Protective effects of Pai-Du-Yang-Shen formula on chronic renal failure in rats

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Abstract
This study aimed to elucidate the therapeutic effects of a traditional Chinese medicine, Pai-Du-Yang-Shen (PDYS) Formula, in 5/6 nephrectomy-induced chronic renal failure (CRF). The CRF model was established via 5/6 nephrectomy (Nx) in rats. Then, PDYS (0.9 and 1.8 g/kg/day), or an equal amount of normal saline, was administrated to the rats in respective groups. Our results showed that PDYS treatment improved degeneration, fibrosis, and histopathological abnormalities of renal tissues in 5/6 Nx rats. Furthermore, the increase in serum creatinine (Scr), blood urea nitrogen (BUN), and total urinary protein (TUP) in 5/6 Nx rats was reduced by PDYS treatment. In addition, the levels of tumor necrosis factor (TNF), fibronectin (FN), and laminin (LN) that were induced in 5/6 Nx rats were also decreased by PDYS treatment. Finally, relative mRNA expression of α-smooth muscle actin (α-SMA), FN, transforming growth factor-β (TGF-β), and TNF as well as protein levels of TGF-β, NF-κB p65, and phosphorylated NF-κB p65 (p-p65) in renal tissues correlated with the concentration of TNF. In conclusion, PDYS shows therapeutic effects against CRF as determined by a reduction in markers for inflammation and fibrosis.

Keywords
5/6 nephrectomy, chronic renal failure, inflammation, NF-κB signaling, Pai-Du-Yang-Shen formula

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Introduction
Chronic renal failure (CRF) is a global problem and one of the main causes of morbidity and mortality.1 Patients with CRF are typically characterized by irreversible loss of renal tissue and function and higher serum urea and creatinine levels, which may lead to various other dangerous diseases.2–4 The pathological characteristics of CRF mainly manifests as glomerulosclerosis, mesangial matrix increases, and tubulointerstitial fibrosis. The main pathological feature of renal fibrosis is a sustained production and accumulation of extracellular matrix (ECM) proteins such as fibronectin (FN) and collagen.5,6 In essence, the treatment for CRF and the improvement of renal function is achieved by ameliorating the degree of renal fibrosis.7

Unfortunately, there are currently no effective clinical strategies for patients with renal fibrosis.8 Transforming growth factor-beta (TGF-β) has been universally regarded as a crucial mediator of kidney fibrosis and is heavily implicated in the progression of kidney diseases.9,10 TGF-β has also been shown to be an important cytokine in the development of tubulointerstitial and glomerulosclerosis.
fibrosis and a crucial inducer of epithelial–mesenchymal transition (EMT).\textsuperscript{11,12} In addition to TGF-β signaling, nuclear factor κB (NF-κB) was also found to play a pivotal role in the maintenance and induction of EMT.\textsuperscript{13,14}

Pai-Du-Yang-Shen (PDYS) formula (Invention Patent of the People’s Republic of China ZL 2007 10191384.X) is a traditional Chinese medical formulation that has been clinically applied to treat CRF in China and has shown good clinical outcomes.\textsuperscript{15} However, the specific molecular mechanism of action of PDYS remains unknown. Thus, in this study, we established a CRF rat model via 5/6 nephrectomy (Nx) and assessed the therapeutic effects of PDYS in rats. The roles of TGF-β and NF-κB signaling were investigated in this process.

### Materials and methods

#### Materials

A hematoxylin–eosin (H&E) staining kit as well as serum creatinine (Scr), blood urea nitrogen (BUN), and total urinary protein (TUP) biochemical assay kits were obtained from Beyotime Biotechnology (Shanghai, China). Tumor necrosis factor-alpha (TNF-α), FN, and laminin (LN) enzyme-linked immunosorbent assay (ELISA) kits were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). TRIzol reagent and maxima first-strand complementary DNA (cDNA) synthesis kit were obtained from Thermo Fisher (MA, USA). Primary antibodies against TGF-β1 (1:500), GAPDH (1:1000), NF-κB p65 (1:1000), and phosphorylated p65 (p-p65; 1:1000) were obtained from Abcam Biotech (Cambridge, MA, USA).

### 5/6 Nx and drug therapy in rats

Male Sprague–Dawley (SD) rats (6 weeks old, 175–185 g) were obtained from Shanghai Sippr BK Laboratory Animals, Ltd. (Shanghai, China) and housed under a 12 h light/12 h dark cycle with food and water ad libitum in a temperature-controlled environment (24°C–26°C). After the adaptive period of 1 week, rats were randomly divided into four groups (n=6): sham group, 5/6 Nx group, and PDYS formula treatment groups (0.9 and 1.8 g/kg/day). CRF rat models were established via 5/6 Nx as previously reported.\textsuperscript{16} Briefly, rats were anesthetized and uninephrectomy of the right kidney was performed, followed by amputation of the poles of the remaining kidney 3 weeks later. Sham operation was administrated in the sham group.

#### PDYS formula

PDYS formula contains six components, as shown in Table 1. All components were supplied by the Traditional Chinese Medicine (TCM) Pharmacy of our hospital. The PDYS formula was decocted and crude drug was extracted at a concentration of 1000 mg/L. The decoction was stored at 4°C after sterilization; 7 days after the final operation, the decoction (0.9 and 1.8 g/kg/day) was administrated by means of intragastric administration daily, for 12 weeks, to the rats in PDYS formula treatment groups. In addition, rats in the sham group and 5/6 Nx group were treated with an equal amount of normal saline. Blood and urine samples were collected at 0, 4, 8, and 12 weeks after surgery. Finally, all rats were sacrificed and tissue samples were collected.

#### Measurement of Scr, BUN, and TUP as well as TNF-α, FN, and LN

The concentration of Scr, BUN, and TUP in each group was tested using the biochemical assay kits according to the manufacturer’s protocol. Blood

### Table 1. Constituents of PDYS formula.

| Chinese name | Scientific name | Weight (g) |
|--------------|-----------------|------------|
| Da Huang     | Rheum palmatum L | 8.3        |
| Huang Qi     | Astragalus membranaceus (Fisch.) | 20.8 |
| Di Huang     | Rehmannia glutinosa (Gaert.) Libosch | 10.4 |
| Dang Shen    | Codonopsis pilosula (Franch.) Nannf | 12.4 |
| Zi Dan Shen  | Salvia miltiorrhiza Bunge | 12.4 |
| Ba Ji Tian   | Morinda officinalis How | 10.4 |
| Liu Yue Xue  | Serissa japonica (Thunb.) | 12.4 |
| Shan Yu Rou  | Cornus officinalis Sieb. et Zucc. | 4.2 |
| Ze Xie       | Alisma plantago-aquatica L | 6.2 |
| Hong Hua     | Carthamus tinctorius L | 2.5 |

PDYS: Pai-Du-Yang-Shen.
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(or 24 h urinary) samples were enriched by refrigerated centrifugation (18,000×g for 20 min at 4°C). Then, relative concentrations of Scr, BUN, and TUP were measured using an automatic biochemistry analyzer (HITACHI 7170S, Japan).

The concentrations of TNF-α, FN, and LN were determined using ELISA assays kits according to the manufacturer’s instructions.

**Quantitative real-time polymerase chain reaction analysis**

Total RNA of renal tissue was prepared using TRIzol reagent. Subsequently, 2 µg of RNA was reverse transcribed into cDNA using the maxima first-strand cDNA synthesis kit. Quantitative polymerase chain reaction (qPCR) was performed using primers and SYBR Green reagents (Thermo, USA) on an ABI 7500 system. Primers: TNF-α, forward-5′-ATGGGCTCTCCACCTCAGT-3’, reverse-5’-CAAGGGCTCTTGATGGCGA-3’. TGF-β, forward-5’-GGACTCTCCACCCTGAAGAC-3’, reverse-5’-CGTGTTGCTCACAGTGAC-3’. α-SMA, forward-5’-AGTGAAAGCAGTCAGTTTGGC-3’, reverse-5’-CTCTCTGTTCAGTTTGGAC-3’. FN, forward-5’-GATGAGCTTCCCCAACTGGT-3’, reverse-5’-GTGGTTCGCTAAGGCCATGT-3’. GAPDH, forward-5’-GGTGGACCTCATGGCCTACA-3’, reverse-5’-CTCTTGTACACCTCGAAGCGA-3’. TGF-β1, forward-5’-GACAGTTTGGAC-3’, reverse-5’-TCTCTTCATCAGTGACT-3’. TGF-β2, forward-5’-GACAGTTTGGAC-3’, reverse-5’-TCTCTTCATCAGTGACT-3’. Data were assessed using 2−ΔΔCt relative quantification analysis against referenced GAPDH expression. ΔΔCt = (Ct, target gene in treatment group − Ct, GAPDH in treatment group) − (Ct, target gene in sham group − Ct, GAPDH in sham group).

**Western blotting**

Total protein was prepared for western blot analysis. After being boiled for 5 min in loading buffer, proteins (35 µg) were separated on 8% Tris-glycine gels and transferred onto polyvinylidene difluoride (PVDF) membrane. Western blotting was performed using primary antibodies of TGF-β1 (1:500), GAPDH (1:5000), NF-κB p65 (1:1000), and p-p65 (1:1000), followed by incubation with horseradish peroxidase (HRP)–conjugated secondary antibody. GAPDH was used as a loading control.

**Statistical analysis**

All data were expressed as mean ± SD (n = 6). For statistical analysis, analysis of variance (ANOVA) and Duncan’s multiple range test were performed on SPSS 19.0 (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered as a statistical difference.

**Result**

**Histopathological changes in renal tissues**

Histopathological changes in renal tissues were detected via hematoxylin–eosin (H&E) staining at the end of the animal experiments. As shown in Figure 1(a), opening and closing of the abdomen did not affect the tubular and glomerular structures (sham group) and there were no obvious histopathological changes in the renal tissues in the sham group. However, obvious fibrosis and degeneration were observed in the 5/6 Nx group. These features included a thick glomerular basement membrane and disordered glomerular structure. However, fibrosis and diffuse glomerular sclerosis were effectively improved by PDYS formula treatment (both in the 0.9 and 1.8 g/kg/day treatment groups).

**Effect of PDYS formula treatment on Scr, BUN, and TUP in rats**

The Scr, BUN, ALB, and TUP contents in each group were determined at 0, 4, 8, and 12 weeks after surgery to evaluate renal function. The results show that Scr, BUN, and TUP levels were induced by 5/6 Nx in rats in a time-dependent manner. The increase in urinary and blood biochemical indexes was decreased by PDYS formula treatment. In addition, an obvious density effect of PDYS formula was observed in this study (Figure 1(b)).

**Effect of PDYS formula treatment on TNF-α, FN, and LN concentration in renal tissues**

The TNF-α, FN, and LN levels were tested by ELISA kits to assess the inflammation and accumulation of ECM in renal tissues. As shown in Figure 2(a), the increase in concentrations of TNF-α, FN, and LN in 5/6 Nx rats was decreased by PDYS formula treatment.

**Effect of PDYS formula treatment on mRNA expressions of α-SMA, FN, TNF-α, and TGF-β in renal tissues**

Relative mRNA expressions of α-SMA, FN, TNF-α, and TGF-β in 5/6 Nx rats were determined to
investigate the potential mechanism of PDYS formula on CRF. Relative mRNA expressions of α-SMA, FN, TNF-α, and TGF-β were upregulated in 5/6 Nx rats but was reversed PDYS formula treatment (Figure 2(b)).

**Discussion**

CRF is chronic and progressive kidney damage caused by various factors. Renal fibrosis, a common pathological feature of CRF, is characterized by the loss of renal units instead of ECM. In this study, PDYS formula, a traditional Chinese medicine, has shown certain therapeutic effects on CRF, specifically manifesting as an effective reduction in the inflammation and ECM deposition in 5/6 Nx rats.

TGF-β is the most common pro-fibrotic cytokine during chronic kidney disease (CKD) via non-Smad and Smad-dependent signaling pathways and results in various downstream biological effects. The
activation of TGF-β-Smads can stimulate myofibroblasts to enhance the production of ECM proteins by upregulating α-SMA, FN, LN, and collagen as well as tissue inhibitors of metalloproteinases (TIMPs) expression while inhibiting matrix metalloproteinases (MMPs), which promotes the degeneration of ECM. Inflammatory response has been regarded as the driving force for renal fibrosis as inflammation is known to initiate fibrogenesis. The NF-κB pathway has been shown to mediate cellular inflammatory reactions and is activated in hypoxic epithelia cells. Activation of NF-κB promotes fibrosis in liver, renal, and brain tissues via regulation of target adhesion molecules, pro-inflammatory enzymes, and inflammatory cytokines. Blockade of NF-κB signaling has been reported to revert cells to epithelioid morphologies in peritoneal mesothelial cells and inhibit the expression of transcription factor, Snail1, an efficient inducer of EMT. In this study, the expression of TGF-β and

**Figure 2.** (a) Effects of PDYS formula treatment on TNF-α, FN, and LN content in renal tissues. (b) Effects of PDYS formula treatment on mRNA expression of α-SMA, FN, TNF-α, and TGF-β in renal tissues.

*P < 0.05 and **P < 0.01, compared with the sham group. $P < 0.05$ and $$P < 0.01$, data in PDYS formula treatment group compared with those in 5/6 Nx group (n = 6).

**Figure 3.** Effects of PDYS formula treatment on TGF-β, NF-κB p65, and p-p65 protein levels in renal tissues.

*P < 0.05 and **P < 0.01, compared with the sham group. $P < 0.05$ and $$P < 0.01$, relative protein levels in PDYS formula treatment group (L: 0.9 g/kg/day; M: 1.8 g/kg/day) compared with those in 5/6 Nx group (n = 6).
activated NF-κB were downregulated by PDYS formula treatment in 5/6 Nx rats.

Taken together, PDYS formula shows an ideal therapeutic effect against 5/6 Nx-induced CRF in rats by decreasing inflammation and ECM deposition. In addition, the effects of PDYS may be through downregulation of TGF-β and NF-κB signaling.

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