Original Paper

HuangQi Decoction Ameliorates Renal Fibrosis via TGF- β /Smad Signaling Pathway *In Vivo* and *In Vitro*

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Key Words

HuangQi decoction • TGF- β /Smad signaling pathway • UUO model • Renal interstitial fibrosis

Abstract

Objective: Traditional Chinese Medicine compound HuangQi decoction is widely used in clinical treatment of chronic kidney disease, but its role on renal interstitial fibrosis and the underlying mechanism remains unclear. The aim of this study is to investigate the effect of HuangQi decoction on renal interstitial fibrosis and its association with the TGF- β /Smad signaling pathway. **Methods:** A total of 120 C57/BL mice were randomly divided into six groups: sham group, sham plus high-dose HuangQi decoction (1.08g/kg) group, unilateral ureteral obstruction (UUO) model group, and UUO model plus low to high doses of HuangQi decoction (0.12g/kg, 0.36g/kg and 1.08g/kg respectively) groups. Animals were sacrificed 14 days after the administration and ipsilateral kidney tissue was sampled for pathologic examinations. Immunohistochemistry, PCR and western blot were used to detect the expressions of related molecules in the TGF- β /Smad signaling pathway. TGF- β 1 was used in *in vitro* experiments to induce human kidney proximal tubule epithelial cells (HK2). **Results:** HuangQi decoction improved ipsilateral kidney fibrosis in UUO mice and downregulated the expressions of TGF- β 1, T β RI, T β RII, Smad4, Smad2/3, P-Smad2/3, α -SMA, collagen type I, III and IV in a dose-dependent manner while upregulated the expression of Smad7 in the same fashion. Similar results were found in *in vitro* studies. **Conclusion:** The protective effect of HuangQi decoction for unilateral ureteral obstruction kidney damage in mice was mediated by downregulating the TGF- β /Smad signaling pathway.

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Introduction

Renal interstitial fibrosis is a common pathway of chronic kidney disease up until end stage renal disease (ESRD) [1-3], which is a key indicator in renal prognosis reflecting the severity of the renal function deterioration. Tubular epithelial-mesenchymal transition (EMT) plays a key role in the development and progression of renal interstitial fibrosis [4]. When EMT is activated, the kidney fibroblasts could transdifferentiate into myofibroblasts (MFBs) that express α -SMA and fibronectin and secrete excess extracellular matrix (ECM), the deposition of which within the tissue would eventually cause fibrosis. Transforming growth factor- β 1 (TGF- β 1) is currently the strongest known fibrogenic factor, which can induce EMT activation in renal tubular epithelial cells, promote the synthesis of extracellular matrix components and inhibiting its degradation; On the other hand, it could stimulate the differentiation of tubular epithelial cells into MFBs, and thus play an important role in the pathogenesis of renal interstitial fibrosis [5]. Although not reversible at the late stage, renal interstitial fibrosis, which may have great significance in the prognosis of the disease, can be ameliorated and renal function could be improved provided with early and timely diagnosis and treatment.

Chinese medicine believes that "spleen-kidney deficiency" is an internal condition and key factor in the development of renal interstitial fibrosis. Therefore, "tonifying the spleen and the kidney" is the main principle of the ancient Chinese physicians in the treatment of renal interstitial fibrosis, and HuangQi decoction is one of the most classic recipes in the clinical treatment of chronic kidney disease. HuangQi decoction was first recorded in Yang shiying's Renzhai Zhizhi Fang Volume 17 dating back to Northern Song Dynasty. The whole prescription consists of 7 kinds of Chinese medicine including *Radix astragali*, *Wolfiporia extensa*, *Fructus trichosanthis*, *Radix ophiopogonis*, *Shizandra*, *Liquoric root*, and *Radix rehmanniae*. HuangQi decoction is widely used in Traditional Chinese Medicine (TCM) practices due to its satisfactory efficacy, and although our published study has showed that the compound is a promising candidate for renal interstitial fibrosis [6], the mechanism of action still need to be further explored. In this study, we investigated the effect of HuangQi decoction in three doses, from low to high, on renal interstitial fibrosis in mice models with unilateral ureteral obstruction and its association with the TGF- β /Smad signaling pathway. In addition, we induced human kidney proximal tubule epithelial cells (HK2) *in vitro* with TGF- β 1 to explore the mechanism of action of HuangQi decoction for interstitial fibrosis.

Materials and Methods

Drugs and reagents

All crudes of HuangQi decoction drugs were purchased from Shanghai Hua Yu Chinese Herbs Co., LTD. The gradient of the decoction consisted of 2 kg of *Radix astragali* (lot No. H2013031806), 2 kg of *Wolfiporia extensa* (lot No. H2012082001), 2 kg of *Fructus trichosanthis* (lot No. H2011011706), 2 kg of *Radix ophiopogonis* (lot No. H2012101604), 1 kg of *Shizandra* (lot No. H2013050303), 1 kg of *Liquoric root* (lot No. H2013052901), and 3 kg of *Radix rehmanniae* (lot No. H2012091801).

Antibodies to TGF- β 1, T β RI, T β RII, Smad4, α -SMA, collagen I, III and IV were purchased from Abcam (Cambridge, MA, USA); Smad7 was purchased from R&D Systems (Minneapolis, USA); Smad2/3, p-Smad2/3, fibronectin, β -actin and GAPDH, as well as goat anti-rabbit and goat anti-mouse antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). BCA protein quantification kit was purchased from Pierce (Rockford, IL, USA). ECL developing solution was purchased from Billerica (MA, USA).

The real-time quantitative PCR kit was purchased from Tiangen Biochemical Technology (Beijing, China), including RNeasy Mini Kit (QIAGEN, Valencia, CA), AMV First Strand cDNA Synthesis kit (QIAGEN, Valencia, CA) and SYBR green PCR master mix (QIAGEN, Valencia, CA). The primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

Animals and modeling

Animal experiments were approved by the ethics committee of Putuo Hospital, Shanghai University of Traditional Chinese Medicine. A total of 120 male C57/BL mice (body weight 18 ± 2 g) purchased from Shanghai Sippr-BK experimental animal Co., Ltd. with SPF-class breeding. After one week of adaptive feeding, mice were randomly divided into six groups. The selective doses of HuangQi decoction for animal study were calculated basing on the human optimal equivalent dose of 12 g raw herbs. A total amount of 1.0 g of extract powder that was about 13.3% of the raw herb was obtained. Similar with our previous study, HuangQi decoction at doses of 0.12, 0.36, and 1.08 mg/kg (dilutes with 0.5% sodium carboxymethyl cellulose) were chosen [6]. Mice in each group ingested the suspensions by gavage for 14 days after completion of modeling. Pure sham group and model group were given 0.5% sodium carboxymethyl cellulose. Mice were fasted the day before modeling. Anesthesia was done by intraperitoneal injection of 3% sodium pentobarbital, an incision on the left side of the abdomen was made to expose and dissect the left ureter, which was then ligated with 4-0 silk. The remnant middle ureter was sheared off. The sham group only underwent laparotomy and exposure of the left ureter. Blood samples and left kidney specimens were taken. The kidney specimen was cut open sagittally, half of which was fixed in 10% formalin, and the remaining half was stored at -80°C .

Cell treatment

HK2 cells were the courtesy of Dr. Zhang Xuemei from the School of Pharmacy, Fudan University. HK2 cells were cultured using complete RPMI 1640 medium (Corning, USA) containing 10% heat-inactivated fetal bovine serum (Gibco, USA). Cells were incubated in at 37°C with 5% carbon dioxide. HK2 cells in the log phase of growth was taken and digested by 0.25% trypsin-EDTA (Sigma-Aldrich) before mixing with the culture medium into cell suspension (concentration of about $4 \times 10^4/\text{ml}$), which was then seeded in 96-well plates with 100 μl for each well. After 12 hours the culture medium was aspirated before HuangQi decoctions in concentration gradient of 0, 1, 3, 10, 30, 100, 300, 1000, 3000 and 10000 $\mu\text{g}/\text{ml}$ were added. After 24 hours, the drug solution was discarded before CCK8 was added. The cells were then cultured for 1-4 hours, the absorbance at 450 nm was measured using a microplate reader. The HK2 cells were pre-treated with HuangQi decoction at concentrations of 100, 300 and 1000 $\mu\text{g}/\text{ml}$ for 30 min, and then TGF- $\beta 1$ at 2.5 ng/ml was added. After treatment with HuangQi decoction for 24h, protein expression was measured. Briefly, the samples were lysed with D-hanks (1 ml lysate was added to every 10 μl PMSF) by sonication on ice. The supernatant was collected after centrifugation at 12,000 rpm at 4°C for 5 min. Protein quantification was determined by BCA kits instructions.

Histology and immunohistochemistry

The kidney tissue was fixed in 10% neutral formalin. After embedded in paraffin, sections were stained with H&E staining and Masson's Trichrome staining. The assessment of tissue injury in the obstructed kidney after H&E staining was in accordance with the scoring criteria described by Debelle et al. [7] with a slight alteration: A total of 10 unrepeatable cortical areas were randomly selected (observed under 400x light microscope), the renal interstitial injury was assessed according to the 4 parameters and 4 grades (Table 1). Kidney injury after ureter obstruction under Masson's Trichrome staining was assessed by the method described by Mizuguchi et al. [8]. Blue staining was considered the positive signal: 0 = normal; 1 = lesion range from 0 to 25% of; 2 = lesion range from 25% to 50% (moderate damage); 3 = lesion range more than 50% (severe damage) [9]. Immunohistochemical detection was done using the ABC method as previously reported [10, 11]. Collagen IV (1: 100), P-Smad2/3 (1: 200) and α -SMA (1: 100) were added after rinsing with PBS. The sheep anti-rabbit, goat anti-mouse secondary antibodies were then added. Image Pro Plus analysis software was used to calculate the mean optical density value.

Table 1. The scoring criteria of kidney tissue injury

Score	Tubular atrophy	Tubular necrosis	Lymphocyte infiltration	Interstitial fibrosis
0	None	None	None	None
1	Mild, <25%	Mild, <25%	Mildly local	Mild, <25%
2	Moderate, 25%-50%	Moderate, 25%-50%	Mildly diffused or moderately local	Moderate, 25%-50%
3	Moderate, >50%	Moderate, >50%	Severely diffused	Moderate, >50%

Table 2. Primers used for quantitative Real-time PCR

Genes	Forward primer	Reverse primer
TGF- β 1	5'-TGCCAGATCCTGTCCAAAC-3'	5'-GCGGGTGACCTCTTTAGCAT-3'
α -SMA	5'-GTTTCTCGCAGTCTCCTCT-3'	5'-CAGGCAGTTCGTAGCTCTTC-3'
T β RI	5'-ACCGCCGATGAATATGGGTCT-3'	5'-TGGCCTGTCTCGAGGAATTAG-3'
T β RII	5'-ACGTTCCCAAGTCGGATGTG-3'	5'-TGTCGTTCTTCTCCACACG-3'
Smad-2	5'-TCGGCACACGGAGATTCTAAG-3'	5'-ACCAGAATGCAGGTTCCGAG-3'
Smad-3	5'-CCGTGGAACCTACAAGGCGAC-3'	5'-GGCAGCAAATTCCTGGTTGT-3'
Smad-4	5'-ATCAACGGAGACGTACAGCG-3'	5'-TCCTCCGAAGGATCCACAT-3'
Smad-7	5'-TCTCAAACCAACTGGCTGTCC-3'	5'-AGAGCCTCCCCACGCGA-3'
Collagen I	5'-CATGGCCAAGAAGACATCCCT-3'	5'-CATTGCATTGCACGTCATCG-3'
Collagen III	5'-AAGGCTGCAAGATGGATGCT-3'	5'-GTGCTTACGTGGGACAGTCA-3'
Collagen IV	5'-ATGCACTCCAAGACTGCTCC-3'	5'-TTTGCTTTGCAGACACAGC-3'
GAPDH	5'-AGGAGAGTGTTCCTCGTCC-3'	5'-ATGGGCTTCCCGTTGATGAC-3'

Western blot analysis

Total protein extraction tissue: 1mg tissue was lysed in 1mL of RIPA lysis buffer before centrifuged for 15 min. The supernatant was then extracted to determine protein concentration. Tissue protein samples (sample volume of 20 μ g) were separated by SDS-PAGE. After blocking with 5% BSA, anti-TGF- β 1 (1: 1000 dilution), T β RI (1: 1000 dilution), T β RII (1: 1000 dilution), Smad 2/3 (1: 1000 dilution), p-Smad 2/3 (1: 1000 dilution), Smad 4 (1: 1000 dilution), α -SMA (1: 1000 dilution), Smad 7 (1: 800 dilution), GAPDH (1: 1000 dilution), Collagen I/III (1: 5000 dilution) and Collagen IV (1: 1000 dilution) antibodies were added and incubated overnight at 4 °C, the membrane was washed before secondary antibodies were added and incubated for 1 h. TBST was used to wash the membrane and a solution of ECL developer was added. The gray value of the bands of interest was analyzed using Image J software.

Real-time quantitative PCR

Primers were designed with the Primer 5.0 software (Table 2). Total RNA was extracted with the RNeasy Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's protocol. Briefly, 1 μ g of total RNA was reverse transcribed using the AMV First Strand cDNA Synthesis kit (QIAGEN, Valencia, CA) and synthesized complementary DNA was amplified by a standard PCR protocol using SYBR green PCR master mix (QIAGEN, Valencia, CA). Primers were synthesized from Sangon Biotech Co. Ltd. (Shanghai, China). The sequences of rat-specific primers for TGF- β 1, α -SMA, T β RI, T β RII, Smad-2, Smad-3, 6, Smad-4, Smad-7, Collagen I, III, IV and GAPDH used in the study were enlisted in Table 2. Cycling conditions were: 15 min preincubation at 95°C, 10 sec denaturation at 95°C, 31 sec annealing at 58°C for 40 cycles using ViiA7 Standard 96 sequence detection system (Applied biosystems, USA). The PCR products from each primer pair were subjected to a melting curve analysis in order to confirm amplification specificity. Each reaction was amplified in triplicate and the threshold cycles (Ct) were calculated using the $2^{-\Delta\Delta C_t}$ method. Relative gene expression was normalized with GAPDH as an internal reference.

Statistical Analysis

The GraphPad Prism 5.0 software (San Diego, CA, USA) was used for statistical analysis. The *t* test was used to compare the difference between two individual groups, and One-Way Analysis of Variance (ANOVA) was used to compare the difference among multiple groups; *P* < 0.05 was considered statistically significant.

Results

HuangQi decoction alleviated UUO matrix accumulation and improved renal interstitial damage in mice

After 14 days of unilateral ureteral obstruction, renal fibrosis on the obstructed side was significant. As shown in Fig. 1A, H&E staining exhibited tubular atrophy with partial

Fig. 1. Effect of HuangQi decoction on obstructed kidney tissue in UUO mice model. A. H&E staining; B. Scoring criteria for renal interstitial damage; C. Masson's Trichrome staining; D. Masson's Trichrome staining with statistical analysis. a. Sham group; b. Sham group with high dose (1.08g/kg) of HuangQi decoction; c. UUO group; d. UUO group with low dose of HuangQi decoction (0.12g/kg) e. UUO group with medium dose of HuangQi decoction (0.36g/kg); f. UUO group with high dose of HuangQi decoction (1.08g/kg). *** $P < 0.001$ compared to Sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared to UUO group. $n = 12-15$.

necrosis, renal tubular collapse, structural damage, tubulointerstitial widening, fibrotic hyperplasia, inflammatory cell infiltration, and extracellular matrix deposition in UUO kidneys compared with those in sham-operative kidneys. Additionally, Masson's Trichrome staining showed large number of collagen fiber streaks staining blue with prominent collagen fiber hypertrophy in the obstructed kidney (Fig. 1C). However, treatment with HuangQi decoction led to less tubular epithelial cell degeneration and inflammatory cell infiltration compared with UUO group, and the collagen fiber streak (blue-stained area) in the treatment group also decreased in a dose-dependent manner significantly ($P < 0.05$). TGF- $\beta 1$ is considered the most critical factor in the pathogenesis of renal interstitial fibrosis and can be found in a variety of chronic kidney diseases [9, 12-14]. Collagen I, III and IV, α -SMA and fibronectin were all markers for kidney myofibroblasts and extracellular matrix accumulation. They could be elevated in various types of chronic kidney damages [15-19]. In this study, we observed that the protein expression levels of TGF- $\beta 1$, Collagen I, III, IV and α -SMA, fibronectin (Fig. 2) and expressions of TGF- $\beta 1$ mRNA (Fig. 3) in mice of the ureteral obstruction group were both significantly higher than the basal expression levels in the sham group ($P < 0.05$). After HuangQi decoction in low-to-high concentrations were given, mRNA levels and protein expression of TGF- $\beta 1$ in the treatment group decreased dose-dependently compared with those in the UUO model group ($P < 0.05$).

HuangQi decoction inhibited TGF- β -induced epithelial-mesenchymal transdifferentiation of HK2 cells

TGF- $\beta 1$ could activate the transdifferentiation ability of renal tubular epithelial cells, which, in turn, can lead to α -SMA expression and synthesis of extracellular matrix such

Fig. 2. Effect of Huang-Qi decoction on expressions of TGF- β 1, Collagen I, III, IV, and α -SMA detected by Western blot. A. Representative photographs showing protein expressions of TGF- β 1, Collagen I, III, IV, and α -SMA. B-F. Statistical analyses versus A. ***P<0.001, compared to Sham group; #P<0.05, ##P<0.01, ###P<0.001, compared to UUO group. n=12-15.

Fig. 3. Effect of Huang-Qi decoction on relative expressions of TGF- β 1, Collagen I, III, IV, α -SMA and fibronectin mRNAs detected by PCR. A-F. Relative mRNA levels of TGF- β 1, Collagen I, III, IV, α -SMA and fibronectin expression in kidneys. ***P<0.001, compared to sham group; #P<0.05, ##P<0.01, ###P<0.001, compared to UUO group. n=12-15.

as Collagen I, III, IV and thus be involved in renal interstitial fibrosis. In our experiment, TGF- β 1 (2.5 ng/ml) alone was used to treat HK2 cells, and western blot detected the protein

Fig. 4. Effect of HuangQi decoction on expressions of Collagen IV and α -SMA in the left kidney of the mice detected by immunohistochemistry. A and C. Representative photomicrographs of Collagen IV and α -SMA immunohistochemistry on kidney sections. B and D. Integrated optical density analysis of Collagen IV and α -SMA expression from sections similar to those shown in A and C. *** $P < 0.001$, compared to Sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared to UUO group. $n = 12-15$.

expressions of TGF- β 1, Collagen I, III, IV and α -SMA increased significantly with TGF- β 1 treatment (Fig. 5A-F, $P < 0.05$), indicating the activation of EMT mechanism and accumulation of extracellular matrix. After HuangQi decoction of different concentrations (100 μ g/ml, 300 μ g/ml and 1000 μ g/ml) were given, protein expressions TGF- β 1, Collagen I, III, IV and α -SMA decreased in a concentration-dependent fashion ($P < 0.05$).

HuangQi decoction could downregulate the TGF- β /Smad signaling pathway

Immunohistochemical detection showed increased P-Smad2/3 expression in the ipsilateral kidney of UUO mice model compared to the sham group (Fig. 6A-B), the difference was statistically significant ($P < 0.05$). After the treatments of HuangQi decoction in low-to-high concentrations, expression of P-Smad2/3 decreased in a dose-dependent fashion. We also found the mRNA (Fig. 8A-E) and protein (Fig. 7A-F) expressions of T β RI, T β RII, Smad2/3, P-Smad2/3 and Smad4 in mice of the UUO group were higher than the sham group. After the treatments of HuangQi decoction in low-to-high concentrations, expressions of T β RI, T β RII, Smad2/3, P-Smad2/3 and Smad4 decreased in a dose-dependent fashion. The expressions of Smad7 mRNA (Fig. 8F) and protein (Fig. 7A, G) in UUO mice models were significantly lower than those in the sham group ($P < 0.05$), and Smad7 increased in a dose-dependent manner after HuangQi decoction of different concentrations were given. Western blot of *in vitro* experiments detected increased T β RI, T β RII, Smad2/3, P-Smad2/3 and Smad4 levels after TGF- β 1 (2.5 ng/ml) stimulation of HK2 cells (Fig. 9A-F, $P < 0.05$). This expression decreased after HuangQi decoction treatment in a concentration-dependent fashion. Meanwhile, the expression of Smad7 protein significantly decreased after stimulation (Fig. 9A, G, $P < 0.05$), and increased dose-dependently after HuangQi decoction were given.

Fig. 5. Effect of HuangQi decoction on expressions of TGF- β 1, Collagen I, III, IV, and α -SMA in HK2 cells detected by Western blot. A. Representative photographs showing protein expressions of TGF- β 1, Collagen I, III, IV, and α -SMA. B-F. Statistical analyses versus A. ***P<0.001, compared to Sham group; #P<0.05, ##P<0.01, ###P<0.001, compared to UUO group, n=3-5.

Fig. 6. Effect of HuangQi decoction on expressions of P-Smad2/3 detected by immunohistochemistry. A. Expression of P-Smad2/3 in the left kidney; B Statistical analysis versus A. ***P< 0.001, compared to Sham group; #P< 0.05, ##P<0.01, ###P<0.001, compared to UUO group. n=3-5.

Discussion

Modern pharmacological studies have demonstrated that each single drug in HuangQi decoction could be beneficial in chronic kidney disease. For instance, astragalus could alleviate macrophage infiltration tubular interstitial fibrosis in the renal tissue of a rat

Fig. 7. Effect of HuangQi decoction on expressions of T β R I, T β R II, Smad2/3, P-Smad2/3, Smad4 and Smad7 detected by Western blot. A. Protein expressions after treatment. B-G, statistical analyses. ***P<0.001, compared to Sham group, #P<0.05, ##P<0.01, ###P<0.001, compared to UUO group. n=3-5.

model, and thus delaying the process of chronic kidney disease [20]; By regulating TIMP-1, MMP and expression of TGF- β 1, it could improve the renal interstitial fibrosis in Adriamycin-induced rat models [21]; Astragaloside could improve renal fibrosis in UUO rat models by downregulating TGF- β /Smad and wnt/ β -catenin signaling pathway in *in vivo* experiments [22-24]. Poria could slow down the progress of chronic kidney disease by the intervention on certain metabolic pathways (e.g. metabolism of adenine and amino acid) [25, 26]. Melon was proved to be able to improve myocardial fibrosis by regulating TGF- β /Smad signaling pathway [27] and could thereby alleviate the cisplatin-induced kidney damage proved by *in vitro* and *in vivo* experiments [28]. The main monomer of Radix, ruscogenin, was found with an anti-fibrotic effect for diabetic nephropathy in rats [29], Schisandrin, which is rich in Chinese medicine Schisandra, could inhibit TGF- β signaling pathway to achieve an anti-fibrotic effect as well [30, 31]. Licorice could downregulate TGF- β 1/Smad signal transduction, and thus play a therapeutic role in the processes of interstitial fibrosis and glomerulosclerosis [32]. Meanwhile, Radix rehmanniae was proved to be able to alleviate hematuria and proteinuria associated with chronic glomerulonephritis [33], as well as bring improvement in diabetic nephropathy [34]. The present study demonstrated the role of HuangQi decoction in improving renal interstitial fibrosis, reducing extracellular matrix

Fig. 8. Effect of HuangQi decoction on mRNA and protein expressions of TβRI, TβRII, Smad2/3, P-Smad2/3, Smad4 and Smad7 detected by PCR. A-F. mRNA and protein expressions. *** $P < 0.001$, compared to Sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared to UUO group. $n = 3-5$.

accumulation, and downregulating TGF- β /Smad signaling pathway in unilateral ureteral obstruction mice models.

Renal interstitial fibrosis is a key process in the progression of chronic kidney disease to end-stage renal disease. Compared to glomerulosclerosis, interstitial fibrosis plays a more critical role in the consequence of nephron loss. TGF- β 1 is a pleiotropic fibrosis-inducing cytokine recognized in the development of renal interstitial fibrosis [2, 35-37]. TGF- β 1 may induce excessive accumulation of extracellular matrix, mainly through the following approaches: (1) Smad3-dependent or -independent pathways leading to the accumulation of collagen types I, IV and fibronectin; (2) Renal tubular epithelial-mesenchymal cells transdifferentiation, which is considered to have a direct effect on a myofibroblasts pool during kidney damage, as well as the most critical cell types for matrix accumulation in the renal interstitium.

Renal tubular epithelial-mesenchymal cell transdifferentiation is characterized by the loss of epithelial genetic phenotype and expression of interstitial fibrotic features. Renal interstitial fibroblasts are the major effector cells contributing to EMT in renal interstitial fibrosis, whose proliferation and activation serve the pilot role for ECM. Activated fibroblasts may undergo functional and phenotypic changes and transform into myofibroblasts expressing smooth muscle actins (SMAs). The ability of the myofibroblasts in ECM synthesis would be markedly enhanced and thus continuous accumulation of interstitial matrix would ensue. Therefore, α -SMA is one of the most important proteins in the EMT process,

Fig. 9. Effect of HuangQi decoction on expressions of T β R I, T β R II, Smad2/3, P-Smad2/3, Smad4 and Smad7 in HK2 cells Smad-dependent signaling pathway detected by Western blot. A. Protein expressions after treatment. B-G. Statistical analysis versus A ***P<0.001, compared to Sham group; #P<0.05, ##P<0.01, ###P<0.001, compared to UUO group. n=3-5.

and its expression in activated EMT would be substantially increased [38, 39]. In animal experiments, we observed increased α -SMA expression in the obstructed kidneys of UUO model mice using immunohistochemistry, RT-PCR and Western blot; α -SMA expression was also significantly increased in cell experiments when HK2 cells were stimulated by TGF- β 1, suggesting a close association between the development of EMT and renal fibrosis. Collagen is a major component of ECM and under normal conditions the synthesis and degradation of collagen are in a dynamic balance. In addition, we have observed increased expressions of Collagen I, III, IV protein in the obstructed kidney tissue of mice models and HK2 cells in both *in vitro* and *in vivo* experiments, suggesting the involvement of EMT increased renal extracellular matrix accumulation. After HuangQi decoction was given, decreased Collagen I, III, IV protein levels were observed. This experiment proved that HuangQi decoction could alleviate EMT and extracellular matrix accumulation by inhibiting Collagen I, III, IV protein expression in a certain extent, and thereby improve renal interstitial fibrosis.

Smad-dependent signaling pathway is considered the most classic signaling pathway in TGF- β 1-induced fibrosis [21, 40, 41]. Smad2/3 levels would be higher than normal in patients with chronic kidney disease as well as animal models [42-44]. Compromised Smad3 gene in renal tubular epithelial cells would reduce Ang II-induced matrix production [45]. Smad3 gene knockout was also proved to be able to reduce the Ang II-induced matrix production and improve renal fibrosis [46, 47]. In this experiment, we observed significantly

increased Smad2/3 expression in the obstructed kidney tissues of UUO mice through immunohistochemistry, RT-PCR and Western blot, which was reversed dose-dependently after treatment with HuangQi decoction. TGF- β 1-induced Smad2/3 expression elevation in HK2 cells was also significantly reduced by high dose of HuangQi decoction. Same changes were found in the level of P-Smad2/3, phosphorylation product of Smad2/3. Smad7 is one type of inhibitory Smad protein, whose expression in normal renal tissue is mainly located in the glomeruli and cortical tubular epithelial cells, with only a small amount in medullary tubules. Smad7 serves as an autoregulative negative feedback signal in TGF- β 1 signaling, with the ability to adjust TGF- β 1 signal intensity and duration. Intracellularly, endogenous Smad7 prevents the phosphorylation of R-Smads and thereby inhibiting the transcription of target genes by competitive binding with TGF- β 1. Meanwhile, Smad7 promote the degradation and ubiquitination of the receptor and thus reducing the reactivity of the TGF- β signal [48, 49]. While in the nucleus, Smad7 can inhibit gene transcription by reducing histone acetylation, thereby blocking the formation of the functional TGF- β -induced Smad-DNA complex. In this experiment, we found that HuangQi decoction can increase TGF- β 1-induced Smad7 expression in the obstructed kidney tissue in UUO mice and HK2 cells, thus playing a renal protective role in renal interstitial fibrosis. This experiment also proved that HuangQi decoction could improve renal interstitial fibrosis by affecting the TGF- β /Smad signaling pathway and inhibiting EMT and the consequent extracellular matrix accumulation.

Conclusion

In this study, we observed a dose-dependent improvement of renal interstitial fibrosis in unilateral ureteral obstruction mice models by HuangQi decoction. Both *in vivo* and cell experiments found that the decoction could downregulate the TGF- β /Smad signaling pathway, inhibit EMT, and reduce cell accumulation of extracellular matrix, thereby delaying the progression of renal interstitial fibrosis.

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Disclosure Statement

No.

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