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Original Article

Adenosine signaling pathway regulated bladder fibrosis caused by outlet obstruction in rats

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Abstract: This study is to investigate the role of adenosine signaling pathway in bladder fibrosis caused by outlet obstruction. A bladder fibrosis model was established in female Wistar rats. Immunohistochemistry and real-time fluorescent quantitative polymerase chain reaction (RT-PCR) were used to measure the changes of fibrosis-related gene expression in bladder tissues, including collagen-I, collagen-III, transforming growth factor-β1 (TGF-β1), and ecto-5'-nucleotidase (CD73) genes. Adenosine receptor blocker theophylline was injected intraperitoneally to confirm the potential role of adenosine in the pathogenesis of bladder fibrosis. A surgery sham and a normal control groups were also included in the study. Compared with the normal control and sham groups, the expression levels of collagen-I, collagen-III, and TGF-β1 genes in the bladder outlet obstruction (BOO) group were significantly increased, while CD73 gene expression was significantly decreased ($P < 0.05$). After injection of theophylline, the expressions of collagen-I, collagen-III, and TGF-β1 genes were significantly decreased, while CD73 gene was significantly upregulated ($P < 0.05$). In conclusions; Adenosine signaling pathway plays an important role in the pathogenesis of bladder fibrosis caused by BOO. These findings suggest a potential intervention for bladder fibrosis in patients with a similar condition, such as benign prostatic hyperplasia.

Keywords: Bladder fibrosis, bladder outlet obstruction, adenosine, adenosine receptor

Introduction

Adenosine, the main metabolite of adenosine triphosphate (ATP) and adenosine monophosphate (AMP), is a nucleoside widely distributed in human tissues and cells [1]. It is normally generated by hydrolysis of s-adenosyl-l-homocysteine (SAH). However, adenosine is mainly produced by CD73 catalyzing AMP under ischemic and hypoxic conditions [2]. CD73 is an ectonucleotidase, a type of glycoprotein formed from glycosyl-phosphatidylinositol, which is stably and ubiquitously expressed and has the activity of 5'-nucleotide hydrolase.

Adenosine protects and repairs tissues upon its binding to different adenosine receptors (ARs) by increasing oxygen supply, enhancing anti-inflammatory effects, and stimulating angiogenesis [3]. The physiological concentration of adenosine is very low in tissues and cells; however, its concentration is dramatically elevated under stress, such as inflammation, ischemia, trauma, and anoxia. High level of adenosine was found to activate fibrosis pathways through ARs, resulting in liver and lung tissues fibrosis [4-6]. Our previous studies have also shown that adenosine can promote fibrosis in the penis and the kidneys after chronic anoxic injuries [7, 8]. These findings indicate that adenosine plays an important role in tissue fibrosis.

Prior studies have shown that transforming growth factor-β1 (TGF-β1), as a downstream effector of adenosine, may be involved in tissue fibrosis. Mice with adenosine deaminase deficiency displayed high concentrations of adenosine in the penis, which further increased TGF-β1 expression and caused penile fibrosis

[7]. Elevated expression of TGF-β1 was also observed under ischemic and anoxic conditions in bladder tissues of rabbits [9]. Bladder fibrosis is a common histopathological lesion caused by increased bladder pressure in conditions such as bladder outlet obstruction [10]. However, the underlying mechanism of bladder fibrosis has not been elucidated. Previous studies have shown that high bladder pressure resulted in ischemia and anoxia in the bladder [11, 12], accompanied by elevated ATP and adenosine levels in the surrounding tissues [13-15]. These observations indicated that adenosine is associated with bladder tissue fibrosis caused by increased pressure inside of the bladder. However, the role of adenosine signaling in the pathogenesis of bladder fibrosis has not been fully understood.

The present study used an animal model to investigate the relationship between adenosine and bladder fibrosis caused by bladder outlet obstruction. We found that adenosine pathway plays an important role in the pathogenesis of bladder fibrosis.

Materials and methods

Animal model

Six-week-old female Wistar rats (150-180 g) were purchased from the Laboratory of Animal Resources at Xiangya School of Medicine of Central South University. The animals were housed at the Animal Experimental Center of the Third Xiangya Hospital of Central South University. The animal experiments were approved by the institute Ethics Committee.

After one week of acclimatization, the animals were randomly divided into 4 groups with 6 rats in each group, including a normal control group, sham surgical (sham) group, bladder outlet obstruction (BOO) group, and a Theophylline (Tocris, UK) intervention group (BOO + Theo).

Male rats can reflux urine into the seminar vesicles *via* the vas deferens, resulting in a pressure decrease inside of the bladder. Therefore, female rats were chosen for the present study. The BOO animal model was established using the proximal urethral ligature method [16]. Briefly, after anesthesia with 10% chloral hydrate, a midline incision was made at the lower abdomen to fully expose the bladder and the proximal urethra. The proximal urethra was carefully separated from the surround tissues. Then a polyethylene catheter with a diameter of 1.0 mm was inserted into the bladder cavity through the external orifice of urethra. The proximal urethra along with the catheter was tightened with a 3-0 silk thread, and then the catheter was removed from the urethra, resulting in a proximal urethra 1.0 mm in diameter. After surgery, the rats were treated with gentamycin sulfate by intramuscular injection for 3 days. For the sham-treated rats, the proximal urethra was freed, but not ligated. Theophylline (10 mg/kg) was administered to rats of the BOO + Theo group *via* intraperitoneal injection 2 times per day for 6 weeks after ligation of the proximal urethra. The control group did not have surgery.

Urodynamic measurement

The rats were anesthetized with 10% chloral hydrate intraperitoneally at 6 weeks after surgery. The bladder was fully exposed through a midline pelvic incision. A small hole was made on the top of the bladder. A polyethylene catheter with a diameter of 1.0 mm was then inserted into the bladder cavity. The catheter was fixed in position with a suture. After closing the pelvic wall incision, the catheter was connected to the urodynamic instrument (Andromeda, Germany) and a microperfusion pump *via* a three-way connector. The abdomen was gently pressed to empty the bladder. The abdominal pressure was maintained at 0 during urodynamic measurement, which was monitored by inserting a piezometric tube into the rectum. A 0.9% saline solution was then infused into the bladder through the catheter with a velocity of 1 mL/min until the rat began to urinate. The change of bladder pressure was then recorded.

Histology and Sirius red staining of the bladder

After measuring urodynamic change, the rats were euthanized by cervical dislocation, and the bladders were collected. The bladder tissues were then cut into parts for different purposes as described below:

For histological examination, the bladder tissues were processed, according to standard histological methods. The tissue slides were then stained with hematoxylin and eosin (H&E),

or Sirius red F3B, and used for immunohistochemical assay. Upon Sirius red F3B staining (Sigma, USA), collagen-I protein showed strong birefringence (either yellow or red), while collagen-III protein exhibited weak birefringence (green) under the microscope.

Immunohistochemical staining was conducted, according to the instructions provided with the Avidin/Biotin Complex (ABC) Kit (Vector Elite, USA). The primary antibodies, including collagen-I, collagen-III (Abcam, UK), and TGF-β1 (Boster, China) were used for staining. After staining, the tissue sections were viewed under a microscope. The images were semi-quantitatively analyzed with Image Pro Plus 6.0 image analysis software (Cybernetics, USA). Brown particles in the extracellular matrix were considered to be positive staining. The optical density and positive staining area were calculated to obtain the mean optical density (MOD). The average of MODs from 5 different microscopic fields represented the MOD for each individual tissue section.

Real-time quantitative polymerase chain reaction (RT-PCR)

Total RNA from a portion of bladder tissues was extracted using TRIZOL® Reagent kit, according to the manufacture's protocol (Invitrogen, USA). The SYBR Green I RT fluorescent quantitative PCR kit (Applied Biosystems, USA) was used to detect cDNA. RT-PCR was carried out as instructed by the company (Fermentas, Lithuania). Actin was used as the reference gene. The actin primers were 5'-CATCCTGCGTCTGGACCTGG-3' (upstream) and 5'-TAATGTCACGCACGATTTCC-3' (downstream). The amplified product was 147 bp. The TGF-β1 primers were 5'-TGAGTGGCTGTCTTTTGACG-3' (upstream) and 5'-TTGGGACTGATCCCATTGAT-3' (downstream). The amplified product was 168 bp. The CD73 primers were 5'-CCTGGGAGAACCTGGCTGCTGT-3' (upstream) and 5'-AGGCTCATAGATGGGCACTCGA-3' (downstream). The amplified product was 239 bp. The collagen-I primers were 5'-CGAGTATGGAAGCGAAGGT-3' (upstream) and 5'-CCACAAGCGTGCTGTAGGT-3' (downstream). The amplified product was 230 bp. The collagen-III primers were 5'-CCTCCCAGAACATTACATACCAC-3' (upstream) and 5'-ACTGTCTTGCTCCATTCACCAG-3' (downstream), and the amplified product was 193 bp. The reaction conditions for TGF-β1 were as follows: 95°C for 7 min for one cycle, then 95°C for 5 s and followed by 60°C for 30 s for 40 cycles. The reaction conditions for the other primers were 97°C for 7 min for one cycle, and then 95°C for 5 s and followed by 58°C for 30 s for 40 cycles. The relative expression levels of the target genes were determined, according to the threshold method with the derivation formula of $2^{-\Delta\Delta Ct}$ [17].

Statistical analyses

Statistical Package Prism 5 for Windows (version 5.01) software was used for data analysis. The data were presented as $\bar{x} \pm s$. All data were analyzed by one-way ANOVA. Comparisons among groups were carried out by Newman-Keuls method. $P < 0.05$ was considered significantly different.

Results

Irregular bladder contraction was observed in rats with bladder outlet obstruction

Female rats in the BOO and BOO + Theo groups displayed irregular bladder contractions during the bladder infusion process (**Figure 1C** and **1D**), in which the cystometrograms showed fluctuating lines. However, rats treated with Theophylline had mild irregular bladder contraction compared with the Boo group. Normal bladder contractions were observed in in the normal control and Sham groups (**Figure 1A** and **1B**).

Bladder outlet obstruction caused bladder fibrosis in female rats

Rats in the BOO and BOO + Theo groups showed much thinner bladder walls, muscle fiber hypertrophy in the bladder walls, and irregular arrangement compared with the control and sham groups (**Figure 2**). Sirius red staining showed increased expression of collagen fibers (mostly collagen-III, green) under the mucous in the BOO and BOO + Theo groups, demonstrating a successful bladder fibrosis model.

Immunohistochemistry of fibrosis related protein in bladder tissues

TGF-β1, collagen-I, and collagen-III protein levels were significantly increased in rats of the BOO and BOO + Theo groups compared to

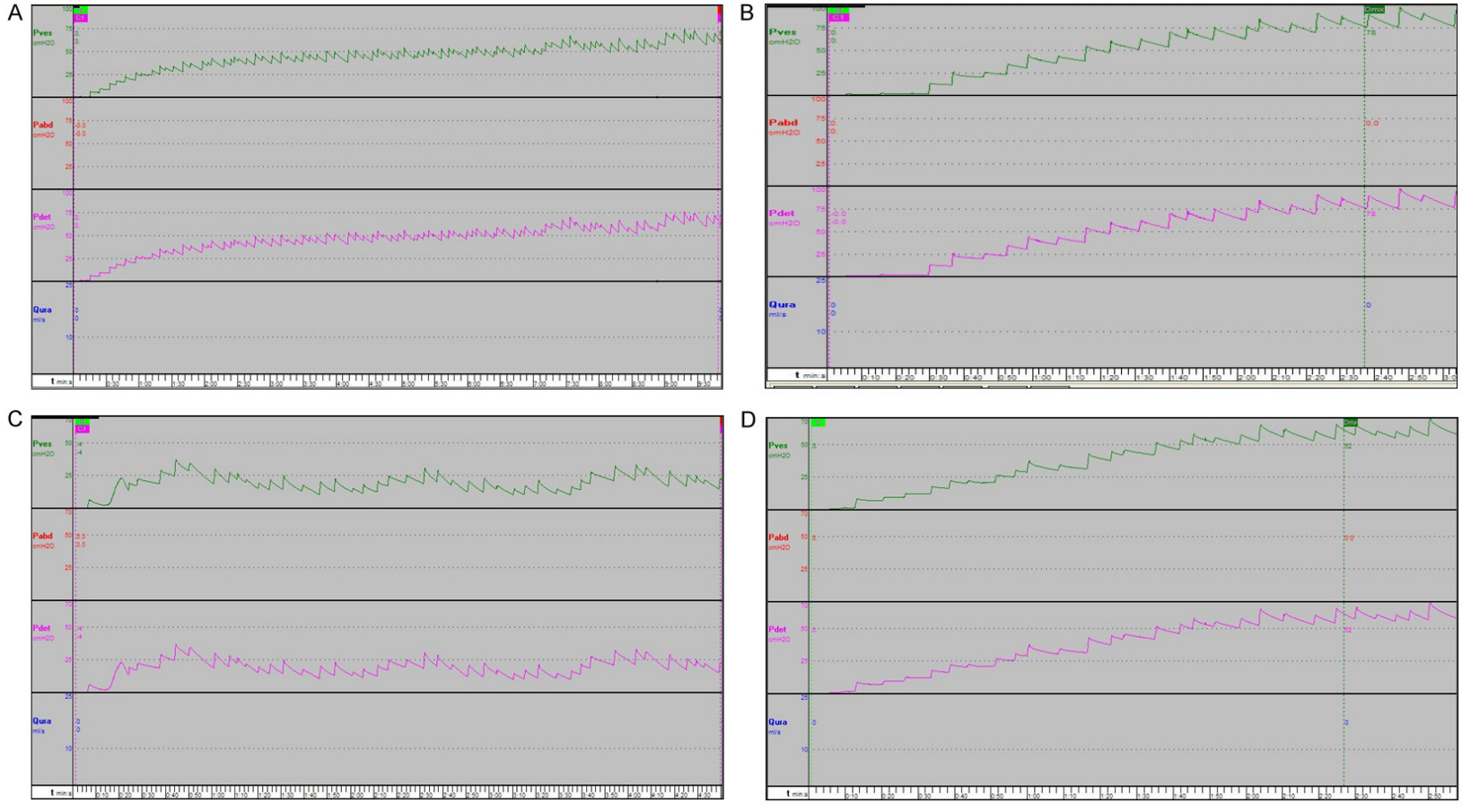

Figure 1. Cystometrogram in rats during bladder infusion with saline solution. A. The normal control rat; B. The sham rat; C. The BOO group; D. The BOO + Theo group.

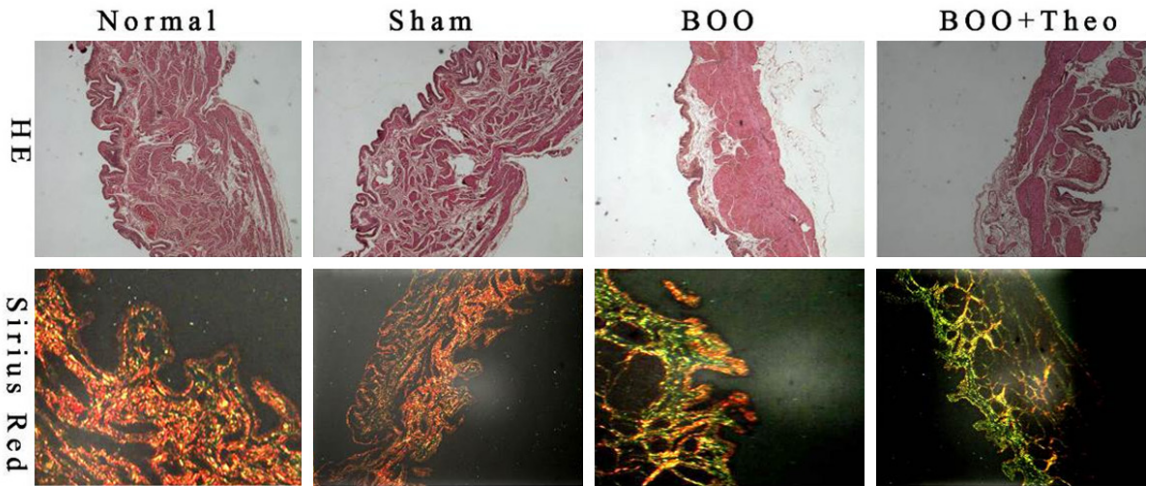

Figure 2. Histological examination of bladder tissues from rats in different groups (20×).

those in the control and sham groups ($P < 0.05$, **Figure 3**). There was no significant difference in these protein levels between the control and sham groups ($P > 0.05$). However, the MODs for TGF-β1, collagen-I, and collagen-III staining in the BOO + Theo group were significantly lower than in the BOO group ($P < 0.05$, **Figure 3**).

Gene expression levels of fibrosis-related genes in bladder tissues

The gene expression levels of TGF-β1, collagen-I, and collagen-III mRNA in the bladder tissues were significantly higher in the BOO and BOO + Theo groups than in in the normal control and sham groups as shown in **Figure 4** ($P < 0.05$). Compared with the BOO group, TGF-β1, collagen-I, and collagen-III gene expression levels in the bladder tissues were significantly decreased in the BOO + Theo group.

CD73 mRNA expression level was significantly lower in the BOO and BOO + Theo groups compared to the control and sham groups as shown in **Figure 4** ($P < 0.05$). Rats in the BOO + Theo groups showed significantly higher CD73 mRNA expression level compared to the BOO group, as shown in **Figure 4** ($P < 0.05$).

Discussion

We successfully established a bladder fibrosis rat model in the present study. Using this animal model, we found that genes related to tissue fibrosis (TGF-β1, collagen-I, and collagen-II) were highly expressed in the bladder tissues. The levels of these gene expressions were significantly changed when adenosine receptor (AR) was blocked with Theophylline. These findings suggest that adenosine plays an important role in the pathogenesis of bladder fibrosis due to bladder outlet obstruction.

A significant increase in the expression of TGF-β1 in bladder tissues was shown to promote the production of collagen and to accelerate bladder fibrosis [18-20]. TGF-β1 was found to induce tissue fibrosis by promoting the differentiation and growth of fibroblasts [21]. It also induces the proliferation of scar fibroblasts by increasing synthesis of collagen, which can be prevented by TGF-β1 inhibitors [22]. Increased collagen expression (primarily collagen-I and collagen-III) in rat bladder tissue caused bladder fibrosis due to urethral constriction [9, 23, 24]. TGF-β1 was also found to accelerate fibrosis in myocardial cells by increasing the level of collagen in neonatal Sprague Dawley rats [25]. Our previous study has found that high levels of adenosine can increase TGF-β1 levels in penile tissues of mice deficient with ADA gene. Adenosine analogue, 5'-N-ethylcarboxamidoadenosine (NECA), can markedly increase the level of TGF-β1 expression in corpus cavernosal fibroblast cells. After addition of TGF-β1 antibodies, the corpus cavernosal fibroblast cells were no longer able to produce collagen [7], indicating that TGF-β1 can regulate synthesis of collagen. Therefore, the molecular mechanism of our bladder fibrosis animal model could relate to the upregulated expression levels of TGF-β1, collagen-I and collagen-III in bladder tissues.

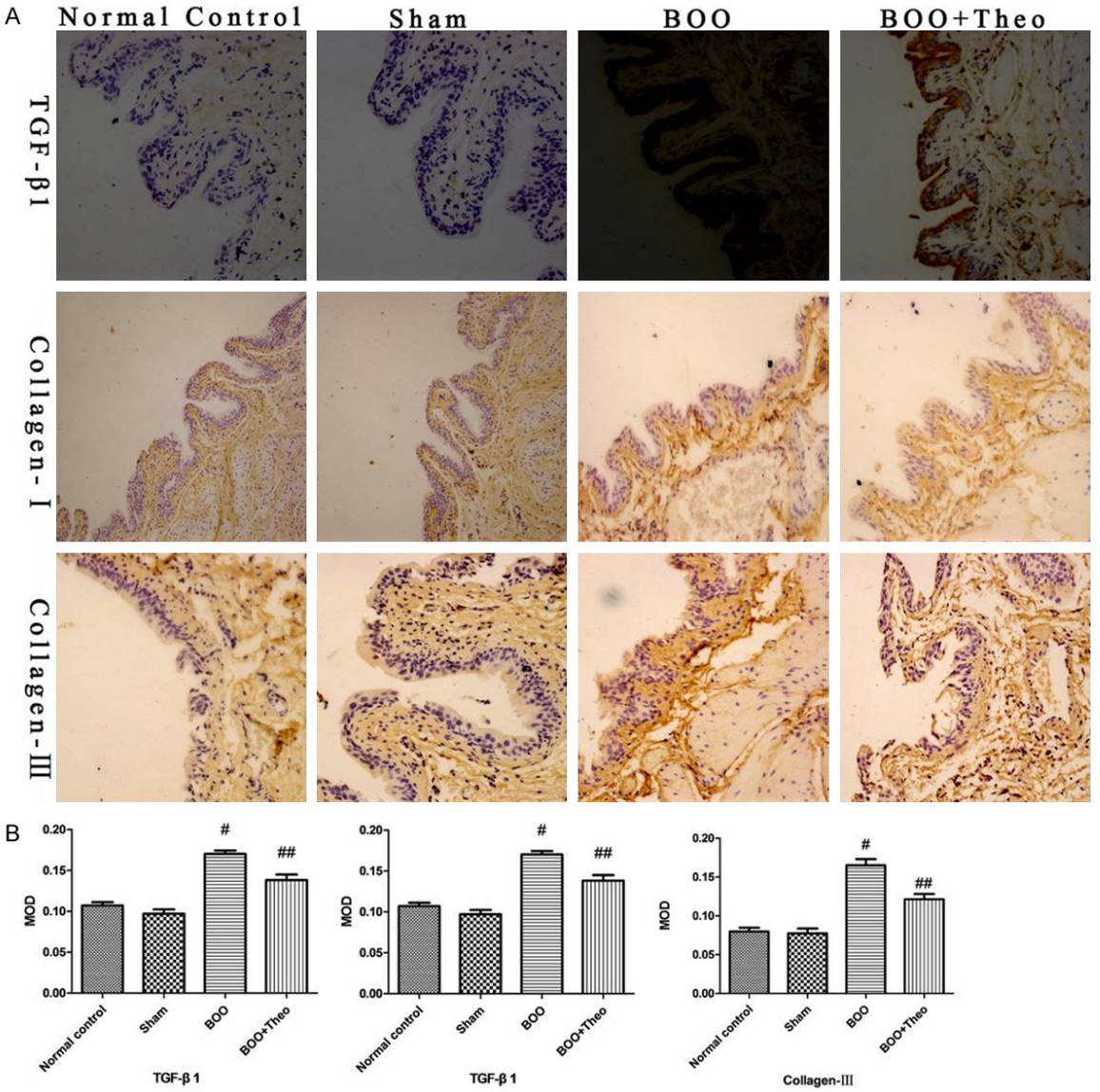

Figure 3. A. Immunohistochemical images of TGF-β, Collagen-I, and Collagen-III in the bladder tissues from different groups. B. Semi-quantitation of TGF-β, Collagen-I, and Collagen-III protein expression in the bladder tissues. Data are expressed as means ± SEM (n = 6). #, $P < 0.05$ compared to the normal control group; ##, $P < 0.05$ compared to the BOO group.

Chronic bladder outlet obstruction can increase pressure within the bladder cavity, resulting in hypoxia or ischemia in the bladder wall [11, 12]. Hypoxia can increase production of adenosine, which further inhibits inflammatory responses, alleviates the calcium load, minimizes the effect of catecholamines, regulates energy metabolism, reduces the oxygen consumption of cells, and as a result protects tissues and organs [26-28]. However, chronic accumulation of adenosine could result in overexpression of the AR and/or down-regulation of AR sensitivity, consequently causing pulmonary injury and fibrosis [6]. Accumulation of adenosine due to chronic anoxia is considered to be harmful to tissues [29]. Therefore, it is possible that ischemia and anoxia in our animal model may contribute to the pathogenesis of bladder fibrosis.

CD73 increases the level of cAMP in the cytoplasm under hypoxic condition, which in turn increases the expression of CD73 through positive feedback. Adenosine can also regulate the transcription and expression of CD73 through a feedback mechanism [30]. However, the pres-

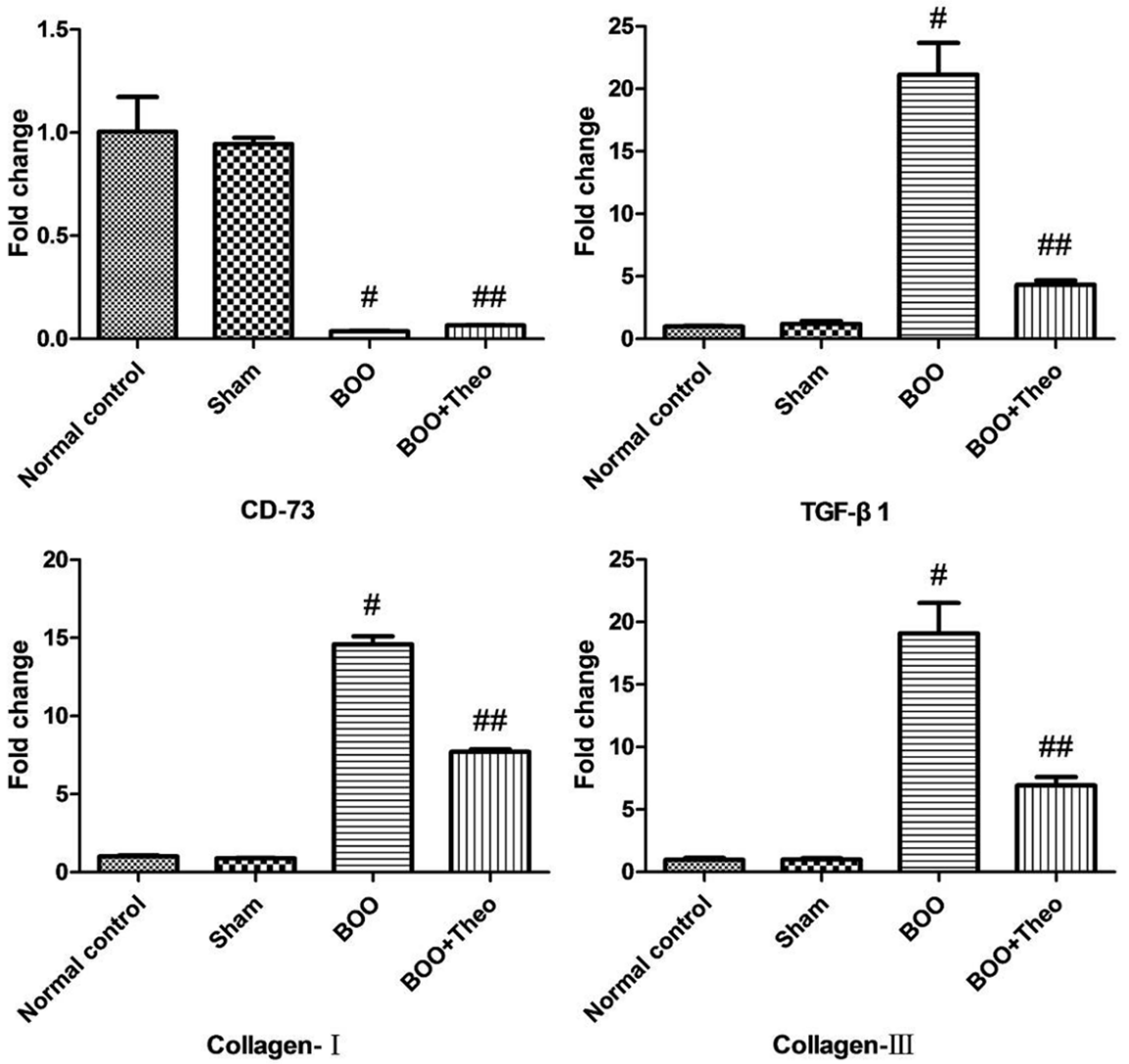

Figure 4. TGF-β1, CD73, collagen-I, and collagen-III mRNA expression levels in bladder tissues in different groups. Data are expressed as mean ± SEM (n = 6). #, P < 0.05 compared to the normal control rats; ##, P < 0.05 compared to rats in the BOO group.

ent study showed a decreased level of CD73 mRNA in the bladder tissues in the BOO and Theo-treated rats. A similar finding was also observed in cerebral microvascular endothelial cells in hypoxic mice [31]. In this study, CD73 mRNA level was significantly increased during the first 4-8 h of hypoxia, but decreased after 16 h or longer of hypoxia. CD73 mRNA level in mouse cerebral microvascular endothelial cells returned to normal levels at 1 h and significantly increased at 8 h after restoration of oxygen [31]. These findings suggest that change in the CD73 levels induced by hypoxia is time-dependent, i.e., short-term hypoxia promotes, but chronic hypoxia inhibits CD73 expression. In the current study, bladder tissues under a hypoxic condition for a long time (6 weeks), resulting in decreased CD73 levels. However, we cannot exclude the possibility of negative feedback between adenosine and CD73. Under long-term increased pressure and hypoxic conditions, the bladder tissues were constantly stimulated by adenosine, which could inhibit CD73 mRNA expression. This negative feedback was blocked by an AR inhibitor, as a result that CD73 mRNA expression was up-regulated in bladder tissue.

In summary, the present study found that the adenosine signaling pathway is involved into the pathogenesis of bladder fibrosis caused by outlet obstruction in a rat model, and the effect

of adenosine on tissue fibrosis can be alleviated by blocking the AR.

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Disclosure of conflict of interest

None.

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