Effect of Bushen Qudu Decoction on TGF-β1/Smads signal transduction pathway in rats with chronic renal failure

Weirong Shi1*, Qiang Wu1, Qiuhong Wu2, Juan Tang1
1Department of Nephrology, 2Department of Health Management Center, The Second People’s Hospital Affiliated to Fujian University of Traditional Chinese Medicine, Fuzhou, PR China

*For correspondence: Email: fujianshitou@163.com

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Abstract

Purpose: To study the impact of Bushen Qudu Decoction on renal function, renal tissue morphology and TGF-β1/Smads signal transduction pathway in adenine-induced chronic renal failure rats.

Methods: 76 rats were assigned to normal group, model group, uremic clearance group, benazepil group, Bushen Qudu decoction gushan A group and Bushen Qudu decoction Guiza group. Renal failure was induced in rats using intragastric administration of adenine for 30 days. The expressions of TGF-β1, Smad2, Smad3 and Smad6 were determined with immunohistochemistry. Real-time quantitative PCR was employed to assay the mRNA expression of TGF-β1 in each group, while protein expressions of Smad2, Smad3 and Smad6 were determined with western blot. The scores of glomerular and tubulointerstitial lesions in each group were obtained by histopathological examination.

Results: Bushen Qudu decoction significantly reduced BUN and creatinine in rats with chronic renal failure; furthermore, it also lowered the protein expressions of TGF-β1 and SMAD2/3, but Smad6 protein, relative to control (p < 0.05). The TGF-β1 mRNA expression was down-regulated (p < 0.05), relative to control group.

Conclusion: Bushen Qudu decoction reduces renal interstitial fibrosis in rats and delays the progression of chronic renal failure by regulating TGF-β1/Smads signaling pathway. These findings provide some insight that should facilitate the development of new drugs that would delay the onset of chronic renal failure.

Keywords: Chronic renal failure, renal interstitial fibrosis, TGF-β1/Smads signal transduction pathway, Bushen Qudu decoction

INTRODUCTION

In modern medicine, the pathological manifestations of chronic renal failure are mainly glomerulosclerosis, interstitial fibrosis and renal tubular atrophy. The late stage of chronic kidney disease causes renal degeneration, high blood viscosity and coagulation, leading to reduced blood supply. Modern pharmacological studies on Sanqi in Bushen Qudu decoction suggest that Sanqi increases renal blood flow, improves renal circulation, reduces blood viscosity, dilates capillaries and relieves arteriolar spasm. Moreover, Salvia miltiorrhiza suppressed the multiplication of fibroblasts in renal tissues, reduced renal interstitial fibrosis, decreased...
blood viscosity, and inhibited platelet aggregation and adhesion [1-4].

While the combination of Panax notoginseng, Salvia miltiorrhiza, cowherb seed and Achyranthes bidentata is used to dredge blood vessels and reduce renal tissue fibrosis, rhubarb is an essential drug for the treatment of chronic renal failure [5-7]. Rhubarb cleanses turbidity, detoxifies and removes blood stasis, calms the five viscera, and weeds out the old to let the new emerge. The present research was aimed at investigating the impact of Busshen Qudu decoction on TGF-β1/Smads signal transduction pathway, and also to determine the mechanism involved in its anti-renal interstitial fibrotic effect.

EXPERIMENTAL

Animals

Seventy-six healthy female SD rats weighing 150 ± 30 g were purchased from Shanghai Experimental Animal Co. Ltd by the Experimental Animal Center of The Second People’s Hospital of Fujian Province [Certificate of Qualification: SCXX (Shanghai)]. The rats were raised in clean grade SPF laboratory, and were housed singly in cages. They were provided ad libitum access to feed and water at room temperatures (23 - 28 ºC and humidity (50 - 60 %).

Ethical approval

This research received approval from the ethics Committee of our institution according to “Principles of Laboratory Animal Care” (NIH publication no. 85-23, revised 1985) [8] (the approval number was 2020089).

Drugs and reagents

The drugs and reagents used, and their suppliers (in brackets) were: adenine (Beijing Jingke Hongda Biotechnology Co. Ltd., molecular weight 135.14, batch number: 3220C121); uremic clearance granules (Oriental pharmacy, produced by Guangzhou Kangchen Pharmaceutical Co. Ltd, National medicine approval number = Z20073256); Gunshan group A kidney Tonifying and Toxin Removing Soup (Pharmacy of TCM of the Second People’s Hospital of Fujian Province; it was composed of 15 g of mulberry jisheng, 15 g of Eucommia ulmoides, 9 g of Cornus officinalis, 15 g of Dangshan, 15 g of Astragalus, 15 g of Poria fulva, 15 g of Niu genu, 9 g of raw rhubarb, 12 g of Angelica, 15 g of Danshen, 15 g of vinegar turtle shell, 6 g of gunshan shell, and 6 g of panax notoginseng powder).

The other drug was Bushen qudu decoction tortoise shell group (Pharmacy TCM of Fujian Second People’s Hospital. It was composed of 15 g of parasite, 15 g of Eucommia ulmoides, 15 g of Cornus officinalis, 15 g of angshen, 15 g of astragalus, 15 g of Poria cocos, 9 g of raw rhubarb, 12 g of angelica, 15 g of S. miltiorrhiza, 15 g of A. bidentata, 20 g of decocted vinegar tortoise shell, 15 g of decocted vinegar tortoise shell, and 2 g of panax notoginseng powder.

The reagents used were polylysine (Sigma P8920); TGF-β1 antibody, SMAD2/3, Smad6, Smad7, MMP-9 and TIMP-1 (all from Boorson BS-0103R); secondary antibody SP-9001 (Invitrogen SP-9001), DAB (Beijing Zhongshan ZLI-9032), TGF Beta 1 antibody (Boolsen, BS-0103R), SMAD2/3 antibody (Bosco, BA-1395), Smad6 antibody (Bosco, BA-1398), Smad7 antibody (Boolsen, BS-0566R), Beta-Action internal reference antibody (Zhongshan, TA-09), Horse radish-labeled goat anti-rabbit secondary antibody (Zhongshan, ZB-2301), Biozol Reagent (Hangzhou, BioRT), cDNA First Strand Synthesis Kit (Hangzhou, BIOER), BioRT cDNA First Strand Synthesis Kit BIOER, and RNase-free ddH2O (Hangzhou, BIOER).

Animal grouping

A total of 76 SD rats were fed adaptively for 1 week after weighing. Thereafter, the rats were randomly assigned to 6 groups: Control, Model, Uric poisonous clear, Cannon Mountain first division, Tortoise shell, and Benazepril.

Establishment of rat model of chronic renal failure

The rat model of chronic renal failure was established by administering adenine to the animals (200 mg/kg bwt/day) via intragastric route for 30 days. The adenine was solubilized in H2O, and the volume administered was 2 mL. The amount of adenine was adjusted according to body weight, and the cumulative total dose was not more than 1.5 g/rat. Rats in normal control group received 2 mL of normal saline through the intragastric route.

Treatments

The rats with chronic renal failure were divided into five groups: model group (untreated), uric poisonous clear group, Cannon mountain first division, tortoise shell group and benazepril
(standard drug) group. Rats in the standard group were given benazepril (9.2 \( \times 10^{-4} \) g/kg). The medicine was given once a day at the fixed regular time for 30 days.

**Biochemical analysis**

In the morning, 3 ml of fasting venous blood was taken, and the serum was separated via centrifugation. Then, SCr and BUN in each group were determined enzymatically with Hitachi 7600-010 automatic biochemical analyzer. The reagents were provided by Zhejiang Dongou Biological Engineering Co. Ltd.

**H & E staining**

The tissues were completely immersed in formalin solution and fixed. Then, H&E staining and paraffin embedding were performed for pathological sections, and immunohistochemical staining was performed at the same time. After paraffin sections were dewaxed and hydrated, they were sequentially immersed in PBS solution, incubated overnight with primary antibody, washed with PBS, labeled with secondary antibody, washed with buffer solution, stained with DAB, rinsed fully with tap water, redyed, dehydrated, cleared and dried, and sealed. The presence of brownish-yellow granules in cytoplasm was a positive reaction. Semi-quantitative analysis of immunohistochemical staining results was performed using Image Pro-plus 6.0, and integrated optical density (IOD) values were calculated. Each immunohistochemical section was observed under 400\( \times \) field of vision and images were collected. Five fields were randomly selected for image collection. The IOD values of positive staining were determined, and the mean value was used to reflect the expression level. Results of H&E staining were used to ascertain changes in histomorphology in each group. The scores of glomerular and tubulointerstitial lesions in each group were obtained from histopathology.

**Assessment of expression of transforming growth factor (TGF-\( \beta1 \))**

The mRNA expression of TGF-\( \beta1 \) was determined using RT-PCR based on primer sequences synthesized by Beijing Saibaisheng Gene Technology Co. Ltd. The heart tissue was cut into pieces and ground in liquid nitrogen. Total RNA was extracted from heart tissue using TRIZol. Then, RT-PCR was used to measure TGF-\( \beta1 \) mRNA in each group. Table 1 shows the primer sequences used.

**Table 1: The primer sequences used in RT-PCR**

| Variable | Primer sequence |
|----------|-----------------|
| Forward  | TCAAGTCAACTGTGGAGCAACG; |
| Reverse  | CGAAAGCCCT GTATTCCGTCTCC. |

**Determination of Smad2, Smad3 and Smad6**

These were determined using western blotting. Rat hearts were excised, rinsed with PBS at 4 °C, and preserved in liquid nitrogen at -80 °C. Total protein was extracted using RIPA lysis buffer (100 ul/mg of tissue). The protein contents of the supernatants obtained after centrifugation at 12000 rpm were measured. Then, the proteins were subjected to SDS-PAGE, followed by electro-transfer to PVDF membranes and sequential incubation with primary and secondary antibodies. Relative protein expressions were determined using ImageJ software.

**Statistical analysis**

Statistical analysis was done with SPSS13.0 statistical software. All data are expressed in the form of mean ± standard deviation (SD). Measurement data consistent with normal distribution and homogeneity of variance were compared with \( t \)-test, while \( \chi^2 \) test was used for counting data. Statistical significance of difference was assumed at \( p < 0.05 \).

**RESULTS**

**Changes in body weight**

During the study, the experimental rats were weighed every morning. The weights of the rats in the various groups were compared at initial time, 3 and 5 weeks (Table 2).

**BUN and Scr levels**

The concentrations of BUN and Scr are presented in Table 2.

**Effect of Bushen Qudu decoction on 24-h urine protein in rats with adenine-induced chronic renal failure**

The quantity of 24-h urine protein in model rats increased gradually with time. At week 5, 24-h urinary protein was markedly decreased, relative to model rats. At week 6, 24-h urine protein concentration was markedly reduced, relative to model (Table 4).
Table 2: Weights of rats in each group at various time points (g)

| Group                        | Initial time       | At 3 weeks          | At 5 weeks          |
|------------------------------|--------------------|---------------------|---------------------|
| Control                      | 212.89±16.10       | 246.44±21.54        | 267±26.17           |
| Model                        | 214.17±13.26       | 231.85±24.41        | 243.38±24.06        |
| Uric poisonous clear         | 216.46±11.32       | 236.45±16.78        | 247.67±22.20        |
| Cannon mountain first division| 221.29±13.76       | 241.25±0.10         | 254.00±13.01        |
| Tortoise shell               | 219.57±12.92       | 236.45±16.78        | 251.08±22.96        |
| Benazepril                   | 217.61±4.25        | 235.15±24.31        | 243.81±35.15        |

a,b,cP < 0.05, compared with blank group; d,e,fP < 0.05, compared with model group; g,h,iP < 0.05, compared with uriduqing group.

Table 3: Renal parameters of rats in each group

| Group                        | N  | Scr (μmol/L) | BUN (mmol/L) |
|------------------------------|----|--------------|--------------|
| Blank                        | 9  | 50.01±8.25   | 8.06±1.67    |
| Model                        | 12 | 360.07±34.33 | 57.05±9.35   |
| Uric poisonous clear         | 12 | 289.56±23.00 | 47.81±10.87  |
| Cannon mountain first division| 12 | 216.19±14.12 | 36.15±4.39   |
| Tortoise shell               | 11 | 233.15±57.53 | 40.21±4.09   |
| Benazepril                   | 13 | 293.08±37.84 | 48.83±8.67   |

aP < 0.01, vs control; bP < 0.05, vs model; cP < 0.05, vs uriduqing.

Table 4: 24-h urinary protein levels (mg/24 h)

| Group                        | N  | At 5 weeks n | At 6 weeks n |
|------------------------------|----|--------------|--------------|
| Blank                        | 9  | 19.10±5.15   | 32.17±13.36  |
| Model                        | 12 | 50.60±11.48  | 87.22±21.47  |
| Uric poisonous clear         | 12 | 50.31±18.39  | 85.24±13.90  |
| Cannon mountain first division| 12 | 48.48±19.09  | 84.61±21.24  |
| Tortoise shell               | 11 | 49.98±17.53  | 84.90±13.68  |
| Benazepril                   | 13 | 47.88±19.05  | 82.61±23.54  |

*Values that have different superscripts vertically differ significantly.

Effect of *Bushen Qudu* decoction on renal histopathology of rats with chronic renal failure

Under a light microscope, five horizons were randomly selected from kidney slides, and the gray values of four areas were measured in each view. Positive signals within the areas were distinguished through gray adjustment vision, and the total area of positive interstitial fibrosis was scored for each group of rats. Interstitial lesion scores were compared amongst the groups. The results are shown in Table 5.

Effect of *Bushen Qudu* decoction on protein expressions of TGF-β1/Smad2 and 3/Smad6 in rat kidney

The differences in expressions of these proteins in rat kidney tissue are shown in Tables 6 and 7.

Protein expression levels of SMAD2/3 and Smad6

*Bushen Qudu* decoction reduced the protein expression of SMAD2/3, but upregulated that of Smad6, as shown in Table 8.

Table 5: Semi-quantitative renal interstitial fibrosis scores in each group

| Group                        | N  | Mean score of renal interstitial lesions |
|------------------------------|----|----------------------------------------|
| Blank                        | 5  | 0.200±0.447                             |
| Model                        | 8  | 2.500±0.535                             |
| Uric poisonous clear         | 8  | 2.250±0.707                             |
| Cannon mountain first division| 8  | 1.750±0.707                             |
| Tortoise shell               | 8  | 2.125±0.641                             |
| Benazepril                   | 9  | 2.125±0.835                             |

*Values that have different superscripts vertically differ markedly.
Table 6: Expression level of TGF-β1 protein in each group after treatment

| Group                    | N | TGF-β1                | Mean surface density (%) | Mean optical density |
|--------------------------|---|-----------------------|--------------------------|----------------------|
| Blank                    | 5 | 20.33±1.71<sup>ab</sup> | 0.15±0.005               |
| Model                    | 8 | 37.03±1.50<sup>a</sup>  | 0.253±0.010              |
| Uric poisonous clear     | 8 | 34.88±2.49<sup>a</sup>  | 0.216±0.112              |
| Cannon mountain first division | 8 | 25.65±3.40<sup>b</sup>  | 0.187±0.008              |
| Tortoise shell           | 7 | 32.13±2.70<sup>a</sup>  | 0.199±0.011              |
| Benazepril              | 9 | 34.66±2.33<sup>ab</sup> | 0.232±0.016              |

*Values that have different superscripts vertically differ significantly.

Table 7: Protein levels of SMAD2/3 and Smad6 in each group after treatment

| Group                    | Smad2/3 | Smad6                | Mean surface density (%) | Mean optical density |
|--------------------------|---------|-----------------------|--------------------------|----------------------|
| Blank                    | 29.49±0.375<sup>ab</sup> | 39.078±0.222<sup>ab</sup> | 0.12±0.003 | 0.218±0.151 |
| Model                    | 37.156±0.965 | 31.032±0.469 | 0.186±0.005 | 0.122±0.003 |
| Uric poisonous clear     | 35.668±0.439<sup>b</sup> | 33.806±0.477<sup>bc</sup> | 0.147±0.004 | 0.128±0.005 |
| Cannon mountain first division | 32.819±0.834<sup>c</sup> | 36.329±0.465<sup>bc</sup> | 0.137±0.005 | 0.140±0.005 |
| The tortoise shell       | 34.414±0.534<sup>b</sup> | 34.199±0.334<sup>b</sup> | 0.143±0.004 | 0.133±0.005 |
| Benazepril              | 36.474±0.559<sup>abc</sup> | 32.967±0.407<sup>bc</sup> | 0.154±0.007 | 0.125±0.008 |

*Values that have different superscripts vertically differ significantly.

Table 8: Quantitative protein expressions of SMAD2/3 and Smad6 in each group after treatment

| Group                    | Smad2/3 | Smad6                | Mean surface density (%) | Mean optical density |
|--------------------------|---------|-----------------------|--------------------------|----------------------|
| Blank                    | 0.6471±0.2316<sup>ab</sup> | 1.956±0.2811<sup>ab</sup> | 1.568±0.4740 | 1.308±0.1405 |
| Model                    | 1.1469±0.2442<sup>a</sup>  | 1.736±0.2367<sup>a</sup>  | 0.7470±0.2669<sup>ab</sup> | 1.943±0.2021<sup>ab</sup> |
| Uric poisonous clear     | 1.0289±0.1086<sup>ab</sup> | 1.815±0.1388<sup>ab</sup> | 1.3152±0.4208<sup>abc</sup> | 1.6032±0.1824<sup>abc</sup> |

*p < 0.01, vs model; ^p < 0.01, vs uriduqing; ^p < 0.01, vs Gunshan group A

Effect of Bushen qudu decoction on TGF-β1 mRNA expression in rat kidney tissue

These results are presented in Table 8.

Table 9: mRNA expression level of TGF-β1 in each group after treatment

| Group                    | TGF-β1mRNA |
|--------------------------|------------|
| Blank                    | 0.144±0.004<sup>ab</sup> |
| Model                    | 0.257±0.004 |
| Uric poisonous clear     | 0.204±0.005<sup>a</sup>  |
| Cannon mountain first division | 0.182±0.005<sup>ab</sup> |
| Tortoise shell           | 0.192±0.005<sup>ab</sup> |
| Benazepril              | 0.213±0.007<sup>a</sup>  |

*Values that have different superscripts vertically differ significantly.

DISCUSSION

Serum creatinine and urea nitrogen are not only important reference indicators for clinical diagnosis of chronic renal failure, but also important indicators for clinical treatment efficacy [9]. When serum urea nitrogen and creatinine levels rise, clinical manifestations of digestive system discomfort such as nausea and vomiting are seen. Symptoms of discomfort in the digestive system may impair the production of red blood cells, resulting in a vicious cycle of low immunity [10].

The present clinical study showed that Bushen Qudu decoction significantly reduced serum creatinine and urea nitrogen levels, when compared with the control group. The results showed that after treatment, the urea nitrogen and creatinine levels of Bushen Qudu decoction gunshan A group and tortoise shell group were markedly decreased, relative to model group. Clinical and experimental results confirmed that Bushen Qudu decoction reduced serum urea nitrogen and creatinine levels in patients with chronic renal failure, and delayed the progression of renal failure.

In the Bushen Qudu decoction formula, Salvia miltiorrhiza, Panax notoginseng, Angelica and other components which promote blood
circulation and remove blood stasis also improve kidney circulation and enhance the excretion of metabolic substances, thereby enhancing renal function.

It has been shown that kidney tubule EM trans-differentiation is involved in kidney interstitial fibrotic lesions [11,12]. Moreover, TGF-β1 mediates renal tubular epithelial-mesenchymal trans-differentiation [13]. Cytokines which affect renal interstitial fibrosis comprise those that promote renal interstitial fibrosis and those that exert anti-renal interstitial fibrotic effects. Increased expression of TGF-β1 is an important factor that increases the formation of renal interstitial fibrosis [14]. The Smads proteins are transcoding molecules that transmit TGF-β1 family signals from receptors to the nucleus. The TGF-β1/Smads pathway is the ultimate common pathway in renal fibrosis. In the TGF-β1/Smad signal route, SMad2/3 forms active transcription complexes in the nucleus, while Smad6 inhibits TGF-β1 signal transduction [15].

The findings in this research indicate that, relative to the model group, Bushen Qudu decoction reduced protein levels of TGF-β1 and SMAD2/3 in the renal tissues of kidney failure rats, and increased the expression of Smad6 protein, thereby inhibiting the transduction of TGF-β1 signal into the nucleus, and delaying renal interstitial fibrosis. This study used adenine to induce chronic kidney failure model. Although the lesion process is similar to that in humans, it does not fully conform with the progress of human chronic renal failure lesions and TCM treatment of syndrome differentiation. Thus, there is need to evolve a method for establishing chronic kidney failure disease in an appropriate animal model.

Currently, blood creatinine and urea nitrogen are used to diagnose chronic renal failure in rats through comparison with normal rats. There are no stages in chronic kidney disease in rats, so it is impossible to improve kidney function in different stages, relative to what is done in traditional Chinese medicine. In this study, it was preliminarily confirmed that TGF-β1 was elevated in the renal tissue of rats with chronic renal failure. The use of the decoction increased Smad2/3 and decreased Smad6, thereby invigorating the spleen, tonifying kidney, and removing dampness and blood stasis. This study provides possible ways for preventing and controlling kidney disease, and provides new ideas and methods for its clinical treatment. However, there is need for further investigation on other pathways for inhibiting renal interstitial fibrosis.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Weirong Shi designed the study, supervised the data collection, and analyzed the data. Weirong Shi interpreted the data and prepared the manuscript for publication. Qiang Wu, Qiuhong Wu and Juan Tang supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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