Scientific Research Publishing

Open Journal of Nephrology, 2020, 10, 323-337
https://www.scirp.org/journal/ojneph
ISSN Online: 2164-2869
ISSN Print: 2164-2842

Shenning II Decoction Inhibits Epithelial-Mesenchymal Transition of Renal Tubular Epithelial Cells via Regulation of Wnt/β-Catenin Signaling

Shengfang Xie[1*], Fengfeng Ge[1], Meixiao Sheng[2], Yuanzhang Yao[1], Liming Fang[1], Houcai Huang[3], Wei Zhang[4]

[1]Department of Nephrology, Jiangsu Provincial Academy of Traditional Chinese Medicine, Nanjing, China
[2]Department of Nephrology, Jiangsu Provincial Hospital of Traditional Chinese Medicine, Nanjing, China
[3]Department of Experimental Animal Center, Jiangsu Provincial Academy of Traditional Chinese Medicine, Nanjing, China
[4]Chinese Medicine Quality and Metabolism Study Group, Jiangsu Provincial Academy of Traditional Chinese Medicine, Nanjing, China
Email: *Xieshengfang@163.com

How to cite this paper: Xie, S.F., Ge, F.F., Sheng, M.X., Yao, Y.Z., Fang, L.M., Huang, H.C. and Zhang, W. (2020) Shenning II Decoction Inhibits Epithelial-Mesenchymal Transition of Renal Tubular Epithelial Cells via Regulation of Wnt/β-Catenin Signaling. *Open Journal of Nephrology*, **10**, 323-337.
https://doi.org/10.4236/ojneph.2020.104032

Received: October 20, 2020
Accepted: November 15, 2020
Published: November 18, 2020

Abstract

This study was conducted to investigate the effect of Shenning II decoction on renal function, renal pathology, epithelial-to-mesenchymal transition (EMT), and Wnt/beta-catenin signaling in unilateral ureteral obstruction (UUO) renal fibrosis. Sprague-Dawley rats were randomly divided into blank control, sham-operated, UUO model (untreated), and UUO with Shenning decoction treatment (high, medium, and low dose) groups. Renal function was evaluated based on blood urea nitrogen (BUN) and serum creatinine (Scr) levels. Histopathological analysis of rat kidney tubular tissue was carried out and E-cadherin, fibronectin, vimentin, Wnt4, glycogen synthase kinase (GSK)β, low-density lipoprotein receptor-related protein (LRP)5, LRP6, β-catenin, Snail, and fibroblast-specific protein (FSP)1 expression was evaluated by immunohistochemistry, western blotting, and real-time PCR. BUN and Scr were found to be increased in UUO when compared with the sham rats ($P < 0.05$), but this was reversed (albeit non-significantly) in rats treated with high and medium doses of Shenning II ($P > 0.05$). Shenning II decoction decreased histopathological scores relative to the UUO rats ($P < 0.05$). Protein expression of E-cadherin was increased, whereas that of vimentin, Wnt4, β-catenin, and fibronectin was decreased in Shenning I-treated rats, when compared with the untreated UUO rats, as determined by immunohistochemistry and western blotting ($P < 0.05$). Wnt4, β-catenin, GSK-3β, LRP5, LRP6, Snail, and FSP1 mRNA levels were also downregulated by Shenning II decoction treatment ($P < 0.05$). Shenning II decoction, therefore, protects

against renal fibrosis by blocking renal tubular EMT via suppression of Wnt/beta-catenin signaling.

Keywords

Chronic Kidney Disease, Renal Fibrosis, Wnt/Beta-Catenin Signaling Pathway, EMT, Traditional Chinese Medicine

1. Introduction

Chronic kidney disease (CKD) can progress to end-stage renal disease (ESRD), leading to cardiovascular events, decreased quality of life, high medical costs, and death. The global prevalence of CKD among adults over the age of 20 is 10.4% for males and 11.8% for females [1]; among Chinese adults, the prevalence is 10.8% [2]. CKD is characterized by renal fibrosis, which involves epithelial-to-mesenchymal transition (EMT) of renal tubular epithelial cells; this begins with loss of adhesion of tubular epithelial cells owing to the downregulation of E-cadherin and upregulation of α-smooth muscle actin (α-SMA), vimentin, and other mesenchymal cell markers, with consequent increases in cell migration and invasion [3]. Migrating cells penetrate the ruptured basement membrane and enter the renal interstitium, where they transform into myofibroblasts that secrete collage and promote extracellular matrix (ECM) synthesis, thereby contributing to structural remodeling of the kidney and fibrosis.

The Wnt family comprises secreted, lipid-modified signaling glycoproteins, and includes at least 19 members in mammals. Wnt proteins transmit signals across cell membranes by interacting with Frizzled (Fzd) receptors and their co-receptor low-density lipoprotein receptor-associated protein (LRP)5 or LRP6. Most Wnt and Fzd proteins are expressed in the kidney. In canonical Wnt/β-catenin signaling, the binding of Wnt to Fzd and LRP5/6 results in activation and phosphorylation of the latter, recruitment of Dishvelled and Axin proteins, and inhibition of glycogen synthase kinase (GSK)3β-mediated β-catenin phosphorylation and proteolytic degradation. Stabilized β-catenin accumulates in the nucleus where it binds to T cell factor/lymphoid enhancer factor (LEF) and regulates the expression of Snail, Twist, fibronectin, fibroblast-specific protein (FSP)1, matrix metalloproteinase (MMP)-7, plasminogen activator inhibitor (PAI)-1, Ras, and other target genes that promote EMT. Soluble Fzd-related proteins, Dickkopf, and Klotho are inhibitors of Wnt signaling that block target gene expression [4].

Some Wnt proteins are upregulated in obstructive nephropathy, resulting in β-catenin accumulation in the cytoplasm and nucleus of renal tubular epithelial cells. The expression of Wnt/β-catenin target genes including c-Myc, Twist, LEF1, and fibronectin is related to the β-catenin level in the kidneys [5]. Immunohistochemical analysis of renal fibrotic tissue has revealed that E-cadherin is downregulated, whereas the interstitial markers vimentin and fibronectin are

upregulated in tubular epithelial and non-damaged renal tubulointerstitial epithelial cells; additionally, Wnt5b and β-catenin levels were found to be increased in interstitial inflammation and fibrosis and in stromal cells [6].

Shenning II decoction can alleviate unilateral ureteral obstruction (UUO) renal fibrosis and mercuric chloride- and gentamicin-induced renal failure in animal models [7]. It was shown to inhibit renal tubular epithelial cell transdifferentiation and suppress the expression of transforming growth factor (TGF)-β1 and α-SMA in UUO rat kidneys, leading to ECM degradation [8].

Based on the above reports, we speculated that Shenning II decoction can exert anti-fibrotic effects to prevent kidney injury via regulation of the Wnt/β-catenin signaling pathway. To test this hypothesis, we used a rat model of UUO to simulate renal fibrosis. The rats were treated with Shenning II decoction and changes in renal function, urinary protein levels, renal histology, and expression of Wnt/β-catenin pathway components and EMT-associated factors were evaluated.

2. Materials and Methods

2.1. Materials and Reagents

Shenning II decoction is composed of *Astragalus membranaceus* (20 g), *Radix Polygoni Multiflori Preparata* (20 g), *prepared rhubarb* (10 g), *Ligusticum chuanxiong* (12 g), *Hirudo* (3 g), *stir-baked Crataegus pinnatifida* (20 g), *Exocarpium Benincasae* (30 g), *Semen Persicae* (10 g), *Serissa serissoides* (15 g), and *Lycopus lucidus* (10 g). The herbs were provided by Jiangsu Provincial Academy of Traditional Chinese Medicine, and different concentrations of Shenning II decoction—*i.e.*, low dose (1.25 g crude herb/ml), medium dose (2.5 g crude herb/ml), and high dose (5 g crude herb/ml) aqueous solutions—were prepared in our laboratory. Antibodies against the following proteins were used in this study: Wnt4 (bs-6134r; Beijing Bo Ao Sen Biotechnology Co., Beijing, China); β-catenin (KG11116; Jiangsu KGI Biotechnology Co., Nanjing, China); E-cadherin (3195) and vimentin (5741) (both from Cell Signaling Technology, Danvers, MA, USA); and fibronectin (sc-9068; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.2. Animal Model

Specific pathogen-free male Sprague-Dawley rats (n = 75, 7 - 8 weeks old, weighing 220 - 250 g) were purchased from Beijing Victoria Tonghua Experimental Animal Technology Co. (Beijing, China; license number SCXK [Beijing] 2012-0001) and were maintained under standard conditions at the Experimental Animal Center of Jiangsu Provincial Academy of Traditional Chinese Medicine.

The animals were allowed to adapt to their environment for 1 week before the experiment. After anesthetization with 5% chloral hydrate (0.3 ml/100 g) by intraperitoneal injection, the rats were fixed in the prone position on the operating table and a longitudinal incision was made along the lower edge of the left

lower back. The skin and abdominal muscle were cut to expose the kidneys and left ureter; the latter was ligated with 4-0 silk in the proximal renal pelvis, and the abdomen was closed layer by layer. Sham operation involved the same procedure except that the ureter was not ligated. For the first 3 days after surgery, 4×10^5 IU/day of penicillin was administered to the rats by intraperitoneal injection to prevent infection.

2.3. Grouping and Treatment

Five rats were randomly selected as the blank control group and the remaining 70 were divided into five groups: sham operation (n = 10), UUO model (n = 15), and UUO with Shenning II decoction treatment (low, medium, and high doses; n = 15 rats each). Rats in the treatment groups received Shenning II decoction (1 ml/100g body weight) by intragastric administration on the first day after the operation. Rats in the blank control, sham-operated, and UUO model groups were given normal saline. After 21 days of treatment, urine specimens were collected to measure urinary protein creatinine ratios, and orbital blood samples were collected for detection of serum creatinine (Scr) and blood urea nitrogen (BUN) levels. The left kidney was harvested and cut along the sagittal plane into two parts; one was preserved in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining, Masson's trichrome staining, and immunohistochemistry; and the other was flash frozen in liquid nitrogen and stored at −80˚C for western blotting and real-time (RT-)PCR analyses.

2.4. Histological and Immunohistochemical Analyses

Fixed renal tissue samples were cut into sections at a thickness of 3 μm; the sections were stained with H & E and Masson's trichrome and examined by light microscopy. The extent of injury was quantified by assigning a pathological score as follows: normal, 0 points; < 5%, 0.5 points; 5% - 15%, 1 point; 15% - 50%, 2 points; 50% - 75%, 3 points; and ≥75%, 4 points.

For immunohistochemical detection of fibronectin, E-cadherin, vimentin, Wnt4, and β-catenin expression, 3-μm-thick paraffin tissue sections were deparaffinized and rehydrated by a conventional method, and antigen retrieval was carried out at 90˚C - 100˚C in citric acid solution for 8 - 10 min. Endogenous peroxidase was inactivated with 3% H_2O_2-methanol solution and the sections were blocked in goat serum at room temperature for 20 min. The sections were then incubated with primary antibody at 37˚C in a humid chamber for 2 h, then washed with phosphate-buffered saline (PBS). After incubation with appropriate secondary antibodies for 30 min at 37˚C, immunoreactivity was detected with diaminobenzidene solution and sections were counterstained with hematoxylin for 10 min. After dehydration through a graded series of ethanol (5 min at each concentration) and xylene, the sections were mounted and observed by microscopy. Three images of each section were obtained at 400× magnification and the immunopositive area and integrated optical density (IOD) (average density × area) were computed using Image-Pro Plus software (Media Cybernetics, Rock-

ville, MD, USA).

2.5. Western Blotting

Renal tissue samples were homogenized in radioimmunoprecipitation assay lysis buffer using a glass homogenizer. The homogenate was centrifuged at 10,000 g for 10 min at 4˚C, and the supernatant was collected and mixed with loading buffer. After denaturation of the proteins by boiling for 5 min in a 0.25× volume of 5× sample buffer, total protein concentration was determined with the Bradford method. After the samples had cooled to room temperature, approximately 20 μg of protein were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis at a constant voltage of 110 V for 80 min in running buffer containing 3 g Tris base, 14.4 g glycine, and 1 g SDS per 1 l of distilled water. The gel was stained with Ponceau red to visualize the separation of proteins, which were transferred to a nitrocellulose membrane that was incubated in 50 ml PBS with 0.1% Tween-20 (PBST) containing 5% skimmed milk powder at room temperature for 2 h. After rinsing with PBST for 5 min, the membrane was incubated overnight at 4˚C with primary antibodies against E-cadherin, vimentin, Wnt4, and β-catenin. After washing three times with TBST and incubating with secondary antibody at 37˚C for 2 h, the membrane was washed three times with TBST and protein bands were visualized by enhanced chemiluminescence. Protein band intensity was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.6. Real-Time (RT-)PCR

RNA was extracted from renal tissue (50 mg) in 1 ml precooled TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and precipitated with chloroform/isopropanol. The RNA was resuspended in 30 - 50 μl RNase-free water. The concentration was determined at 260 and 280 nm on a spectrophotometer. RNA (2 μg), oligo dT (50 μM), and double-distilled water were combined for a total volume of 12.5 μl and after heating at 65˚C for 5 min, the mixture was placed on ice for 5 min before adding RNase inhibitor (40 U/μl), 0.5 μl, 5× reaction buffer (4 μl), 10 mM dNTPs (2 μl), and Moloney murine leukemia virus (1 μl) to generate cDNA. After centrifugation at 2000 rpm for 20 s, the sample was incubated at 42˚C for 1 h, 70˚C for 10 min, and then placed on ice for 5 min. The following components were added to a final volume of 20 μl for the PCR reaction: 10 μl of 2× RT-PCR Master Mix (SYBR Green), 2 μl of 10-fold diluted cDNA, 2 μl of primer mix (10 μM each of forward and reverse primers), and 7 μl of 0.1% diethyl pyrocarbonate water. Primer Express 5.0 software was used to design the primer sequences for amplifying target genes. Primers were synthesized by Jiangsu KGI Biotechnology Co. (Table 1).

2.7. Statistical Analysis

SPSS v.17.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis.

Table 1. Sequences of primers used for RT-PCR.

Target gene	Primer sequence
Wnt4	Sense: GCGTAGCCTTCTCACAGTCCTT
	Antisense: CTTCCTGCCAGCCTCGTTGTT
β-catenin	Sense: AGGAATGAAGGCGTGGCAACA
	Antonyms: GCACCAATGTCCAGTCCGAGAT
GSK3β	Sense: AATCGCACTGTGTAGCCGTCTC
	Antisense: GCAGGTGTGTCTCGCCCATTT
LRP5	Sense: ACTGGACAGATGTGAGCGAGGA
	Antisense: CGATGCGGTTGGTCTCTGAGTC
LRP6	Sense: GCCATCCGTCGCTCCTTCATTG
	Antisense: TTCTATCCGATCAGTGCCAGTGTCT
Snail	Sense: TTGCCGACCGCTCCAACCTA
	Antisense: GAGCAGCCAGACTCTTGGTGTT
FSP1	Sense: CTCAGGCAACGAGGGTGACAAG
	Antisense: GGCAATGCAGGACAGGAAGACA

Quantitative data are expressed as a percentage. The t test and Wilcoxon rank-sum test were used to compare normally and non-normally distributed data, respectively. Data are expressed as mean ± standard deviation. Data for multiple groups were evaluated by analysis of variance or the mismatched non-parametric test. $P < 0.05$ was considered statistically significant for all analyses.

3. Results

3.1. Shenning II Decoction Alleviates Renal Dysfunction in UUO Rats

There were no differences in urinary protein concentration between groups ($P > 0.05$; Table 2). BUN and Scr values were higher in the UUO model group than in the blank and sham-operated groups ($P < 0.05$). This increase was reversed in rats treated with middle and high doses of Shenning II decoction, although the differences were not statistically significant ($P > 0.05$). Thus, Shenning II decoction intervention had no effect on proteinuria or renal function.

3.2. Histopathological Changes in the Kidney of UUO Rats Are Reversed by Shenning II Decoction

Light microscopy examination of H & E-stained section revealed that in the blank control group, glomerular volume was normal and there was no expansion of the balloon cavity, or swelling or necrosis of tubular epithelial cells. The renal interstitium showed no edema or inflammatory cell infiltration, and there was no fibrous tissue proliferation. The same was observed in the sham-operated group. UUO model rats showed slight necrosis of tubular epithelial cells, infiltration of inflammatory cells into the renal interstitium, and severe fibrous tissue

Table 2. Urinary protein concentrations and blood and serum parameters in each group ($\bar{X} \pm s$).

Group	n	Total protein concentration (mg/g)	BUN (mmol/l)	Scr (μmol/l)
Blank control	5	1233.33 ± 915.96	6.93 ± 0.64	26.2 ± 2.28
Sham-operated	3	1342.54 ± 1014.26	5.95 ± 0.83	27.2 ± 1.64
UUO model	10	1487.23 ± 895.55	8.08 ± 1.82$^{\#}$	32.50 ± 6.46$^{\#}$
Low dose*	10	1342.01 ± 407.98	10.62 ± 4.91	34.67 ± 6.41
Middle dose*	11	2029.66 ± 1382.33	8.62 ± 1.61	32.91 ± 5.47
High dose*	9	1730.14 ± 878.23	7.51 ± 1.25	29.44 ± 6.93

BUN, blood urea nitrogen; Scr, serum creatinine; UUO, unilateral ureteral obstruction. *Shenning II decoction doses: low (1.25 g crude herb/ml), medium (2.5 g crude herb/ml), and high (5 g crude herb/ml). $^{\#}P < 0.05$ vs. blank control and sham-operated groups.

hyperplasia. These pathological changes were attenuated in a dose-dependent manner in rats treated with high, middle, and low doses of Shenning II decoction (Figure 1). Similar trends were observed by Masson's trichrome staining: in the blank control and sham-operated groups, there was a small amount of fibrotic renal tissue, which was the animal's own connective tissue. The model group showed moderate-to-severe fibrosis in the kidney, which was diminished in a dose-dependent manner in rats treated with Shenning II decoction (Figure 1). In accordance with these findings, pathological scores were higher in UUO model rats than in the blank and sham-operated groups ($P < 0.05$; Table 3); however, the scores were decreased by Shenning II decoction treatment ($P < 0.05$), with the high-dose group showing the most marked reduction ($P < 0.05$). A semi-quantitative analysis of fibrosis as visualized by Masson's trichrome staining revealed the same trends: the score was higher in the model, when compared with the blank and sham-operated groups ($P < 0.05$), and was decreased in the high-dose, when compared with the model group ($P < 0.05$), whereas the middle and low doses had little effect. There was also no difference between high and middle dose groups ($P > 0.05$).

3.3. Activation of Wnt/*β*-Catenin Signaling and Enhancement of EMT in UUO Rats Are Reversed by Shenning II Decoction

E-cadherin was detected in renal tubular epithelial cells by immunohistochemistry; the area and IOD value of E-cadherin were lower in the UUO model group as compared to the blank control and sham-operated groups. Compared to the model group, the IOD value of rats treated with a high dose of Shenning II decoction was higher than that of the middle-dose group ($P < 0.05$). On the contrary, the expression of vimentin, fibronectin, Wnt4, and *β*-catenin was higher in the model than in the blank and sham-operated groups; however, the vimentin and Wnt4 levels were downregulated upon treatment with a high dose of Shenning II decoction. The middle dose reduced the levels of fibronectin and *β*-catenin whereas the low dose suppressed *β*-catenin expression relative to UUO

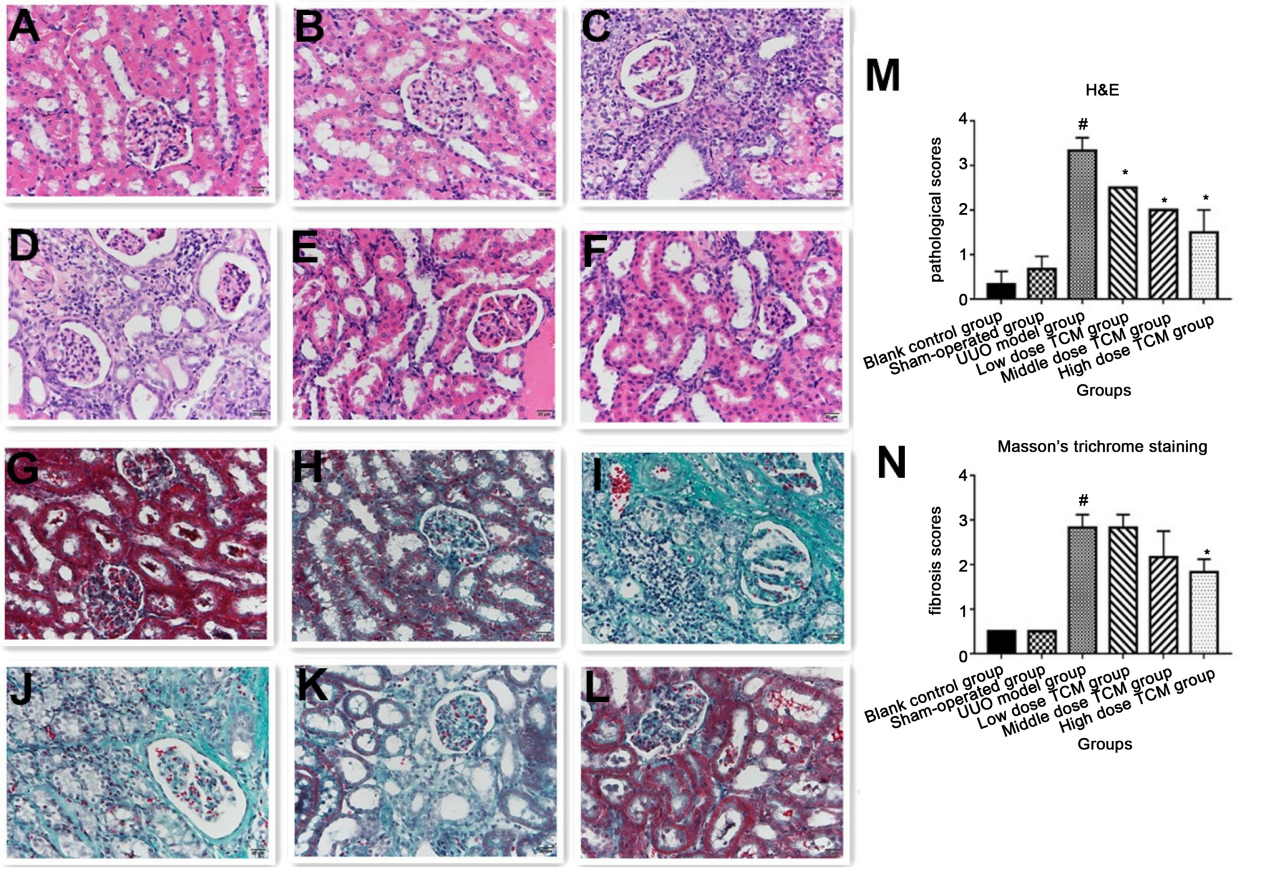

Figure 1. Histopathological analysis of renal tissue (400×). H & E staining: (A) Blank control group. (B) Sham-operated group. (C) UUO model group. (D) Low-dose Shenning II decoction group. (E) Middle-dose Shenning II decoction group. (F) High-dose Shenning II decoction group. Masson's trichrome staining: (G) Blank control group. (H) Sham-operated group. (I) UUO model group. (J) Low-dose Shenning II decoction group. (K) Middle-dose Shenning II decoction group. (L) High-dose Shenning II decoction group. Renal histopathology scores: (M) H & E pathological scores. $^{\#}P < 0.05$ vs. blank control and sham-operated groups; $^{*}P < 0.05$ vs. model group. (N) Masson's trichrome staining fibrosis scores. $^{\#}P < 0.05$ vs. blank control and sham-operated groups; $^{*}P < 0.05$ vs. model group.

Table 3. Renal histopathology scores ($\bar{X} \pm s$).

Group	n	H & E pathological scores	Masson's trichrome staining fibrosis scores
Blank control	3	0.33 ± 0.29	0.50 ± 0.00
Sham-operated	3	0.67 ± 0.29	0.50 ± 0.00
UUO model	3	3.33 ± 0.29$^{\#}$	2.83 ± 0.29$^{\#}$
Low dose†	3	2.50 ± 0.00*	2.83 ± 0.29
Middle dose†	3	2.00 ± 0.00*	2.17 ± 0.58
High dose†	3	1.50 ± 0.50*	1.83 ± 0.29*

H & E, hematoxylin and eosin; UUO, unilateral ureteral obstruction. $^{\#}P < 0.05$ vs. blank control and sham-operated groups; $^{*}P < 0.05$ vs. model group. †Shenning II decoction doses: low (1.25 g crude herb/ml), medium (2.5 g crude herb/ml), and high (5 g crude herb/ml).

rats (Figure 2).

The immunohistochemistry findings were confirmed by western blot analysis of renal tissue lysates. E-cadherin protein level in the kidney was reduced in UUO model rats relative to the blank control and sham-operated groups.

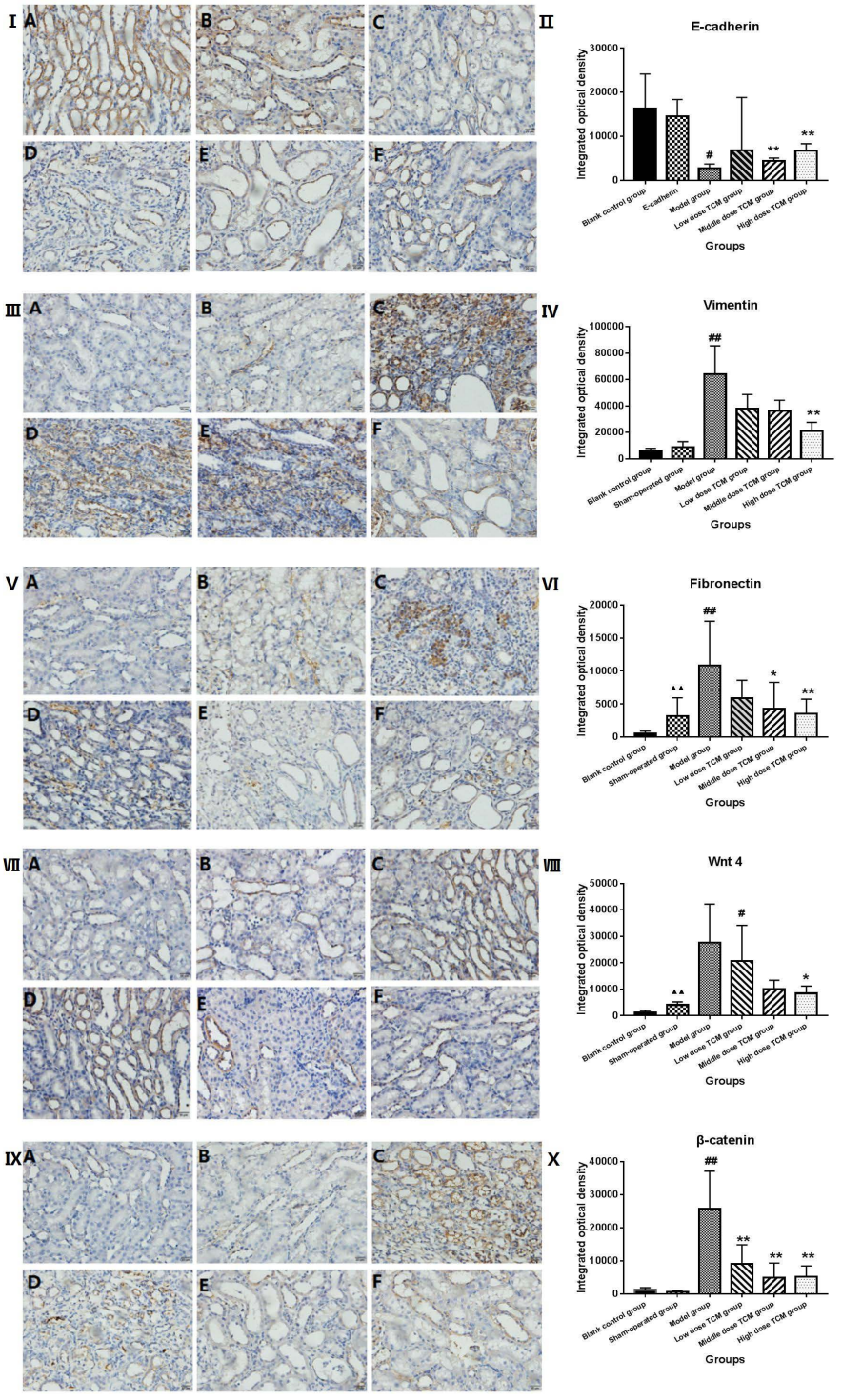

Figure 2. Immunohistochemical analysis of renal tissue sections. Sections were labeled for E-cadherin (I), vimentin (III), fibronectin (V), Wnt4 (VII), and β-catenin (IX). Magnification: 400×. (A) Blank control group. (B) Sham-operated group. (C) UUO model group. (D) Low-dose Shenning II decoction group. (E) Middle-dose Shenning II decoction group. (F) High-dose Shenning II decoction group. IOD values of E-cadherin (II), vimentin (IV), fibronectin (VI), Wnt4 (VIII), and β-catenin (X) were calculated. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. blank control and sham-operated groups; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group; $^{\blacktriangle\blacktriangle}P < 0.01$ vs. blank control group.

Treatment with high and middle doses of Shenning II decoction partly restored E-cadherin level, although the low dose had no effect. On the other hand, vimentin, Wnt4, and β-catenin levels were elevated in UUO rat kidney, but this was reversed in a dose-dependent manner by treatment with high and middle doses of Shenning II decoction (Figure 3).

We also examined the expression of Wnt/β-catenin signaling components and EMT markers in the kidney by RT-PCR. Wnt4, β-catenin, GSK-3β, LRP5/6, Snail, and FSP1 levels were elevated in the renal tissue of UUO rats relative to the levels in the blank control and sham-operated groups. These increases were dose-dependently suppressed by Shenning II decoction, except in the case of LRP5/6 (Figure 4).

4. Discussion

Renal fibrosis is the main pathological manifestation during the progression from CKD to ESRD. It was previously reported that UUO model rats show increased proteinuria after 30 or more days [9]. On day 21 in our study, urinary protein quantity was increased in UUO relative to the blank control and sham-operated rats, although the difference was not statistically significant. It is possible that Shenning II decoction does affect proteinuria, but that this can only be observed by prolonging the observation time. We also found that Scr values were increased in UUO rats; although high and middle doses of Shenning II decoction slightly reduced Scr levels, the differences were not statistically significant, suggesting that short-term administration of Shenning II decoction may be

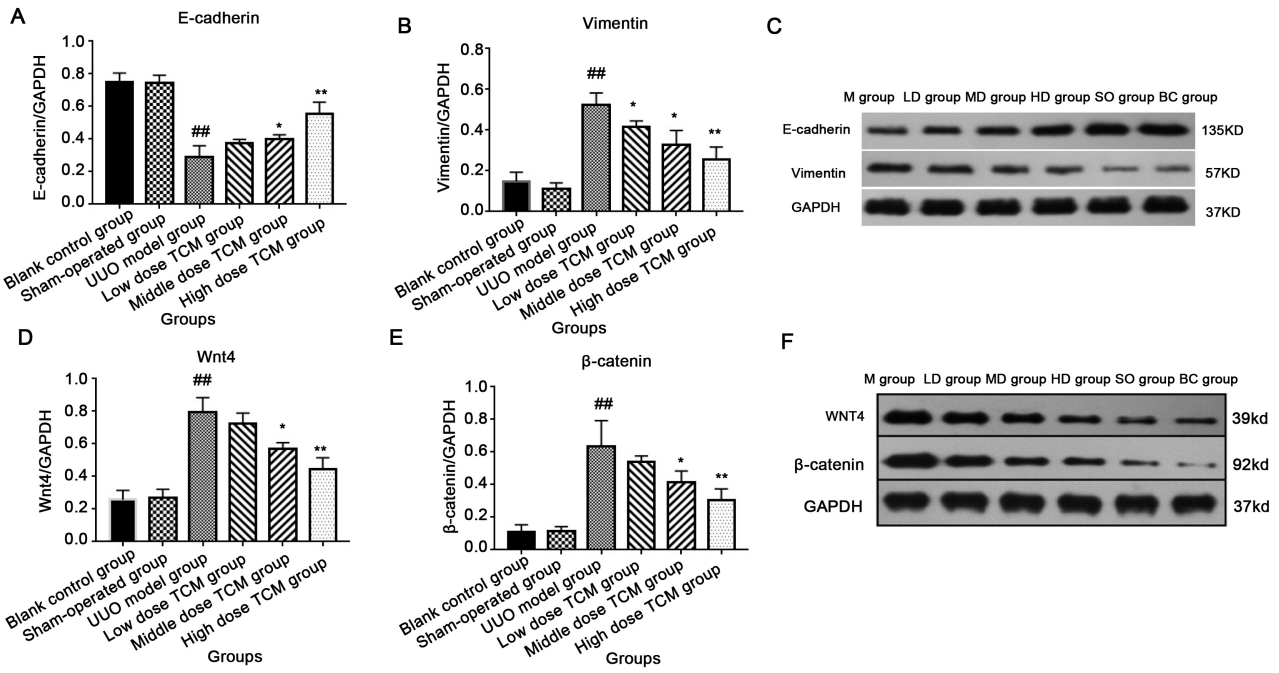

Figure 3. Western blot analysis of Wnt/β-catenin signaling pathway components. $^{\#\#}P < 0.01$ vs. blank control and sham-operated groups; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group. BC, blank control group; HD, high-dose Shenning II decoction group; LD, low-dose Shenning II decoction group; M, model group; MD, middle-dose Shenning II decoction group; SO, sham-operated group.

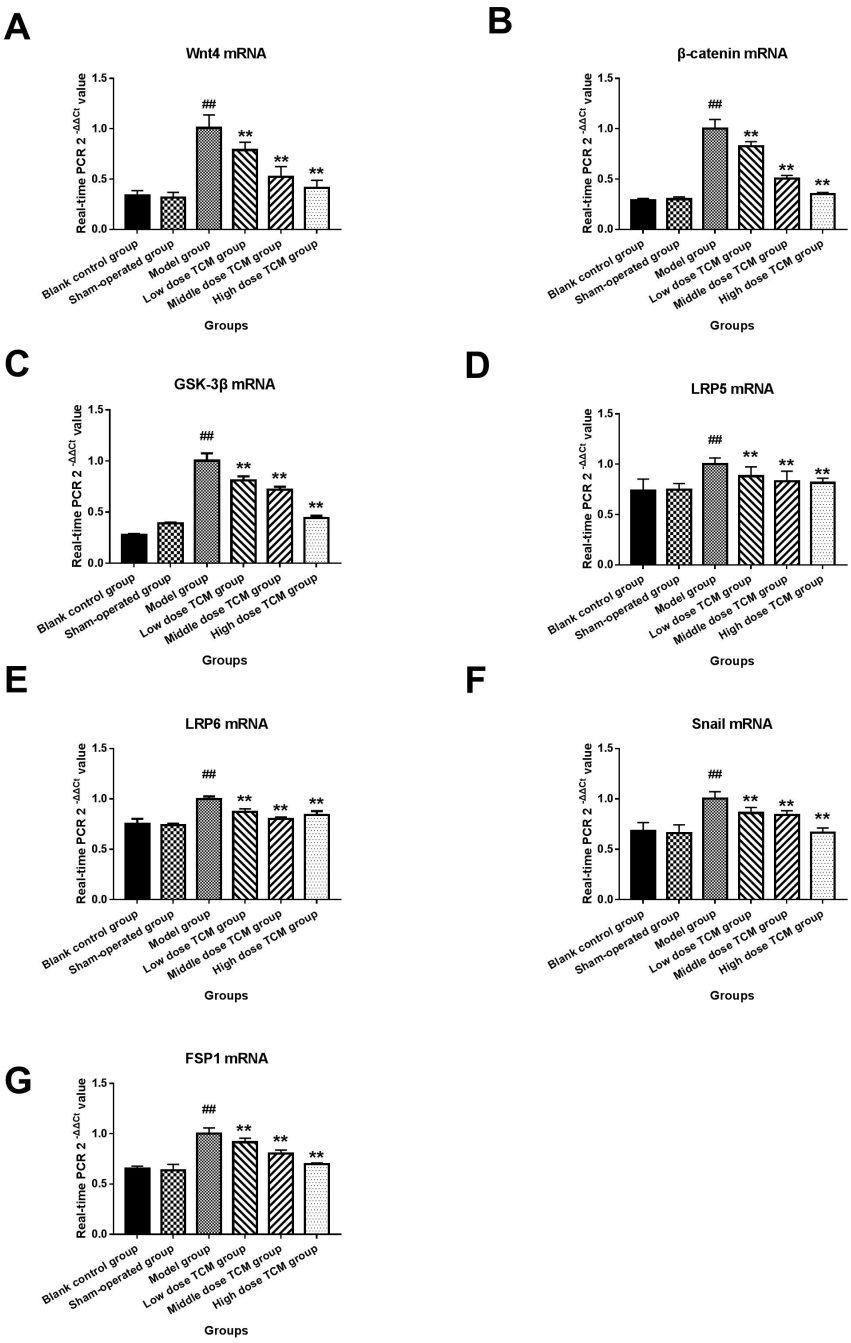

Figure 4. RT-PCR analysis of Wnt/β-catenin signaling pathway components. $^{\#\#}P < 0.01$ vs. blank control and sham-operated groups; $^{**}P < 0.01$ vs. model group.

ineffective against renal dysfunction. In this study, a small number of renal tubular epithelial cells in UUO rat kidney were necrotic; this was associated with infiltration of inflammatory cells into the renal interstitium, expansion of fibrous tissue, and upregulation of fibronectin in tubule epithelial cells and interstitium. Renal lesions were alleviated in a dose-dependent manner by treatment with Shenning II decoction, as evidenced by decreases in the area and IOD value of fibronectin. Thus, Shenning II decoction can reverse the pathological damage

associated with UUO in rats.

EMT of tubular epithelial cells—the source of about 30% - 50% of fibroblasts—is an early pathological feature of renal interstitial fibrosis [10]. In fact, the concept of EMT is based on the fact that tubular epithelial cells can simultaneously express fibroblast markers [11]. One study using an UUO model demonstrated that EMT occurs in renal tubular epithelial cells [12], and an immunohistochemical analysis of renal tissues from patients with renal atrophy revealed that renal tubular epithelial cells showed loss or low levels of E-cadherin but re-expressed vimentin and fibronectin, indicating that EMT occurs in CKD renal fibrosis [6]. Likewise, serum and urine levels of the EMT markers MMP-2 and -9 and E-cadherin were found to be elevated in CKD children [13]. Expression of interstitial cell markers has also been observed in the renal tubules of patients with diabetic nephropathy [14], lupus nephritis [15], oligoimmune complex type crescentic glomerulonephritis [16], IgA nephropathy [17], and chronic allograft nephropathy [18]. Thus, renal tubular EMT is an important mechanism of renal fibrosis and progression. Our immunohistochemistry results showed that E-cadherin and vimentin levels in tubular epithelial cells were unchanged in sham-operated rats but were down- and upregulated, respectively, in the model group, suggesting that EMT occurs in UUO rat kidney. E-cadherin protein expression was increased in rats treated with high and middle doses of Shenning II decoction and vimentin expression was decreased in the high-dose group, indicating that Shenning II decoction suppresses EMT especially at higher doses, which is supported by our western blotting results.

The Wnt/β-catenin signaling pathway is activated during renal fibrosis. Snail1, a key transcription factor that promotes EMT [19], inhibits the transcription of E-cadherin leading to the loss of epithelial adhesion, which is an early event in EMT. Snail1 also induces inhibitor of differentiation 1, which is critical for promoting EMT and renal inflammatory response [20]. Upregulation of Snail1 and β-catenin has been reported in fibroblastic renal tubular epithelial cells, with the latter inducing the expression of the former [21]. MMP-7 cleaves the E-cadherin extracellular domain and regulates apoptosis and inflammation. Urinary MMP-7 can serve as a biomarker for detecting renal Wnt/β-catenin activity and consequently, the severity of renal fibrosis [22]. β-Catenin can induce the expression of PAI-1 in renal tubular epithelial cells, leading to fibrosis, expression of TGF-β1, and recruitment of inflammatory cells and myofibroblasts. β-Catenin was shown to induce Ras expression in renal tubular epithelial cells in vitro and in vivo, thereby stimulating the production of reactive oxygen species, which results in the enhancement of nuclear factor κB and TGF-β expression and contributes to renal fibrosis [23]. In models of obstructive nephropathy, some Wnt proteins are upregulated and β-catenin accumulates in the cytoplasm and nucleus of renal tubular epithelial cells. Wnt/β-catenin target genes such as c-Myc, Twist, LEF1, and fibronectin are closely related to the level of β-catenin in kidney [5]. In renal fibrosis patients, E-cadherin expression was reduced or absent in tubular epithelial cells while the interstitial markers vimentin and fi-

bronectin as well as Wnt5b and β-catenin were upregulated; interstitial inflammation and stromal cells in interstitial fibrosis were also positive for Wnt5b and β-catenin expression [6]. The small peptide ICG-001 can inhibit β-catenin-mediated gene transcription, alleviate fibrosis in obstructive nephropathy, and prevent TGF-β1-mediated fibrosis [24]. Thus, the Wnt/β-catenin pathway is a potential therapeutic target for the treatment of renal fibrosis.

In this experiment, we found that the expression of Wnt4 and the membrane receptor protein LRP5/6 was upregulated in the UUO model group, with a corresponding increase in the protein and mRNA levels of cytoplasmic β-catenin and activation of the downstream effectors Snail and FSP1, indicating that Wnt/β-catenin signaling is involved in the regulation of EMT in renal tubular epithelial cells. Shenning II decoction treatment—especially at a high dose—reversed the above changes, suggesting that it can suppress Wnt/β-catenin signaling and thereby block EMT of renal tubular epithelial cells. On the other hand, GSK-3β—a negative regulator of β-catenin signaling—was upregulated in the UUO model group and downregulated by Shenning II decoction. This can be explained by the fact that although dissociation of the axin/adenomatous polyposis coli (APC)/GSK-3β complex was induced in UUO rats, β-catenin was not degraded, resulting in an increase in free β-catenin levels. Administration of Shenning II decoction led to the formation of the axin/APC/GSK-3β complex, with free GSK-3β decreasing accordingly. The mechanism of action of GSK-3β in Wnt signaling remains controversial [25]. Since our observations were from a single time point (21 days after UUO), it is possible that activation of the Wnt pathway resulted in changes in GSK-3β level that were not detected in our experiments with consequent suppression of β-catenin activity, although more detailed studies are required to validate this possibility.

In summary, the results of this study demonstrate that Shenning II decoction can block EMT and reduce tubulointerstitial fibrosis in UUO rats via a mechanism involving regulation of Wnt/β-catenin signaling pathway components and their downstream target genes. These results suggest that Shenning II decoction can effectively prevent the progression of CKD to ESRD, and thereby improve the outcome of patients with renal fibrosis.

Acknowledgements

This work was supported by the second batch of science and technology projects of Jiangsu Provincial Bureau of Chinese Medicine (no. FY201510), Jiangsu branch of Chinese academy of Traditional Chinese Medicine (no. JSBY1314), the third batch of Jiangsu province TCM clinical outstanding talent training project [Jiangsu science and education of traditional Chinese medicine (2017) no. 18] and the National Natural Science Foundation of China (Grant No. 81603582).

Conflicts of Interest

The authors declare that they have no competing interests.

References

[1] Mills, K.T., Xu, Y., Zhang, W., Bundy, J.D., Chen, C.S., Kelly, T.N., Chen, J. and He, J. (2015) A Systematic Analysis of Worldwide Population-Based Data on the Global Burden of Chronic Kidney Disease in 2010. *Kidney International*, **88**, 950-957. https://doi.org/10.1038/ki.2015.230

[2] Zhang, L., Wang, F., Wang, L., Wang, W., Liu, B., Liu, J., Chen, M., He, Q., Liao, Y., Yu, X., Chen, N., Zhang, J.E., Hu, Z., Liu, F., Hong, D., Ma, L., Liu, H., Zhou, X., Chen, J., Pan, L., Chen, W., Wang, W., Li, X. and Wang, H. (2012) Prevalence of Chronic Kidney Disease in China: A Cross-Sectional Survey. *Lancet*, **379**, 815-822. https://doi.org/10.1016/S0140-6736(12)60033-6

[3] Zhang, Y., Li, H., Zhu, J., Wei, T., Peng, Y., Li, R., Xu, R., Li, M. and Xia, A. (2017). Role of Artesunate in TGF-β1-Induced Renal Tubular Epithelial-Mesenchymal Transdifferentiation in NRK-52E Cells. *Molecular Medicine Reports*, **16**, 8891-8899. https://doi.org/10.3892/mmr.2017.7728

[4] Edeling, M., Ragi, G., Huang, S., Pavenstädt, H. and Susztak, K. (2016) Developmental Signalling Pathways in Renal Fibrosis: The Roles of Notch, Wnt and Hedgehog. *Nature Reviews Nephrology*, **12**, 426-439. https://doi.org/10.1038/nrneph.2016.54

[5] He, W., Dai, C., Li, Y., Zeng, G., Monga, S.P. and Liu, Y. (2009) Wnt/β-Catenin Signaling Promotes Renal Interstitial Fibrosis. *Journal of the American Society of Nephrology*, **20**, 765-776. https://doi.org/10.1681/ASN.2008060566

[6] Kim, M.K., Maeng, Y.I., Sung, W.J., Oh, H.K., Park, J.B., Yoon, G.S., Cho, C.H. and Park, K.K. (2013) The Differential Expression of TGF-β1, ILK and Wnt Signaling Inducing Epithelial to Mesenchymal Transition in Human Renal Fibrogenesis: An Immunohistochemical Study. *International Journal of Clinical and Experimental Pathology*, **6**, 1747-1758.

[7] Yao, Y.Z. (1999) Observation on the Pharmacodynamic Effect of Shenning II Decoction. *China Journal of Traditional Chinese Medicine Information*, **6**, 40-42.

[8] Fang, L.M., Wei, L., Zhang, M., Ge, F.F., Liu, B.B., Yao, X.M., Cao, P. and Yao, Y.Z. (2016) Effect of Bushen Jianpi Huoxue Decoction on Expression of TGF-β1 and α-SMA in Rats with Unilateral Ureteral Obstruction. *Chinese Medicine Herald*, **22**, 10-15.

[9] Chaabane, W., Praddaude, F., Buleon, M., Jaafar, A., Vallet, M., Rischmann, P., Galarreta, C.I., Chevalier, R.L. and Tack, I. (2013) Renal Functional Decline and Glomerulotubular Injury Are Arrested but Not Restored by Release of Unilateral Ureteral Obstruction (UUO). *American Journal of Physiology-Renal Physiology*, **304**, 432-439. https://doi.org/10.1152/ajprenal.00425.2012

[10] Iwano, M., Plieth, D., Danoff, T.M., Xue, C., Okada, H. and Neilson, E.G. (2002) Evidence That Fibroblasts Derive from Epithelium during Tissue Fibrosis. *Journal of Clinical Investigation*, **110**, 341-350. https://doi.org/10.1172/JCI0215518

[11] Strutz, F., Okada, H., Lo, C.W., Danoff, T., Carone, R.L., Tomaszewski, J.E. and Neilson, E.G. (1995) Identification and Characterization of a Fibroblast Marker: FSP1. *Journal of Cell Biology*, **130**, 393-405. https://doi.org/10.1083/jcb.130.2.393

[12] Lovisa, S., LeBleu, V.S., Tampe, B., Sugimoto, H., Vadnagara, K., Carstens, J.L., Wu, C.C., Hagos, Y., Burckhardt, B.C., Pentcheva-Hoang, T., Nischal, H., Allison, J.P., Zeisberg, M. and Kalluri, R. (2015) Epithelial-to-Mesenchymal Transition Induces Cell Cycle Arrest and Parenchymal Damage in Renal Fibrosis. *Nature Medicine*, **21**, 998-1009. https://doi.org/10.1038/nm.3902

[13] Musiał, K., Bargenda, A. and Zwolińska, D. (2015) Urine Survivin, E-Cadherin and

Matrix Metalloproteinases as Novel Biomarkers in Children with Chronic Kidney Disease. *Biomarker*, **20**, 177-182. https://doi.org/10.3109/1354750X.2015.1061598

[14] Chang, B., Chen, W., Zhang, Y., Yang, P. and Liu, L. (2018) *Tripterygium wilfordii* Mitigates Hyperglycemia-Induced Upregulated Wnt/*β*-Catenin Expression and Kidney Injury in Diabetic Rats. *Experimental and Therapeutic Medicine*, **15**, 3874-3882. https://doi.org/10.3892/etm.2018.5901

[15] Wang, X.D., Huang, X.F., Yan, Q.R. and Bao, C-D. (2014) Aberrant Activation of the WNT/*β*-Catenin Signaling Pathway in Lupus Nephritis. *PLoS ONE*, **9**, e84852. https://doi.org/10.1371/journal.pone.0084852

[16] Bariety, J., Hill, G.S., Mandet, C., Irinopoulou, T., Jacquot, C., Meyrier, A. and Bruneval, P. (2003) Glomerular Epithelial-Mesenchymal Transdifferentiation in Pauci-Immune Crescentic Glomerulonephritis. *Nephrology Dialysis Transplantation*, **18**, 1777-1784. https://doi.org/10.1093/ndt/gfg231

[17] Nishitani, Y., Iwano, M., Yamaguchi, Y., Harada, K., Nakatani, K., Akai, Y., Nishino, T., Shiiki, H., Kanauchi, M., Saito, Y. and Neilson, E.G. (2005) Fibroblast-Specific Protein 1 Is a Specific Prognostic Marker for Renal Survival in Patients with IgAN. *Kidney International*, **68**, 1078-1085. https://doi.org/10.1111/j.1523-1755.2005.00500.x

[18] Hertig, A., Anglicheau, D., Verine, J., Pallet, N., Touzot, M., Ancel, P.Y., Mesnard, L., Brousse, N., Baugey, E., Glotz, D., Legendre, C., Rondeau, E. and Xu-Dubois, Y.-C. (2008) Early Epithelial Phenotypic Changes Predict Graft Fibrosis. *Journal of the American Society of Nephrology*, **19**, 1584-1591. https://doi.org/10.1681/ASN.2007101160

[19] Grande, M.T., Sánchez-Laorden, B., López-Blau, C., De Frutos, C.A., Boutet, A., Arévalo, M., Rowe, R.G., Weiss, S.J., López-Novoa, J.M. and Nieto, M.A. (2015) Snail1-Induced Partial Epithelial-to-Mesenchymal Transition Drives Renal Fibrosis in Mice and Can Be Targeted to Reverse Established Disease. *Nature Medicine*, **21**, 989-997. https://doi.org/10.1038/nm.3901

[20] Li, Y., Wen, X. and Liu, Y. (2012) Tubular Cell Dedifferentiation and Peritubular Inflammation Are Coupled by the Transcription Regulator Id1 in Renal Fibrogenesis. *Kidney International*, **81**, 880-891. https://doi.org/10.1038/ki.2011.469

[21] Hao, S., He, W., Li, Y., Ding, H., Hou, Y., Nie, J., Hou, F.F., Kahn, M. and Liu, Y. (2011) Targeted Inhibition of *β*-Catenin/CBP Signaling Ameliorates Renal Interstitial Fibrosis. *Journal of the American Society of Nephrology*, **22**, 1642-1653. https://doi.org/10.1681/ASN.2010101079

[22] He, W., Tan, R.J., Li, Y., Wang, D., Nie, J., Hou, F.F. and Liu, Y. (2012) Matrix Metalloproteinase-7 as a Surrogate Marker Predicts Renal Wnt/*β*-Catenin Activity in CKD. *Journal of the American Society of Nephrology*, **23**, 294-304. https://doi.org/10.1681/ASN.2011050490

[23] Tan, R.J., Zhou, D., Zhou, L. and Liu, Y. (2014) Wnt/*β*-Catenin Signaling and Kidney Fibrosis. *Kidney International Supplements*, **4**, 84-90. https://doi.org/10.1038/kisup.2014.16

[24] Zhao, Y., Masiello, D., McMillian, M., Nguyen, C., Wu, Y., Melendez, E., Smbatyan, G., Kida, A., He, Y., Teo, J.-L. and Kahn, M. (2016) CBP/Catenin Antagonist Safely Eliminates Drug-Resistant Leukemia-Initiating Cells. *Oncogene*, **35**, 3705-3717. https://doi.org/10.1038/onc.2015.438

[25] Wu, D. and Pan, W. (2010) Gsk3: A Multifaceted Kinase in Wnt Signaling. *Trends in Biochemical Sciences*, **35**, 161-168. https://doi.org/10.1016/j.tibs.2009.10.002