Original Paper

HuangQi Decoction Improves Renal Tubulointerstitial Fibrosis in Mice by Inhibiting the Up-Regulation of Wnt/ β -Catenin Signaling Pathway

Ming-Qian Jiang^a Li Wang^b Ai-li Cao^b Jie Zhao^a Xia Chen^a Yun-man Wang^a
Hao Wang^a Wen Peng^{a,b}

^aDepartment of Nephrology, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China, ^bLaboratory of Renal Disease, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Key Words

HuangQi decoction • Wnt/ β -catenin signaling pathway • UUO model • Tubulointerstitial fibrosis

Abstract

Object: To explore the effects of HuangQi decoction on tubulointerstitial fibrosis in mice and the Wnt/ β -catenin signaling pathway. **Methods:** Unilateral ureteral obstruction (UUO) model was used. A total of 120 C57/BL mice were randomly divided into 6 groups, sham group, sham+HuangQi decoction group (1.08 g/kg), UUO group, UUO+HuangQi decoction group (0.12, 0.36, 1.08 g/kg). Immunohistochemical analysis, RT-PCR and Western blot were employed to examine the proteins and genes related to the Wnt/ β -catenin signaling pathway. **Results:** In UUO mice models, expression levels of Wnt3,4, Frizzled4, LRP5,6, β -catenin, LEF-1, TCF-1, Snail, MMP2,7 genes were positively correlated with the degree of renal tubulointerstitial fibrosis, while expression levels of GSK-3 β , Axin, APC, CK1 were negatively correlated. HuangQi decoction could down-regulate expression levels of Wnt3,4, Frizzled4, LRP5,6, β -catenin, LEF-1, TCF-1, Snail, Twist, MMP2,7 and up-regulate expression levels of GSK-3 β , Axin, APC, CK1 and E-cadherin. **Conclusion:** HuangQi decoction could effectively inhibit the up-regulation of Wnt/ β -catenin signaling pathway induced by UUO, implying a possible role in improving renal interstitial fibrosis.

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M.-Q. Jiang and L. Wang contribute equally to this work.

Professor Wen Peng,
Professor Hao Wang

Department of Nephrology, Laboratory of Renal Disease, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, 164 LanXi Road, Shanghai 200062 (China)
E-Mail pengwen_01@vip.sina.com; E-Mail wang402hao@163.com

Introduction

Renal tubular interstitial fibrosis is the common ending of many end-stage chronic renal diseases, manifested as loss of normal nephrons, proliferation of fibroblasts and myofibroblasts and abnormal deposition of extracellular cell matrix (ECM) [1]. Under the circumstance of mild kidney injury, ECM production is the normal repair process. However, with more severe injury, the ECM production is accelerated, which results in generation of large amounts of fibrinous tissue which in turn worsens the renal injury. A vicious circle is formed, functional nephrons are gradually lost and renal functions gradually fail [2]. Current studies show that many molecular signaling pathways are involved in the development of renal interstitial fibrosis. However, the exact mechanism is still unclear and it lacks effective treatments and medications for renal interstitial fibrosis in clinical practice [3].

Wnt/ β -catenin is a highly conservative cell signaling system in evolution, playing a vital role in embryonic development, maintenance of homeostasis of organs and tissues and correlating with many human diseases [4]. With the lacking of Wnt signaling, most β -catenin in cytoplasm combines with E-cadherin (E-cad) on the cell membrane and is attached to the cytoskeleton protein-actin, which mediates intercellular adhesion and integrity of cellular barriers [5]. A small amount of β -catenin combines with the tumor suppressing gene encoded APC proteins in the cytoplasm, scaffold protein Axin, glycogen synthase kinase (GSK-3 β) and casein kinase I α (CK I α) to form multi-protein degradation complexes in which GSK-3 β could phosphorylate the N-ending of β -catenin and lead to degradation of β -catenin by ubiquitin. Thus, cellular concentration of β -catenin is kept at a lower level to prevent the translocation of β -catenin into the nucleus [6]. When the classic Wnt signaling pathway is activated, Wnt protein combines with FZD complex and LRP5/6, goes through the cell membrane and dislocates GSK-3 β /APC/Axin/CK I α , which disturbs the degradation balance of β -catenin. Thus, β -catenin is accumulated in the cytoplasm, transferred into the nucleus, combines with the T-cell specific transcription factor (TCF) and/or lymphoid enhancer factor (LEF) and regulates the transcription of down-stream target genes [7]. More and more evidence indicates that Wnt/ β -catenin is involved in many chronic kidney diseases including diabetic renal disease [8], polycystic renal disease [9], lupus nephritis [10] and renal fibrosis [11], implying that abnormal Wnt/ β -catenin signaling plays a crucial role in renal fibrosis. Thus, it could be a new target for prevention of renal interstitial fibrosis.

HuangQi decoction, which was originally recorded in *Renzhai Zhizhi Fanglun* at song dynasty, has been used in China for thousands of years. It is composed of Astragalus, Poria, Trichosanthes roots, Ophiopogon, Schisandra, licorice and Rehmannia. Great progress has been made in the pharmaceutical study of chemical components of the HuangQi decoction. For example, HuangQi monomer astragaloside improves renal tubular interstitial fibrosis through not only regulating TGF- β /smad [12] and wnt/ β -catenin signaling pathways [13], but also inhibiting MAPK signaling pathway [14]. Besides, studies showed that the astragaloside combined with the ferulic acid could ameliorate fibrosis [15]. Poria could effectively improve renal tubular interstitial fibrosis in chronic renal diseases [16]. Ruscogenin extracted from MaiMenDong could effectively ameliorate diabetic renal disease symptoms in rats induced with STZ through inhibiting inflammation and fibrosis [17]. Schisandra extracts could inhibit renal interstitial fibrosis in diabetic mice [18], protect drug-induced kidney injuries [19]. Dried rehmannia roots and their extracts could improve the chronic renal failure induced by excision of 5/6 kidneys and renal functions [20]. Licorice extracts isoflavones could inhibit renal mesangial cells fibrosis and inflammation [21] and prevent mesangial proliferation and extracellular matrix deposition [22]. Therefore, HuangQi decoction is a promising candidate traditional Chinese medicine complex for renal fibrosis prevention, superior to any monomer.

This study uses UUO mice model to explore the target of the Wnt/ β -catenin signaling pathway in renal tubular interstitial fibrosis in cell membrane, cytoplasm and nucleus, thus to elucidate the exact mechanism of the HuangQi decoction for prevention of renal interstitial fibrosis.

Materials and Methods

Herbs and reagents

HuangQi decoction is composed of Astragalus, Poria, Trichosanthes roots, Ophiopogon, Schisandra, licorice and Rehmannia. Herbs were bought from Shanghai Hua Yu Chinese Herbs Co., LTD (Table 1). HuangQi 2kg, Poria 2kg, Trichosanthes root 2kg, Ophiopogon 2kg, Schisandra 1kg, licorice 1kg, Rehmannia 3kg were mixed, added with water of 4 times volume, extracted 3 times and concentrated to thick decoction. Then the concentration of the decoction was adjusted to 70% with ethanol. Overnight the supernatant was collected and dried in a drying oven at 105 °C for 48h. Dry decoction 1730g was obtained. The production rate was 13.3%.

Antibodies of Wnt4, Frizzled4, Axin, TCF-1 and Twist were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Wnt3, MMP2, APC, E-cadherin, β -catenin and α -tubulin were purchased from Epitomics (Burlingame, CA, USA); LRP5, LRP6, GSK-3 β , CK1, Non-Phospho (Active) β -catenin (Ser33/37/Thr41) β -actin, Snail, MMP7 and goat-anti-rabbit secondary antibodies were provided by Cell Signaling Technology (Danvers, MA, USA); LEF-1 was purchased from Prosci (MO, California, USA) and Histone H1 was purchased from Abcam (Cambridge, MA, USA). ECL developer was purchased from Millipore (Billerica, MA, USA); Cytoplasm and nucleus extract kits was purchased from Cayman (Michigan, MO, USA); BCA protein quantity kits and PVDF membranes were purchased from Pierce (Rockford, IL, USA); Immunohistochemistry kits and DAB staining solution were provided by Wuhan Boster Biotech Company Ltd. RT-PCR kits include RNAsimple Total RNA Kit, FastQuant RT Kit (with gDNase) and SuperReal PreMix Plus (SYBR Green) were purchased from TianGen (TIANGEN BIOTECH (BEIJING) CO., LTD, China). Primers were synthesized by Sangon (Shanghai Co. Ltd., China). RT-PCR machine (ViiA7 Standard 96, Applied biosystems, USA).

Animal model establishment

This study was approved by the Ethics Committee of Putuo Hospital, Shanghai University of Traditional Chinese Medicine. Male C57/BL mice (weight 18 $\pm$ 2g, 120) were purchased from Shanghai Super-B&K laboratory animal Corp. Ltd with SPF feeding. Mice were randomly allocated into sham group, sham+HuangQi decoction (1.08g/kg), UUO group, UUO+HuangQi decoction (0.12g/kg) group, UUO+HuangQi decoction (0.36g/kg) group, UUO+HuangQi decoction (1.08g/kg) group. According to body surface area, the HuangQi decoction was mixed with 0.5% sodium carboxymethyl cellulose into 0.12g/ml, 0.36g/ml, 1.08g/ml suspension, which was administered intragastrically on the next day of animal model establishment consecutively for 14 days.

Before the UUO animal model establishment, the mice were given free access to water and anesthetized with 3% sodium pentobarbital. Left abdominal incision was made and skin, muscle of the abdominal wall was incised to expose and dissect the left ureter. In the model group, the left ureter was ligated twice with 4-0 silk thread, one near the lower pole of the left kidney, another below the first ligation. The ureter was cut between two ligations. In the sham group, the left ureter was only dissected with no other manipulations. After 14 days of drug administration, blood was obtained from the eye ball and the left kidneys were harvested and cut into 2 pieces from the sagittal plane. One was submerged into 10% formalin for pathological and immunohistochemical examinations and one was conserved at -80 °C for protein and RNA tests.

Table 1. HuangQi decoction components

Chinese term	Scientific name	Generic Name	Dose (kg)	Product lot
Huangqi	Astragalus membranaceus (Fisch.) Bunge.	Radix astragali	2	H2013031806
Fuling	Poria cocos (Schw.) Wolf	Wolfiporia extensa	2	H2012082001
Maidong	Ophiopogon japonicus	Radix Ophiopogonis	2	H2012101604
Wuweizi	Schisandra chinensis	shizandra	1	H2013050303
Gancao	Glycyrrhiza uralensis Fisch.	Liquoric Root	1	H2013052901
Dihuang	Rehmannia glutinosa (Gaetn.) Libosch. ex	Radix Rehmanniae	3	H2012091801
Gualou	Trichosanthes kirilowii Maxim	Fructus Trichosanthis	2	H2011011706

Renal functions

On the 14th day after UUO, the blood was obtained from the eyeball, allowed to stand for 1h, centrifuged at 2000 g for 15min at 4°C. The serum was collected. Serum creatinine (Scr) and blood urea nitrogen (BUN) were tested among different groups with creatinine colorimetric assay kits (BioVision, USA) and BUN colorimetric detection kits (Arbor, USA).

Histological and immunohistochemical examinations

Renal tissues were placed into 10% formalin for 24 h and dehydrated in gradient, cleared, embedded in paraffin, cut into 3 μ m slices and stained with Hematoxylin-eosin (HE) and Masson. The sections were observed under light microscopy. Ten non-overlapping visions were randomly selected under a 400-times magnification microscope. For Masson staining, renal tubular interstitial fibrosis relative positive area=collagen staining area (blue)/whole vision area.

Immunohistochemical tests of Wnt3, wnt4 and β -catenin: paraffin-embedded sections were deparaffinized and 0.01 mol/L Citrate buffer (pH 6.0) was used to repair. H₂O₂ (0.3%) solution was used for inhibition of endogenous peroxidases for 20 min. The mixture was incubated with 20% goat serum at room temperature for 30 min, Wnt3 (1:1000), wnt4 (1:50), β -catenin (1:1000) at 4°C overnight for another 1h incubation at 37°C. After washed with 0.01 mol/L PBS, goat-anti-rabbit secondary antibodies were added and co-incubated at 37°C for 20min. DAB was used for coloration for 10 min. Hematoxylin staining was for 30 s, flush for 5 min. After conventional dehydration, clearing and mounting with neutral gum, slices were observed under a light microscope. Immunohistochemical sections were observed under a light microscope at 400 times. The Image Pro Plus image analysis software was employed to calculate its mean optical density and then analyzed statistically.

Western blot

Renal tissue total protein extract: RIPA lysis buffer was added in renal tissues (1 mL RIPA lysis buffer/1mg renal tissues) which were lysed with sonication on ice and then put into 2 mL EP tubes. Then the mixture was centrifuged at 12000 rpm at 4°C for 15min and the supernatant was collected. Renal cytoplasmic and nuclear proteins were extracted according to the instructions by Cayman (Michigan, MO, USA). After being weighed, renal tissues were homogenized, added with 1 $\times$ hypotonic buffer containing DTT and Nonidet P40 per gram of tissue and placed on ice for 15 min. Then the homogenate was transferred to centrifuge tubes and centrifuged at 300 rpm at 4°C for 10 min. 1 $\times$ Extraction buffer (containing protease and phosphatase inhibitors) was added, spun for 30 s, placed on ice for 10min and repeated for 6 times. Then the homogenate was centrifuged at 4°C at 14000 rpm for 10min and the supernatant contained the nucleoprotein. In the original renal tissues were added 500 μ L 1 $\times$ hypotonic buffer and 50 μ L 10% Nonidet P40. Then it was centrifuged at 14,000 rpm for 30s at 4°C. The supernatant contained cytoplasmic proteins. The concentration of proteins extracted was measured according to BCA kits instructions. For each tissue protein sample 30 μ g was used. SDS polyacrylamide gels (SDS-PAGE) electrophoresis was performed. Conditions were electrophoretic transfer voltage 80v to 120v, wet transfer, transfer voltage 100mv, 45-70min. 5 % BSA blocking solution was added for 1h and then added with Wnt3 primary antibodies (1:3000 dilution), Wnt4 (1: 100 dilution), Frizzled4 (1:80 dilution), LRP5 (1: 500 dilution), LRP6 (1: 500 dilution), GSK-3 β (1: 500 dilution), Axin (1: 100 dilution), APC (1: 500 dilution), CK1 (1: 500 dilution), β -catenin (1: 5000), Active β -catenin (1: 500 dilution), LEF-1 (1: 500 dilution), TCF-1 (1: 100 dilution), Snail (1: 250 dilution), E-cadherin (1: 500 dilution), Twist (1: 100 dilution), MMP-2 (1: 1000 dilution), MMP-7 (1: 500 dilution), β -actin (1: 1000 dilution), Histone H1 (1: 1000 dilution) and α -tubulin (1: 500 dilution). It was incubated at 4°C overnight and added with TBST buffer to wash the membrane for 3 $\times$ 10 min/times. Then an appropriate amount of ECL developer was added and the Bio-rad Gel Dol EZ imaging system was employed. The Image J software was used to calculate the gray value of the target band.

RT-PCR

Real-time quantitative PCR was performed. According to the gene sequence published in Genbank database, the Primer 5.0 software was used to design the primer (Table 2). According to the instructions of kits, total RNA were extracted and concentrations were measured with a UV spectrophotometer at a wavelength of 260/280. According to kits instructions, RNA were reversely transcribed into cDNA. Polymerase chain reaction system (20 μ L) included 2 $\times$ super Real 10 μ L, upstream and downstream primers 0.6 μ L, reverse

Table 2. Primers used for quantitative Real-time PCR

Genes	Forward primer	Reverse primer
Wnt3	5'-CCCGCTCAGCTATGAACAAG-3'	5'-GTTTCTCCACCATCTCG-3'
Wnt4	5'-GTTTCTCGCAGCTCTCTCT-3'	5'-CCTGCGACTCCTCGTCTC-3'
Fzd4	5'-CGGTTTATGTGCCAATGTGC-3'	5'-ATGTGGTTGTGGTCGTTCTG-3'
LRP5	5'-TACTTTGTCTGCCAGCGTGT-3'	5'-GGGGCTCCACCAACATACTC-3'
LRP6	5'-ATGGCTCAAGTCGCTTCGTT-3'	5'-AGCTTCCGTCCTGATAGTC-3'
GSK-3 β	5'-GAACCACCTCCTTTGCGGA-3'	5'-TGGTTACCTTGCTGCCATCTT-3'
APC	5'-GCTCAACTTCAGAACCTCAC-3'	5'-ATCTGCCTTGCTTCATACTC-3'
Axin	5'-TGCTATTGGAGACTGCTGCC-3'	5'-CAGGGCATAGCCAGAGTTGA-3'
β -catenin	5'-ACTGTTCTACGCCATCACGA-3'	5'-GCTTGCTCTCTTGATTGCCA-3'
TCF	5'-GCTGTGGGGTGAATGAAGTA-3'	5'-AGACCGGCACATTTCATGGGT-3'
LEF	5'-CATCAAATAAAGTGCCCGTG-3'	5'-GGGGTAGAAGGTGGGGATT-3'
Snail	5'-GGATGTGAAGAGATACCACTGC-3'	5'-GCCAGACTCTTGCTGCTTGT-3'
MMP-2	5'-GGGGTCCATTTTCTTCTTCA-3'	5'-CCAGCAAGTAGATGCTGCCT-3'
MMP-7	5'-GCATTTCTTGAGTTGTCC-3'	5'-CACATCAGTGGGAACAGGC-3'
β -actin	5'-GCGTGACATCAAGAGAAGC-3'	5'-CTCGTTGCCAATAGTGATGA-3'
α -tubulin	5'-TCCAGGGCTTCTTGTTTTC-3'	5'-GCTTGGACTTCTTCCGTAA-3'

transcription product 4 μ L, 50 $\times$ ROX 2 μ L and RNase-free water 2.8 μ L. PCR employed the two-step reaction conditions: 95 °C denaturation for 15 min, 40 cycles under the following conditions: degenerative at 5°C for 10s, annealing/extension for 30 s. β -actin was used as an internal control for membrane and cytoplasmic proteins and α -tubulin for nuclear proteins. The melting curve was used to evaluate the reliability of the results. The target gene expression volume was calculated based on CT value (amplification power curve inflection point) and $2^{-\Delta\Delta Ct}$.

Statistical analysis

The GraphPad Prism 5.0 software (GraphPad Prism software Inc., San Diego, Calif, USA) was used for statistical analysis. Results were expressed as mean $\pm$ standard deviation. Data conformed to the normal distribution between the two groups were compared using the t test and paired *t*-test. Multiple comparisons between groups were performed with One-Way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Effects of HuangQi decoction on the pathological and functional changes of kidneys

Under light microscopy with HE staining, in the sham group, no significant changes were found. The glomerular and tubular structures were normal and tubules were packed closely. In UUO model group, the interstitium became significantly widening with infiltration of inflammatory cells. The glomerular capillaries became dilated and tubules underwent necrosis and atrophy. In HuangQi decoction groups, kidney injuries were gradually reduced. In the high dose group, tubular epithelial cell degeneration and mild inflammatory cell infiltration was noticed (Fig. 1A). Masson staining showed that in the sham group, no collagen fiber proliferation was found and the tubular basement membrane was clearly visible. In the UUO group, in renal interstitial tissues, massive stained collagen could be seen and interstitial fibrous tissue showed bundle and reticular hyperplasia. In the low, middle and high dose HuangQi decoction groups, collagen staining area gradually decreased (Fig. 1C). Serum creatinine and blood urea nitrogen tests showed that in the UUO group, serum creatinine and blood urea nitrogen values were higher than those in the sham group and the difference was statistically significant ($P < 0.001$). In UUO groups given low, middle and high dose HuangQi decoction, serum creatinine and blood urea nitrogen levels gradually decreased in a concentration-dependent manner. Renal functions gradually improved with the increasing dose. In the high dose group, compared with the UUO group, serum creatinine

Fig. 1. Effects of HuangQi decoction on pathological changes of renal interstitial fibrosis in UUO mice models. A. HE staining; B. Scales of renal tubular injuries; C. Masson staining; D. Masson staining statistical analysis. a.sham group; b.sham+high dose group (1.08g/kg); c.UUO group; d.UUO+low dose group (0.12g/kg); e.UUO+middle dose group (0.36g/kg); f.UUO+high dose group (1.08g/kg). *** $P < 0.001$, compared with the sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the UUO group. $n = 12-15$.

Fig. 2. Influence of HuangQi decoction on renal functions in UUO mice models. A. Serum creatinine; B. Serum urea nitrogen, *** $P < 0.001$, compared with the sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the UUO group. $n = 12-15$.

and blood urea nitrogen were significantly decreased and the difference was statistically significant ($P < 0.001$) (Fig. 2).

HuangQi decoction inhibited cytomembrane wnt/ β -catenin signaling pathway levels

Immunohistochemical analysis of Wnt3, 4 showed that in the UUO group, Wnt3, 4 expression were significantly increased compared with the sham group and the difference was statistically significant ($P < 0.001$). RT-PCR examined the mRNA levels of Wnt3,4, Frizzled4 and LRP5,6 (Fig. 5A-E). Western blot examined the membrane expression levels of Wnt3,4, Frizzled4, LRP5,6 (Fig. 6). Results showed that Wnt3,4, Frizzled4 and LRP5,6 levels were significantly higher than those of the sham group both for mRNA and protein ($P < 0.001$).

Fig. 3. Influence of HuangQi decoction on Wnt3,4 expression in UUO mice model. A,C. Immunohistochemical tests for Wnt3,4 expression; B, D. Statistical analysis of A, C, *** $P < 0.001$, compared with the Sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the UUO group. $n=12-15$.

Fig. 4. Influence of HuangQi decoction on nuclear β -catenin expression in UUO mice models. A. Immunohistochemical tests for nuclear β -catenin; B. Statistical analysis of A, *** $P < 0.001$, compared with the Sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the UUO group. $n=12-15$; C. Western blot for nuclear β -catenin; $n=3-5$. D. RT-PCR nuclear β -catenin mRNA, $n=3-5$.

In the low, middle and high dose of HuangQi decoction group, mRNA and protein levels of Wnt3,4, Frizzled4, LRP5,6 gradually decreased in a concentration-dependent manner.

Fig. 5. mRNA levels of cytomembrane, cytoplasmic key proteins in UUO mice models. A-H. PCR results of Wnt3,4, Frizzled4, LRP 5,6, GSK-3 β , Axin, APC, *** $P < 0.001$, compared with the sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the UUO group, $n = 3-5$.

HuangQi decoction inhibited cytoplasmic wnt/ β -catenin signaling pathway levels

Immunohistochemical analysis of β -catenin expression showed that in the UUO model group β -catenin level was significantly higher than that of the sham group (Fig. 4A, B) and the difference was statistically significant ($P < 0.001$). RT-PCR examined mRNA levels of β -catenin, GSK-3 β , Axin and APC (Fig. 4D, 5F-H). Western blot examined the cytoplasmic expression levels of GSK-3 β , Axin, APC, CK1, β -catenin and active β -catenin (Fig. 7). Results showed that GSK-3 β , Axin, APC and CK1 levels in UUO group were significantly lower than those of the sham group ($P < 0.001$). The expression of β -catenin and active- β -catenin was significantly higher in the UUO group compared with the sham group ($P < 0.001$). In the

Fig. 6. Influence of HuangQi decoction on expression of Wnt/ β -catenin cytomembrane protein in UUO mice models. A. Effects of HuangQi decoction on expressions of Wnt3,4, Frizzled4, LRP5,6; B-F. Statistical analysis of A. *** $P < 0.001$, compared with the sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the UUO group. $n = 3-5$.

Table 3. Nuclear mRNA levels. *** $P < 0.001$; compared with the sham group; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$, compared with the UUO group

Groups	LEF-1	TCF-1	Snail	MMP-2	MMP-7
Sham	0.902±0.091	0.946±0.141	1.126±0.362	1.024±0.163	1.175±0.381
Sham+high dose	0.538±0.246	0.825±0.189	1.102±0.524	1.329±0.361	0.620±0.285
UUO	10.650±1.091***	8.367±0.881***	10.901±2.179***	13.880±1.935***	8.071±1.655***
UUO+low dose	5.709±0.854#	4.895±0.6971#	6.931±1.395	8.388±2.096	6.459±0.998
UUO+middle dose	4.664±0.877 #	2.964±0.632##	4.089±1.315	5.844±1.333#	2.906±0.611#
UUO+high dose	3.376±0.925 ##	1.760±0.295##	3.001±0.778#	4.716±1.400#	2.041±0.3894#

low, middle and high dose of HuangQi decoction group, mRNA and protein levels of GSK-3 β , Axin, APC and CK1 gradually increased and β -catenin, Active β -catenin levels decreased in a concentration-dependent manner.

Fig. 7. Influence of HuangQi decoction on expression of Wnt/ β -catenin cytoplasmic protein in UUO mice models. A. Expressions of GSK-3 β , Axin, APC, CK1, β -catenin, Active β -catenin; B-G. Statistical analysis of A. *** $P < 0.001$, compared with the Sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the UUO group. $n = 3-5$.

HuangQi decoction inhibited nuclear Wnt/ β -catenin signaling pathway levels

Western blot was used to examine the nuclear expression level of β -catenin (Fig. 4C). In the UUO group, nuclear β -catenin level was significantly higher than that of the sham group ($P < 0.001$). In the low, middle and high dose group, nuclear β -catenin level gradually decreased in a concentration-dependent manner. RT-PCR examined the mRNA level of LEF-1, TCF-1, Snail, MMP2,7 (Table 3). Western blot examined the nuclear expression of LEF-1, TCF-1, Snail, E-cadherin, Twist, MMP2,7 (Fig. 8). Results all showed that mRNA and protein levels of LEF-1, TCF-1, Snail, MMP2,7 in the UUO group were all significantly higher than those in

Fig. 8. Influence of HuangQi decoction on expression of Wnt/ β -catenin nucleus protein in UUO mice models. A. Expressions of LEF-1, TCF-1, Snail, E-cadherin, Twist, MMP2,7; B-H. Statistical analysis of A. *** $P < 0.001$, compared with the Sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the UUO group. $n = 3-5$.

the sham group ($P < 0.001$), while the E-cadherin level in the UUO group was significantly lower than that in the sham group ($P < 0.001$). In the low, middle and high dose groups, mRNA and protein levels of LEF-1, TCF-1, Snail, Twist, MMP2,7 gradually decreased while the E-cadherin protein level gradually increased in a concentration-dependent manner.

Discussion

This study showed that HuangQi decoction could improve the pathological and functional conditions of kidneys in a concentration-dependent manner. It could inhibit cytomembranous, cytoplasmic and nuclear protein expression of Wnt/ β -catenin signaling pathway, implying that HuangQi could block Wnt/ β -catenin signaling pathway to ameliorate the renal interstitial fibrosis.

Proteins in the Wnt family are secretory glycoproteins with rich cysteine residues and highly conservative in evolution. Wnts and FZD receptor genes were expressed on almost all normal mice kidneys, contributing to the normal tissue dynamic balance [23]. Wnt signaling pathway is widely involved in the onset and development of renal diseases, especially in the renal interstitial fibrosis [24]. In UUO model, the Wnt/ β -catenin signaling pathway was abnormally activated in the obstructed kidney. Wnt protein combined with the FZD receptor complex and LRP5/6, which resulted in degradation of β -catenin inhibitors. Thus, β -catenin accumulated in the nucleus and combined with the T-cell-specific transcription factor (TCF) and/or lymphoid enhancer factor (LEF) to regulate the Wnt downstream genes, jointly participating in the pathogenesis of renal interstitial fibrosis [25]. Studies on Wnts and FZD receptor genes in mice with unilateral ureteral ligation found that Wnt3,4 expressed in the largest amount while Fzd4 expression was continually up-regulated after UUO [26]. Our previous studies also demonstrated that Wnt3,4 and Fzd4 were significantly enhanced in the obstructed kidney. Thus Wnt3,4, Fzd4 were selected to explore the effects of HuangQi decoction on the Wnt/ β -catenin signaling pathway. Studies proved that Wnt3, 4 and Fzd4 were activated in mice UUO model and HuangQi decoction could ameliorate renal interstitial fibrosis in a concentration-dependent manner (Fig. 5A-C, 6). LRP5 and LRP6 comprise low density lipoprotein receptors subsets with cytokine and growth factor receptor characteristics and are regarded as receptors of Wnt. After combining with Wnt, LRP5/LRP6 are phosphorylated by cellular kinases to generate Axin combination sites and maintain the stability of intracellular β -catenin which moves into the nucleus and regulates its target genes [25, 27]. Results in this study were consistent with those in previous reports. In UUO model, after Wnt3,4 being activated, LRP5/LRP6 expression was up-regulated and involved in the regulation of Wnt/ β -catenin signaling pathway (Fig. 5D, E, 6).

Regulation of stability and activity of β -catenin is the core event of the Wnt signaling pathway [28]. A series of proteins including Axin, APC, CK1, GSK3 β comprise the degradation complex which controls the transfer of β -catenin into the nucleus through phosphorylation, thus regulating the transcription mediated by TCF/LEF [29]. Axin is a scaffold protein which assembles the Wnt degradation complex and participates in the phosphorylation, serving as an important inhibitor of the Wnt pathway [30]. Casein kinase1 (CK1) is an important protein of the Axin complex and could regulate the Wnt pathway through phosphorylating β -catenin [31]. GSK3 β is a unique multifunctional serine/threonine kinase which negatively regulates the Wnt pathway through phosphorylation. It could combine with Axin, APC and CK1 to form the complex which in turn enhances the activity of GSK3 β and degradation of β -catenin [32]. In the UUO model, the Wnt signaling pathway was activated, which depolymerized the complex of GSK3 β , Axin, APC, CK1 and enhanced the phosphorylation of GSK-3 β on β -catenin. This study proved that HuangQi decoction could inhibit the depolymerization of the degradation complex to some extent and inhibit the phosphorylation of β -catenin, which improved the renal interstitial fibrosis (Fig. 5F-H, 7).

LEF1/TCF1 is the well-known downstream target of the Wnt/ β -catenin pathway. The LEF1/TCF1 transcription factor is the nuclear effector of the Wnt signaling pathway, serving as the ultimate target of the Wnt dependent pathway to regulate the growth and death of cells. When the Wnt signaling pathway is activated, β -catenin is transferred to the nucleus and combined with the amino-terminal of the LEF1/TCF1, which enhances the interaction with chromatin and regulates related genes [33]. This study showed that HuangQi decoction could down-regulate LEF1/TCF1 expression in UUO mice and inhibit the downstream genes expression (Table 3, Fig. 8). A number of studies demonstrated that the epithelial-

mesenchymal transition (EMT) participated in the onset and development of the renal interstitial fibrosis [34]. Decrease or loss of E-cadherin expression is an important marker of the occurrence of the EMT [35]. E-cadherin inhibits β -catenin signaling in a unique adhesion manner. In the kidney, E-cadherin is an important component of the tight junctions between renal tubular epithelial cells, playing a vital role in maintaining the structural and functional integrity of epithelial cells [36]. As a transcription factor, Snail could promote cell migration in fibrosis through decreasing cell adhesion and interact with many target genes among which E-cadherin is regarded as a direct one [37]. Besides, Twist could act on the transcription sequence of the promoter of target genes and modify E-cadherin in transcription [38]. And a recent study suggests that the renal transcript levels of the β -catenin target genes, including *Twist* and *Lef1* were significantly increased in UUO mice [39]. Twist, Snail could jointly inhibit the transcription of E-cadherin and induce EMT, thus contributing to the development of fibrosis. This study showed that HuangQi decoction could down-regulate Twist, Snail expression and up-regulate E-cadherin in a concentration-dependent manner and delay the progression of EMT (Table 3, Fig. 8).

Matrix metalloproteinases (MMPs) are zinc or calcium-dependent endopeptidases, playing an indispensable role in the degradation of almost all ECM. Additionally, MMPs can affect inter-cellular transmission of information between cells and matrix, regulate intercellular adhesion, deplete E-cad, promote epithelial cells transformation [40]. The current studies have found that in UUO MMP2 and MMP7 are significantly up-regulated, commonly participating in the occurrence and development of renal tubular interstitial fibrosis [41, 42]. Additionally, some people believed that LEF1/TCF1 could regulate the expression of MMP2 to promote the endothelial cells migration [43]. This study also proved that HuangQi decoction could decrease MMP2 and MMP7 and increase E-cad expressions in a concentration-dependent manner, inhibit endothelial cells transformation and ameliorate renal interstitial fibrosis through degrading ECM in fibrosis (Table 3, Fig. 8).

In general, HuangQi decoction could not only improve the pathological changes, serum creatinine and urea nitrogen of the obstructed kidney in UUO mice models, but ameliorate renal interstitial fibrosis through inhibiting Wnt/ β -catenin signaling pathway. However, HuangQi decoction cannot fully reverse the kidney injury in UUO models. Thus, how to manipulate and inhibit the accumulation of β -catenin in the cytoplasm in the early stage of renal interstitial fibrosis require further study.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81473480, 81403235); Construct Program of the Key Discipline of State Administration of Traditional Chinese Medicine of the People's Republic of China; Leading Academic Discipline Project of State Administration of Traditional Chinese Medicine of China, Talent Project of Integrative Medicine of Shanghai Municipal Health Bureau (ZYSN XD012-RC-ZXY); Key Medical Discipline Project of Shanghai Municipal Health Bureau (ZK2012A34); Independent Innovation Research Fund of Putuo District Science and Technology Committee (2012PTKW002) and Putuo Hospital Fund (2013SR1231).

Disclosure Statement

No.

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