the detrusor muscle. Conventionally, highly selective M_2 and M_3 receptor antagonists have been used to treat overactive bladder and detrusor hyperreflexia. Commonly used drugs include the M_2 and M_3 receptor antagonist tolterodine and the M_3 receptor antagonists Solifenacin and Darifenacin, all of which are competitive antagonists [3, 4]. An antimuscarinic agent can also be used for alleviating LUTS secondary to BOO, because BOO can also cause detrusor overactivity. In fact, the use of an antimuscarinic agent for treating detrusor

overactivity associated with benign prostatic hyperplasia had a better curative effect than expected. Most patients may develop a new balance between urine storage and voiding after drug administration, and even maintain a good quality of life after drug discontinuation [22, 23]. This suggests that antimuscarinic agents may have more profound effects on the bladder wall. However, a review of the literature revealed that this research direction has not attracted much attention.

The results of the present study preliminarily confirmed the curative effect of antimuscarinic agents on the pathological status of the rat bladder wall ECM in the PBOO model. According to the rat whole-genome microarray and PCR verification analyses, deposition and decomposition of collagen in the bladder wall are not associated with collagen synthesis. By upregulating the expression of MMP to promote the gradual decomposition of deposited collagen in the rat bladder wall in the PBOO model, an antimuscarinic agent is an effective means for changing the bladder wall ECM/cell ratio. This result associates muscarinic receptors with the catabolism of bladder wall ECM. Inhibiting muscarinic receptor activity is no longer a therapeutic strategy for just alleviating symptoms. It can also slow down and even impede damage to the bladder wall ECM caused by BOO, consistent with its clinical efficacy.

We may regard the rat PBOO model as an effective scheme for applying biomechanical force on the bladder wall *in vivo*. In experiments incorporating mechanical strain on bladder smooth muscle cells *in vitro*, we tried to identify the most favorable method and parameters of strain loading for cell proliferation [24–26]. The *in vitro* experimental model is unique, with vast differences in the mechanical environment compared with the *in vivo* condition [27], so it is impossible to describe strain-loading parameters *in vitro* in simple physiological or pathological terms. The rat PBOO model is closer to the real mechanical environment of the bladder wall. Specifically, the sham group can be regarded as a physiological biomechanics model, the PBOO group can be regarded as a pathological biomechanics model, and the PBOO plus tolterodine group can be regarded as a pathological biomechanics model with drug treatment to slow down ECM lesions.

The expression of cell adhesion molecules and matrix receptor proteins under rat bladder wall strain and medication mode showed the following: (1) *Vtn* was downregulated upon PBOO pathological strain, and it was still downregulated after treatment with tolterodine, suggesting that there is no definite relationship between *Vtn* and rat bladder wall function. *Fn1* showed no obvious change upon pathological strain, but its expression was significantly increased after treatment with tolterodine. Furthermore, immunohistochemical staining showed that fibronectin expression increased after treatment with tolterodine, suggesting that fibronectin expression is closely associated with bladder ECM function. (2) Most of the ECM receptor proteins showed no obvious changes upon PBOO pathological strain, but the expression of some matrix receptors (*Cd44*, *Icam1*, *Sell*, *Itga4*, *Itga1* and *Itgb2*) remarkably increased following tolterodine treatment. Immunohistochemical staining showed that the expression of integrin subtypes $\alpha 4$ and $\beta 2$ also increased, suggesting that the expression of matrix receptor proteins is associated with bladder ECM function. The above results indicate that the increased expression of cell adhesion molecules and matrix receptor proteins has important implications in the effect of tolterodine on the bladder wall ECM in ameliorating PBOO.

Conclusion

Antimuscarinic agents can decrease the volume of collagen in the PBOO rat bladder. They are also effective for reducing the ECM/cell ratio under such pathological conditions. Because tolterodine both increased the expression of MMPs and promoted degradation of collagen fibers, we considered that the volume of collagen was decreased via MMPs. Concurrently, antimuscarinic agents can regulate the expression of ECM proteins and receptors. Such a relationship between muscarinic receptors and bladder wall ECM is likely to be associated with the functional status of the bladder wall.

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Disclosure Statement

There are no competing financial interests to declare.

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