

## The Effect of Tadalafil on Renal Fibrosis Induced by Ureteral Obstruction

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### ABSTRACT

**Objective:** It has been reported that phosphodiesterase-5 (PDE-5) inhibitors improve kidney function during acute and chronic renal failure. This study aimed to determine the possible therapeutic effects of tadalafil, a specific PDE-5 inhibitor, on renal fibrosis induced by unilateral ureteral obstruction (UUO).

**Methods:** Male Sprague-Dawley rats were used and randomly divided into three groups (n = 6) as sham-operated, UUO and tadalafil-treated (10 mg/72 hours, ig) UUO (UUO+T) groups. Unilateral ureteral obstruction was induced by complete ligation of the left ureter and 14 days after surgery creatinine clearance, urinary cyclic guanosine monophosphate (cGMP), renal alpha-smooth muscle actin ( $\alpha$ -sma) and transforming growth factor  $\beta$  (TGF- $\beta$ ) levels, as well as histologic changes, were observed in all the animals.

**Results:** Unilateral ureteral obstruction-induced renal fibrosis was confirmed by increased  $\alpha$ -sma level, collagen deposition, tubular dilation, inflammatory cell infiltration and necrosis. An increased renal TGF- $\beta$  level and decreased urinary cGMP level was also observed in obstructed animals in addition to reduced creatinine clearance. Tadalafil treatment, which restored the animals' urinary cGMP level, significantly attenuated the fibrotic changes and TGF- $\beta$  increase in their kidneys.

**Conclusion:** This study suggests that tadalafil treatment ameliorates renal fibrosis by reducing TGF- $\beta$  expression and may have important clinical relevance since tadalafil is currently used clinically to treat erectile dysfunction and pulmonary hypertension.

**Keywords:**  $\alpha$ -sma, cGMP, PDE-5, renal fibrosis, Tadalafil, TGF- $\beta$ , ureter obstruction

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### RESUMEN

**Objetivo:** Se ha reportado que los inhibidores de la fosfodiesterasa-5 (PDE-5) mejoran las funciones renales durante la insuficiencia renal aguda y crónica. Este estudio tuvo por objetivo determinar los posibles efectos terapéuticos del tadalafil – un inhibidor específico de la PDE-5 – sobre la fibrosis renal inducida por una obstrucción ureteral unilateral (OUU).

**Métodos:** Se utilizaron ratas machos Sprague-Dawley, divididas de manera aleatoria en tres grupos (n = 6): operación simulada, OUU y tratamiento con tadalafil (10 mg/72 horas, IG), y OUU (OUU+T). La obstrucción uretral unilateral fue inducida por una ligadura completa del uréter izquierdo y 14 días después de la cirugía, se observaron niveles de monofosfato

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de guanosina cíclico (GMP) urinario, alfa-actina de músculo liso ( $\alpha$ -SMA), y factor de crecimiento transformante  $\beta$  (FCT- $\beta$ ), así como cambios histológicos en todos los animales.

**Resultados:** La fibrosis renal inducida por obstrucción uretral unilateral fue confirmada por un aumento del nivel de  $\alpha$ -SMA, deposición de colágeno, dilatación tubular, infiltración de células inflamatorias y necrosis. También se observó un aumento del nivel de FCT- $\beta$  renal y una disminución del nivel de GMP urinario en los animales con obstrucción, además de una reducción del aclaramiento de la creatinina. El tratamiento con tadalafil, que restauró el nivel de GMP urinario de los animales, atenuó significativamente los cambios fibróticos y el aumento de FCT- $\beta$  en los riñones.

**Conclusión:** Este estudio sugiere que el tratamiento con tadalafil mejora la fibrosis renal al reducir la expresión de FCT- $\beta$  y puede tener una importante relevancia clínica por cuanto el tadalafil se usa hoy día clínicamente para tratar la disfunción eréctil y la hipertensión pulmonar.

**Palabras clave:**  $\alpha$ -SMA, GMP, PDE-5, fibrosis renal, tadalafil, FCT- $\beta$ , obstrucción uretral

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## INTRODUCTION

Renal fibrosis is considered a common outcome of progressive kidney disease, regardless of the primary cause. It is characterized by extracellular matrix (ECM) accumulation, glomerulosclerosis, tubular atrophy, cell death and capillary loss, as well as inflammatory cell infiltration (1–3). Unilateral ureteral obstruction (UO) induced-renal-changes include both functional and structural deterioration and are well characterized by renal fibrosis (1–4). In fact, UO is a highly preferred and well established animal model for renal fibrosis. In clinical practice, urinary tract obstruction is a common and serious problem induced by different conditions including congenital abnormalities, obstructive urolithiasis or age-related changes (2, 4–6).

Cyclic guanosine monophosphate (cGMP) is an important mediator of numerous renal functions, including tubular transport and renal hemodynamics (7–11). It is produced by both receptor-associated guanylyl cyclase which is activated by natriuretic peptides and cytosolic guanylyl cyclase (cGC) which is activated by nitric oxide (NO) or carbon monoxide (10–12). Accumulating evidence suggests that any treatment to increase the cGMP levels may have an anti-fibrotic effect in the kidney. For example, Sun *et al* (2012) showed that treatment with L-arginine, the substrate for nitric oxide synthase enzyme, improved renal fibrosis in rats with obstructive nephropathy; however, the non-specific inhibition of nitric oxide synthase enzymes with L-NAME exacerbates fibrosis (13). Another study on experimental glomerulonephritis, revealed that the activation of cGC decreased the mesangial cell proliferation and matrix accumulation (14).

Cytosolic cGMP level is largely regulated by catabolic processes directed by phosphodiesterases (PDEs). Phosphodiesterase-5 (PDE-5) is a ubiquitously expressed PDE isozyme that specifically degrades cytoplasmic cGMP (15, 16). In clinical practice, PDE-5 inhibitor drugs, which increase cellular cGMP level, are used to treat male erectile dysfunction and pulmonary hypertension (15–19). It has been noted that PDE-5 inhibitors may also have beneficial effects on various forms of kidney injuries associated with acute and chronic renal failure (20–26). In terms of renal fibrosis, the first marketed PDE-5 inhibitor sildenafil had anti-proliferative and regulatory effects on the extracellular matrix in rats with diabetic nephropathy or ureteral obstruction (20, 27).

Sildenafil, tadalafil and vardenafil are PDE-5 inhibitors currently used in clinics and all inhibit PDE-5 with different pharmacokinetic properties (15, 16). It had been reported that among the PDE-5 inhibitors, tadalafil had the highest selectivity and a longer half-life (15). Therefore, this study was designed to determine whether tadalafil had therapeutic potential for UO-induced renal fibrosis in rats.

## SUBJECTS AND METHODS

**Animals and surgical procedures:** Male Sprague-Dawley rats weighing 250–300 g were divided into three groups (n = 6) as sham-operated, unilateral ureteral obstruction (UO) and UO with tadalafil treatment (UO+T; Cialis, 10 mg/ 72 hours, intragastric; Lilly, Indianapolis, Indiana, USA). All the experimental steps, animal handling, sampling and scarification were approved by the

Ethical Committee of Karadeniz Technical University (2003/07-03), Turkey.

The complete obstruction of the left ureter was induced in the UUO and UUO+T groups as described previously (13). Briefly, under general anaesthesia (ketamine, 50 mg/kg, im), laparotomy was performed and the left kidney and ureter were exposed. The left ureter was ligated with 4-0 silk at two points and cut between the ligatures. After ureter ligation, the surgical incision was closed. In the sham-operated group the left ureter was only manipulated. Following the surgery, the animals were put into individual cages and given free access to the normal rat feed and water. Fourteen days after the surgery, animals were placed in metabolic cages for 24 hours to collect their urine samples. On day 15, all the rats were anaesthetized (ketamine, 50 mg/kg, im) and blood samples were taken from their abdominal aorta for biochemical examination. Their obstructed kidneys were then removed and divided; some portions were stored at -80 °C for tissue analysis, while the remainder was fixed in 4% paraformaldehyde for histologic examinations.

#### Biochemical examinations

To study the kidney functions, the serum and urine concentrations of creatinine were determined using an enzymatic method on auto-analyser (Abbott Laboratory, Architect C8000) according to the manufacturer's procedures and creatinine clearance was calculated as an index of glomerular filtration rate (GFR).

The urinary cGMP concentrations in the rats' samples were analysed by a cGMP Enzyme Immunoassay Kit (Cayman Chemical Company, 581021) according to the manufacturer's instructions. The tissue analysis of  $\alpha$ -sma and TGF- $\beta$  were performed in the same kidney tissue homogenates. Before homogenization, the tissues were weighed and rinsed in ice-cold phosphate buffered saline and then homogenized (Witeg Labortechnik GmGH, WiseTis HG-15D Homogenizer). After two freeze-thaw cycles were performed, the homogenates were centrifuged for five minutes at 5000 g (2–8 °C). In the supernatants  $\alpha$ -sma and TGF- $\beta$  levels were assayed using appropriate ELISA kits (Cusabio CSB-E14027r and Boster EK0514, respectively). The analyses were performed according to the product instructions and read by an absorbance microplate reader (BioTek, EL x 800). The concentration of the total proteins in the homogenates was determined by the Bradford method using Coomassie reagent (Thermo scientific; 23200) and bovine serum albumin as the standard.

#### Light microscopy

The kidney samples were fixed in 4% paraformaldehyde, embedded in paraffin and cut into six  $\mu$ m-thick sections, which were stained with haematoxylin-eosin and Masson's trichrome stains to determine their general histology and interstitial collagen deposition. The extent of tubulointerstitial injury was evaluated by counting the percentage of the areas with tubular dilatation, atrophy, lymphocyte infiltration and necrosis per sections of kidney. For the assessment of renal fibrosis, Masson's trichrome staining was carried out and the proportion of the blue-stained fibrotic area of each section was graded semiquantitatively. Scores from 0 to 3 were used: 0, normal interstitium; 1, < 10% of areas injured; 2, 10–50% of areas injured; and 3, > 50% of areas injured. All the histological analyses were done by a pathologist in a blinded manner, and the mean values were calculated.

#### Statistical analysis

All the values were presented as mean  $\pm$  SEM. Statistical comparisons were performed using a one-way ANOVA, with Newman Keuls as a *post-hoc* test. A value of  $p < 0.05$  was considered statistically significant.

#### RESULTS

Fourteen days after the surgery, neither ureteral obstruction nor tadalafil treatment affected the rats' bodyweight, food and water consumption, or urinary volume. However, the obstructed kidney was larger than the contralateral kidney and clearly had hydronephrotic appearance in both the UUO and UUO+T groups.

Tadalafil treatment: Effectiveness of the tadalafil treatment was evaluated with the rats' urine cGMP levels. The rats' ureteral obstruction significantly reduced their urinary cGMP levels in non-treated UUO rats compared to with the sham-operated control group animals (from  $0.224 \pm 0.003$  nmol/mL to  $0.141 \pm 0.034$  nmol/mL,  $p < 0.05$ ). The 14-day tadalafil treatment, significantly restored their urinary cGMP levels to the range of controls ( $0.203 \pm 0.006$  nmol/mL).

Glomerular filtration rate (GFR): When compared with the sham-operated group, reduced renal functions were confirmed by both increased serum creatinine level (from  $0.29 \pm 0.01$  to  $0.47 \pm 0.07$  mg/dL,  $p < 0.001$ ) and decreased GFR (from  $2.42 \pm 0.26$  to  $1.45 \pm 0.11$  mL/minutes,  $p < 0.001$ ) in the obstructed animals. Though there was a slight improvement in their renal functions, the tadalafil treatment did not cause a significant change

in the rats' serum creatinine level ( $0.40 \pm 0.02$  mg/dL) or GFR ( $1.73 \pm 0.15$  mL/min) in the UUO+T group.

Histologic examination: UUO-induced renal fibrosis was evaluated using both histologic and biochemical examinations. According to the histologic examination with Haemotoxylin and Eosin (H&E) staining, the sham group did not show any morphological changes except mild tubular necrosis (Fig. 1a).

In the UUO group, the cytoplasm of the renal tubular epithelial cells was damaged with swelling and vacuolar degeneration. There was severe dilation, deformation and atrophy in the tubular structures in addition to the thickening of the basal membrane and the partial shedding of the epithelial cells. A remarkably widened interstitium and inflammatory cell infiltration were observed in the UUO group (Fig. 1b). However, there was a clear attenuation in the tubular dilatation, deformation, atrophy and interstitial widening, as well as inflammatory

cell infiltration, in the tadalafil treated UUO+T group. Additionally, the thickening of the tubular basal membrane was not observed in this group (Fig. 1c).

For the assessment of collagen deposition as a sign of renal fibrosis, the sections were stained with Masson's trichrome. Then, the histological scores were determined, as indicated, and the average was calculated. When compared with sham operated group with no sign of collagen deposition, increased collagen deposition was observed in the UUO group ( $2.83 \pm 0.17$ ;  $p < 0.01$ ). The severity of the renal fibrosis was attenuated by the tadalafil treatment in the UUO+T animals when compared with the non-treated UUO group ( $1.33 \pm 0.33$ ; vs UUO,  $p < 0.05$ ). However, there was a statistically significant higher collagen deposition in the kidney sections of the UUO+T group when compared with the sham animals ( $p < 0.01$ ).

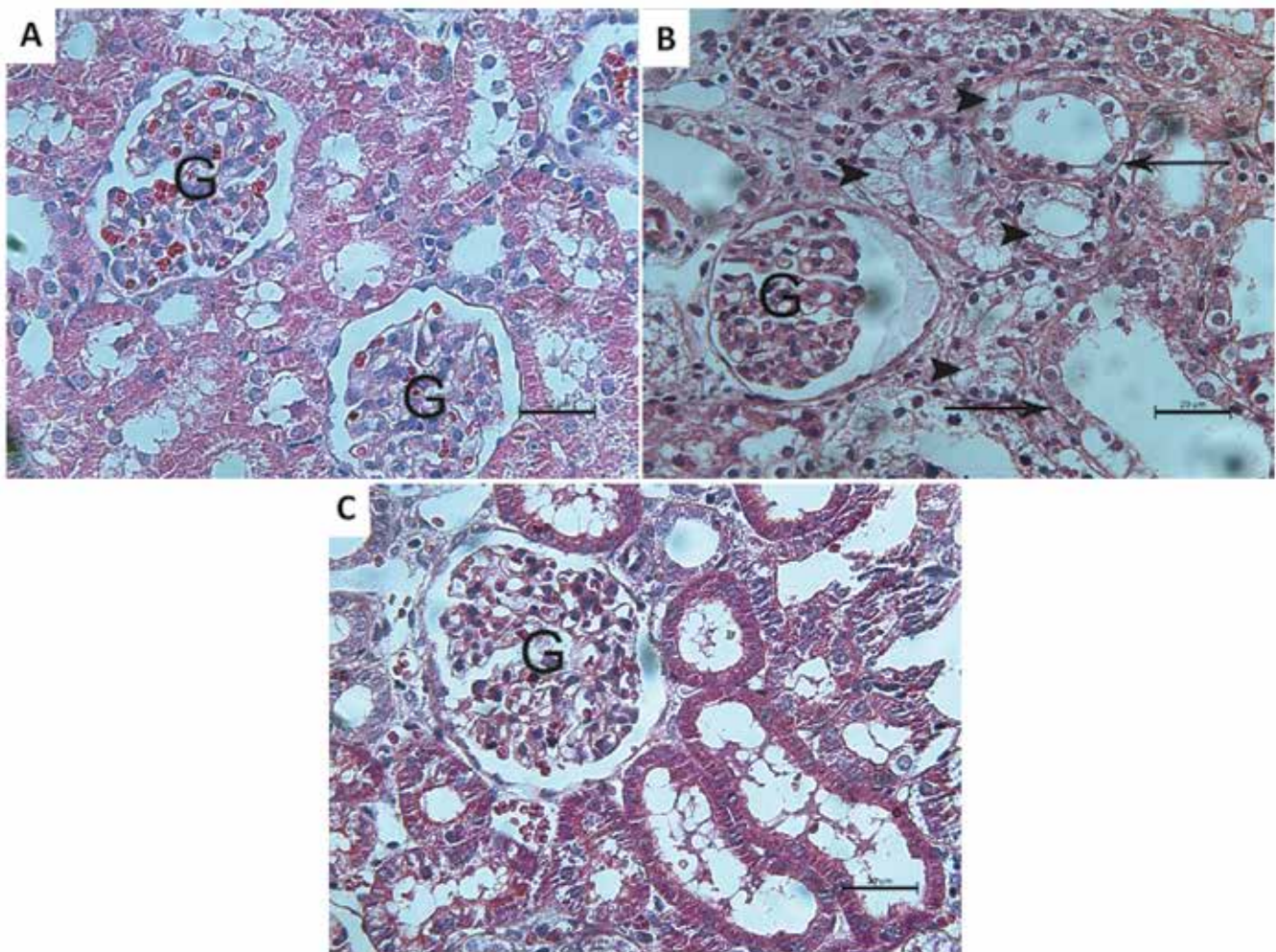


Fig. 1: Representative images showing kidney sections with H&E staining in the sham (A), UUO (B) and UUO+T groups (C) groups. The UUO group showed dilated distal nephron segments, interstitial expansion and thickening of basal membrane (arrows). Arrowheads indicate renal tubular epithelial cells with swelling and vacuolar degeneration. Glomerulus (G).

Renal  $\alpha$ -sma level: As another sign of renal fibrosis, the amount of  $\alpha$ -sma in kidney homogenates was measured by using an appropriate ELISA kit. The tissue level of  $\alpha$ -sma was  $0.41 \pm 0.06$  microgram/milligram protein in the sham-operated rats and significantly increased to  $1.21 \pm 0.12$  microgram/milligram protein in the rats with ureteral obstruction ( $p < 0.001$ ). The UUO-induced  $\alpha$ -sma accumulation in the kidney was significantly alleviated by the tadalafil treatment in the UUO-T rats ( $0.88 \pm 0.13$  microgram/milligram protein;  $p < 0.05$ ), but not completely prevented (Fig. 2).

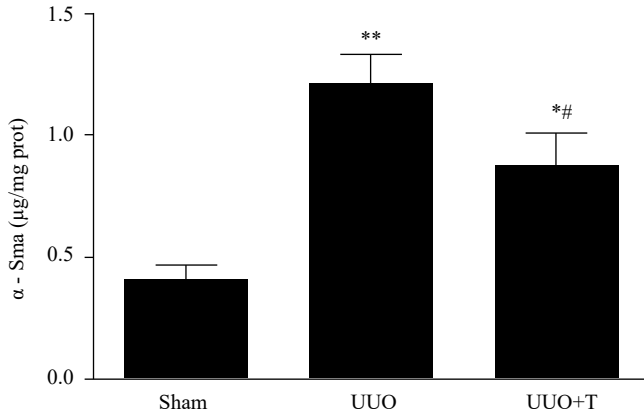


Fig. 2:  $\alpha$ -Sma level in kidney homogenates in sham, UUO and UUO+T groups. Data are presented as mean  $\pm$  SD ( $n = 6$  rat per group). \* $p < 0.01$ , \*\* $p < 0.001$  versus sham, # $p < 0.05$  versus UUO.

Renal TGF- $\beta$  level: TGF- $\beta$  level in the kidney homogenates of the sham group was  $0.16 \pm 0.01$  ng/mg protein. As shown in (Fig. 3), the ureteral obstruction caused a significant elevation in the TGF- $\beta$  level in the UUO group compared with the sham-operated rats ( $0.42 \pm 0.02$  ng/mg protein,  $p < 0.01$ ). Although, the tadalafil treatment could not prevent the TGF- $\beta$  increment, it caused a significant reduction in the TGF- $\beta$  level ( $0.35 \pm 0.02$  ng/mg protein,  $p < 0.01$ ).

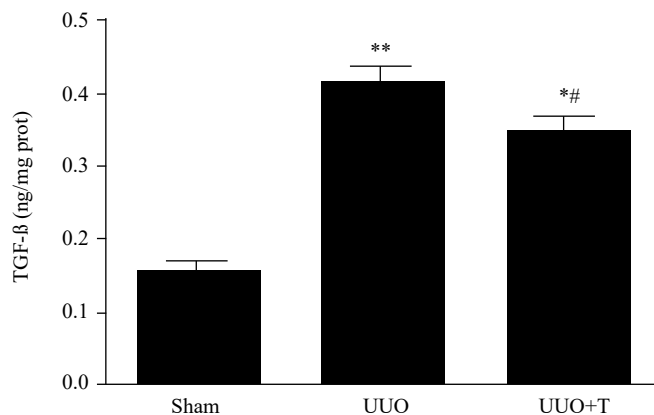


Fig. 3: TGF- $\beta$  level in kidney homogenates in sham, UUO and UUO+T groups. Data are presented as mean  $\pm$  SD ( $n = 6$  rat per group). \* $p < 0.001$  versus sham, # $p < 0.01$  versus UUO.

## DISCUSSION

Fibrotic changes of the renal parenchyma are accompanied by reduced function and result in severe renal failure and may require dialysis or transplantation. Therefore, numerous studies have focussed on investigating the potential mechanisms to prevent or reverse renal damage during fibrosis (1–4). In the present study, the possible therapeutic effects of tadalafil, a specific PDE-5 inhibitor, on renal fibrosis were addressed in a rat UUO model.

Unilateral ureteral obstruction induced renal damage is well characterized by fibrotic changes such as the proliferation of the interstitial myofibroblasts, the increased expression of the  $\alpha$ -sma and ECM proteins, massive infiltration of the inflammatory cells in addition to reduced renal blood flow and GFR (1–4). In our study, the creatinine clearance was calculated 14 days after obstruction as a sign of kidney function and a significant decrease in GFR was identified in the UUO animals. Additionally, the histologic examination of the kidney sections revealed collagen deposition, tubular dilation and atrophy in addition to the inflammatory cell infiltration in the renal interstitium. Consistent with previous results, increased  $\alpha$ -sma level in the kidneys of obstructed animals as well as the above mentioned histologic and functional data, suggested that the UUO model successfully induced renal fibrosis in this study.

The phosphodiesterase-5 inhibitor drugs are primarily marketed to treat male erectile dysfunction. Mechanistically, these drugs inhibit the cGMP degradation and prolong the cGMP signalling pathway which has a significant contribution to many physiological and pathophysiological processes (7–9, 15, 16). Previous research has focussed on their possible extra-sexual effects and evaluated their benefits in pulmonary arterial hypertension and congestive heart failure (17, 28). There is also promising evidence showing the beneficial effect of PDE-5 inhibitors in kidney pathologies such as ischaemia-reperfusion injury (21, 24), septic injury (25), lithium toxicity (26), diabetic nephropathy (20), hypertension (23) and chronic renal failure (22).

The effects of the PDE-5 inhibitors on renal fibrosis, had been investigated in different models including experimental glomerulonephritis (14), diabetes (20, 29) and ureteral obstruction (27). Cui *et al* (2014) revealed the effects of sildenafil treatment in a mouse model and showed a reduction in the UUO-induced collagen accumulation, the  $\alpha$ -sma expression and the macrophage infiltration. They suggested that cGMP-dependent



protein kinase activation might have an important role in the sildenafil induced anti-fibrotic effects (27).

In our study, we used tadalafil, which had the highest selectivity for PDE-5 inhibition (15, 16), to treat UUU-induced renal fibrosis. After the two-week tadalafil treatment, UUU-induced reductions in the cGMP level were restored to the level of the control group. Increased cGMP level was accompanied by a significant attenuation in renal fibrosis as proved by the reduced collagen deposition, the  $\alpha$ -sma level and the inflammatory cell infiltration in the tadalafil treated UUU animals. Although there was a remarkable alleviation in the renal fibrosis in the UUU+T group, it was not accompanied by the same degree of functional improvement, which could be attributed to the fact that the kidneys were still fibrotic.

During renal fibrosis, the over-expression of some pro-fibrotic and inflammatory factors contributed to kidney damage (1–4). Among these factors, TGF- $\beta$  plays a main role by regulating cell growth, differentiation and proliferation, as well as ECM remodelling (3, 30). Consistent with this knowledge, our results revealed that the UUU-induced renal fibrosis was accompanied by elevated TGF- $\beta$  expression in the kidney tissue. Since the treated animals had a lower TGF- $\beta$  level, the tadalafil induced alleviation of renal fibrosis could be attributed to the reduced TGF- $\beta$  expression in the UUU+T animals.

In conclusion, during UUU, tadalafil dependent attenuation of renal fibrotic changes such as the collagen deposition, the  $\alpha$ -sma expression, and the inflammatory cell infiltration, might be mediated by decreased TGF- $\beta$  expression. Since the UUU-induced renal fibrosis was partially inhibited but not prevented by the tadalafil treatment, further research is needed to assess the optimal dose and duration of the treatment. However, our results may have important clinical relevance since tadalafil is currently used clinically to treat erectile dysfunction and pulmonary hypertension. Considering the frequency of ureteral obstruction induced by stones or different pathologies, we suggest that PDE-5 inhibition by tadalafil may have protective effects on kidneys during the diagnosis and treatment of ureteral obstruction.

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