ARTICLE

doi: 10.12032/TMRIM202004013

TMR Integrative Medicine

Effects of Yishenbupi (tonifying-kidney and invigorating-spleen) prescription on the expression of renal fibrosis-associated proteins in unilateral ureteral occlusion rats

Jue-Ying Chen[1], Ji-Wei Qiu[1], Jun-Lin Mo[1], Chun-Li Long[1], Wei Shi[2], Yong-Xiang Xie[2*]

[1]Guangxi University of Chinese Medicine, Nanning 530001, China. [2]First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning 530023, China.

*Corresponding to: Yong-Xiang Xie, Department of Nephrology, First Affiliated Hospital of Guangxi University of Chinese Medicine, No.89-9, Dongge Road, Qingxiu District, Nanning 530023, China. Tel: 18169636318, Email: xyx2218@163.com.

Highlights

Yishenbupi (tonifying-kidney and invigorating-spleen) prescription is an effective compound preparation for inhibiting the progress of renal interstitial fibrosis, which is regulated by multiple targets. The study shows that Yishenbupi prescription can reduce the expression of renal fibrosis-associated proteins, such as α-SMA, fibronectin, and vimentin, thus improving renal interstitial fibrosis and delaying the progress of renal interstitial fibrosis in unilateral ureteral occlusion rats.

Abstract

Objective: To evaluate the effects and mechanism of the Yishenbupi (tonifying-kidney and invigorating-spleen) prescription on the expression of renal fibrosis-associated vimentin, α-SMA, and fibronectin in unilateral ureteral occlusion rats. **Methods:** A total of 48 SD (*Sprague-Dawley*) rats were randomly divided into the model, sham-operated (sham), irbesartan, and Yishenbupi groups, with 12 rats in each group. After the unilateral ureteral occlusion model was established, rats in the model and sham groups were administered normal saline, whereas rats in the Yishenbupi group were administered Yishenbupi prescription (18 g/kg/d) intragastrically and those in the irbesartan group were administered irbesartan (10 mg/kg/d) intragastrically. All rats were sacrificed 21 days later. Pathological changes in rat renal tissue were evaluated by H&E staining. The expression of vimentin, α-SMA, and fibronectin in renal tissues was detected by western blotting. **Results:** Compared with the sham group the model group had renal tubular epithelial cell atrophy, inflammatory cell infiltration accompanied with the proliferation of interstitial collagen fibers, fewer glomeruli, or glomerulosclerosis. Compared with the model group, significantly less renal tubular and glomerular damages, inflammatory cell infiltration, and collagen fibers were observed in different intervention groups, especially in the Yishenbupi group. Compared with the sham group, significantly higher expressions of fibrosis markers, including vimentin, α-SMA, and fibronectin, were observed in the model group. Compared with the model group, the expression of anti-fibrosis markers, including vimentin, α-SMA and fibronectin, was significantly decreased in both the irbesartan and Yishenbupi groups ($P < 0.01$); however, the Yishenbupi group showed higher efficacy than the irbesartan group ($P < 0.05$). **Conclusion:** The Yishenbupi prescription may improve renal fibrosis by reducing the expression of fibrosis-associated vimentin, α-SMA, and fibronectin.

Keywords: Renal interstitial fibrosis, Yishenbupi prescription, Vimentin, α-SMA, Fibronectin

***Abbreviations*:**

RIF, renal interstitial fibrosis; EMT, epithelial-mesenchymal transition; ECM, extracellular matrix; α-SMA, α-smooth muscle actin; FN, fibronectin; TCM, traditional Chinese medicine; UUO, unilateral ureteral occlusion; SPF, specific-pathogen free.

***Ethical approval*:**

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

***Competing interests*:**

The authors declare that there is no conflict of interest.

***Acknowledgments*:**

This work was supported by the Program of Fund for Less Developed Regions of the National Natural Science Foundation of China (No.81460719), the General Program of Guangxi Natural Science Foundation (No.2017GXNSFAA198217), the Key specialty of the National Administration of Traditional Chinese Medicine, the Office of Famous TCM Doctor SHI Wei, and the Zhangjiakou Key Research and Development Program (No.1921136H).

***Citation*:**

Jue-Ying Chen, Ji-Wei Qiu, Jun-Lin Mo, et al. Effects of Yishenbupi (tonifying-kidney and invigorating-spleen) prescription on the expression of renal fibrosis-associated proteins in unilateral ureteral occlusion rats. TMR Integrative Medicine 2020, 4: e20012.

***Executive Editor*:** Shan-Shan Lin.

***Submitted*:** 15 April 2020, ***Accepted*:** 13 June 2020

TMR
doi: 10.12032/TMRIM202004013

Background

Renal interstitial fibrosis (RIF) is the final pathological manifestation of end-stage renal disease. The mechanism of RIF remains unclear and may be related to renal tubular epithelial-mesenchymal transition (EMT), inflammatory cytokines, and excess deposition of extracellular matrix (ECM) [1]. Therefore, finding an anti-fibrotic drug for chronic kidney disease is a major problem. EMT is an important mechanism in RIF. EMT reduces fibronectin expression, obtains new mesenchyme, and increases the expression of vimentin, α-smooth muscle actin (α-SMA) and fibronectin (FN) [2-3]. Renal tubular epithelial cells transdifferentiate into activated fibroblasts and mesenchymal cells, which are then activated by chemokines into myofibroblasts. Myofibroblasts deposit new collagen and elastic fibers and synthesize ECM proteins to induce RIF [4].

Renal fibrosis is a complex mechanism and no effective treatment is present in Western medicine. Therefore, we explored new treatments using traditional Chinese medicine (TCM). RIF can be inhibited by TCM monomers and compound combinations, such as tonic, which promotes blood circulation, removes blood stasis, and softens and resolves hard mass [5]. Our studies show [6-7] that the Yishenbupi (tonifying-kidney and invigorating-spleen) prescription can improve renal function by lowering microinflammation, oxidative stress, and TGF-β1 expression. Therefore, the Yishenbupi prescription was used in this study for the intervention of the unilateral ureteral occlusion (UUO) model. The change in expressions of RIF indexes, such as vimentin, α-SMA, fibronectin, was observed in the pathological process of UUO. The mechanism of the Yishenbupi prescription in anti-renal fibrosis was preliminarily investigated.

Materials and methods

Materials

Animals. A total of 48 healthy male specific-pathogen free (SPF) SD (*Sprague-Dawley*) rats (aged 6–8 weeks) weighing 200 ± 20 g were provided by the Chengdu Dossy Experimental Animals Co., Ltd. All the rats were given access to food and water ad libitum and raised in a quiet environment. All experiments were performed in accordance with the rules of our hospital on the protection and use of experimental animals.

Yishenbupi prescription. The Yishenbupi prescription comprised 12 Chinese herbs: 30 g Huangi (*Radix Astragali*), 20 g Fuling (*Poria*), 20 g Huaishan (*Rhizoma Dioscoreae*), 10 g Dangshen (*radix codonopsis*), 10 g Baizhu (*Rhizoma Atractylodis Macrocephalae*), 10 g Shouwu (*Radix Polygoni Multiflori*), 10 g Tusizi (*Semen Cuscutae*), 6 g Gancao (*Radix Glycyrrhizae*), 10 g Muxiang (*Radix Glycyrrhizae*), 15 g Yiyiren (*Semen Coicis*), 10 g Sanqi (*Radix Notoginseng*), and 10 g Danshen (*Radix Salviae Miltiorrhiae*). All the herbs were obtained from the TCM pharmacy of the First Affiliated Hospital of Guangxi University of Chinese Medicine, prepared by the hospital preparation center into a solution containing 1 g/mL of dried medicinal herbs after physical purification and concentration. The solution was stored at 4°C in the refrigerator until further. Irbesartan tablets: provided by Jiangsu Hengrui Medicine Co., Ltd. (No.H20000513), specification: 150 mg/tablet.

Reagents. The mouse streptavidin-biotin detection system (Lot: SP-9002), rabbit streptavidin-biotin detection system (Lot: SP-9001), and DAB kit (Lot: ZLI-9017) were purchased from China Zhongshan Goldenbridge Biotechnology Co., Ltd. The H&E staining kit (Lot: C0105) was purchased from Beyotime Biotechnology Co., Ltd. The mouse monoclonal anti-α-SMA antibody (Lot: 56856) was purchased from Cell Signaling Technology, USA. The mouse monoclonal anti-fibronectin antibody (Lot: BA1772) and rabbit monoclonal anti-vimentin antibody (Lot: BM0135) were purchased from Boster of USA.

Instruments. High-speed refrigerated centrifuge (Eppendorf, Germany); optical microscope (Olympus, Japan); MultiImager (Bio-Rad, USA); microtome (Leica, Germany); MS-H-S standard magnetic stirrer (Kebosaier, Beijing); tissue homogenizer (Kinematica, Switzerland); and electrophoresis instrument (Bio-Rad, USA).

Establishment of animal model

The UUO rat model was established as described [8]. Briefly, rats were deprived of food and water 24 hours before the operation. Thereafter, the rats were anesthetized by an intraperitoneal injection of pentobarbitone sodium at 45 mg/kg body weight. An incision was made in the skin along the lower rib on the left back to isolate the left kidney. The left ureter was separated, ligated, and sutured layer by layer. The ureter was sutured layer by layer after being separated in the sham group. The entire procedure was performed under sterile conditions.

Grouping and administration

Forty-eight rats were randomly divided into the model, sham, irbesartan, and Yishenbupi groups, with 12 rats in each group. The rats in the Yishenbupi group were intragastrically administered the Yishenbupi prescription (18 g/kg/d) every morning for 21 days, from the day the model was established to the day the rats were sacrificed. The rats in the irbesartan group were administered 10 mg/kg/d irbesartan tablets intragastrically every morning for 21 days, from the day the model was established to the day the rats were

sacrificed. The rats in the model and sham groups were administered equal volumes of normal saline intragastrically once a day from the day the model was established to the day the rats were sacrificed.

Observation methods

H&E staining. From each group, three rat kidneys were selected, fixed, dehydrated, embedded, and sectioned. Thereafter, the sections were stained with Harris' hematoxylin according to the kit instructions, dried, and sealed with neutral balsam.

Expression of α-SMA, vimentin, and fibronectin detected by western blotting. The frozen tissue samples were configured with gel and loaded for electrophoresis. The membranes were transferred and blocked with 5% non-fat milk powder. The primary antibody (1:1000) was blocked at 4°C overnight. Next, non-fat milk powder was used to prepare the secondary antibody diluent (1:3000), which was incubated for 1 hour at room temperature (about 25°C). The Gel-Pro analyzer software was used to analyze the IOD ratio between the target protein band and the internal reference protein.

Statistical methods

Data were analyzed by SPSS 16.0. Differences among groups were compared by analysis of variance or q test; correlations were analyzed by the method of linear correlation analysis. $P < 0.05$ indicates a significant difference and $P < 0.01$ indicates a highly significant difference.

Results

Effects of the Yishenbupi prescription on renal pathology in the rat model

The H&E staining showed that the renal glomerulus and tubule had normal structure, and no inflammatory infiltration of tissues or fibrous hyperplasia was observed around the interstitium in the sham group. Renal tubular epithelial atrophy, less visible glomeruli and glomerulosclerosis, different degrees of renal interstitial edema, and fibrous hyperplasia accompanied with inflammatory cell infiltration were observed in the model group. Compared with the model group, glomerulosclerosis and renal tubular epithelial atrophy were relieved to different degrees and renal interstitial fibrous hyperplasia and inflammatory cell infiltration were reduced significantly in the irbesartan and Yishenbupi groups. The Yishenbupi group showed better efficacy than the irbesartan group, as shown in Figure 1.

Effects on expression of α-SMA, vimentin, and fibronectin in kidney tissues of rats with renal fibrosis

The expression of α-SMA in the renal tissue of each group was observed by immunohistochemistry.

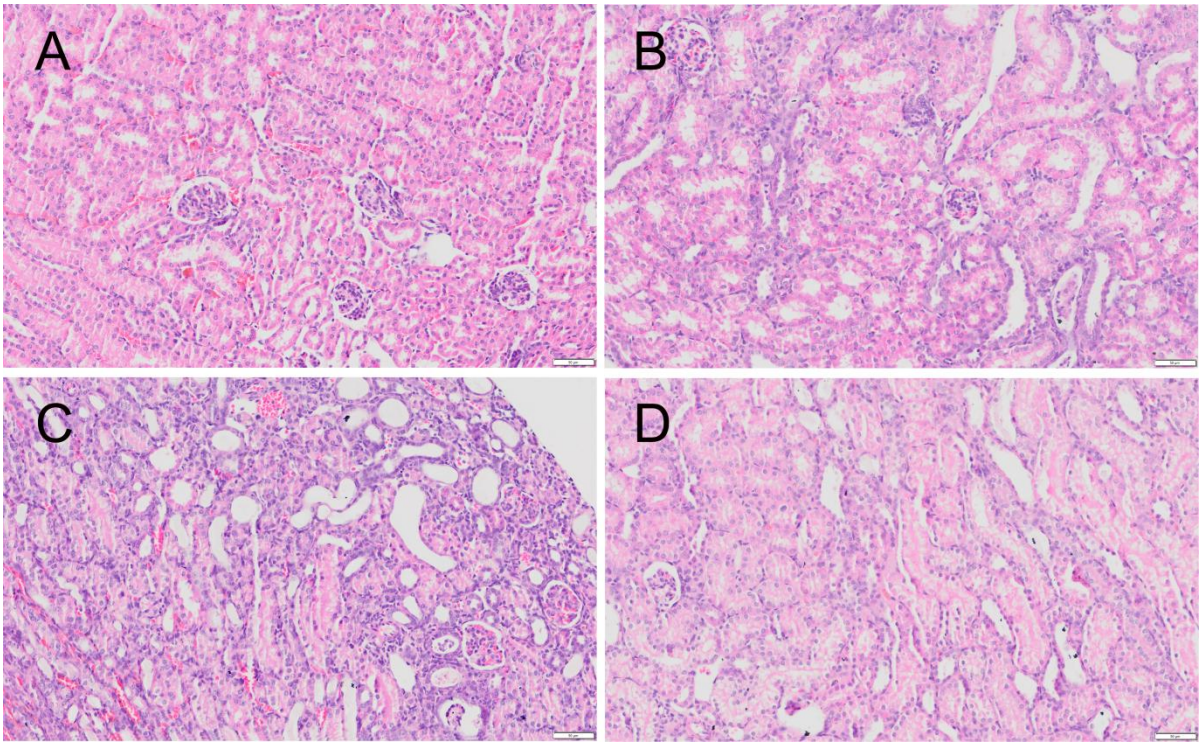

Figure 1 H&E staining of rat kidneys in each group. Compared with the model group, glomerulosclerosis and renal tubular epithelial atrophy were relieved to different degrees and renal interstitial fibrous hyperplasia and inflammatory cell infiltration were reduced significantly in the irbesartan and Yishenbupi groups. The Yishenbupi group showed better efficacy than the irbesartan group. UUO, unilateral ureteral occlusion.

TMR
doi: 10.12032/TMRIM202004013

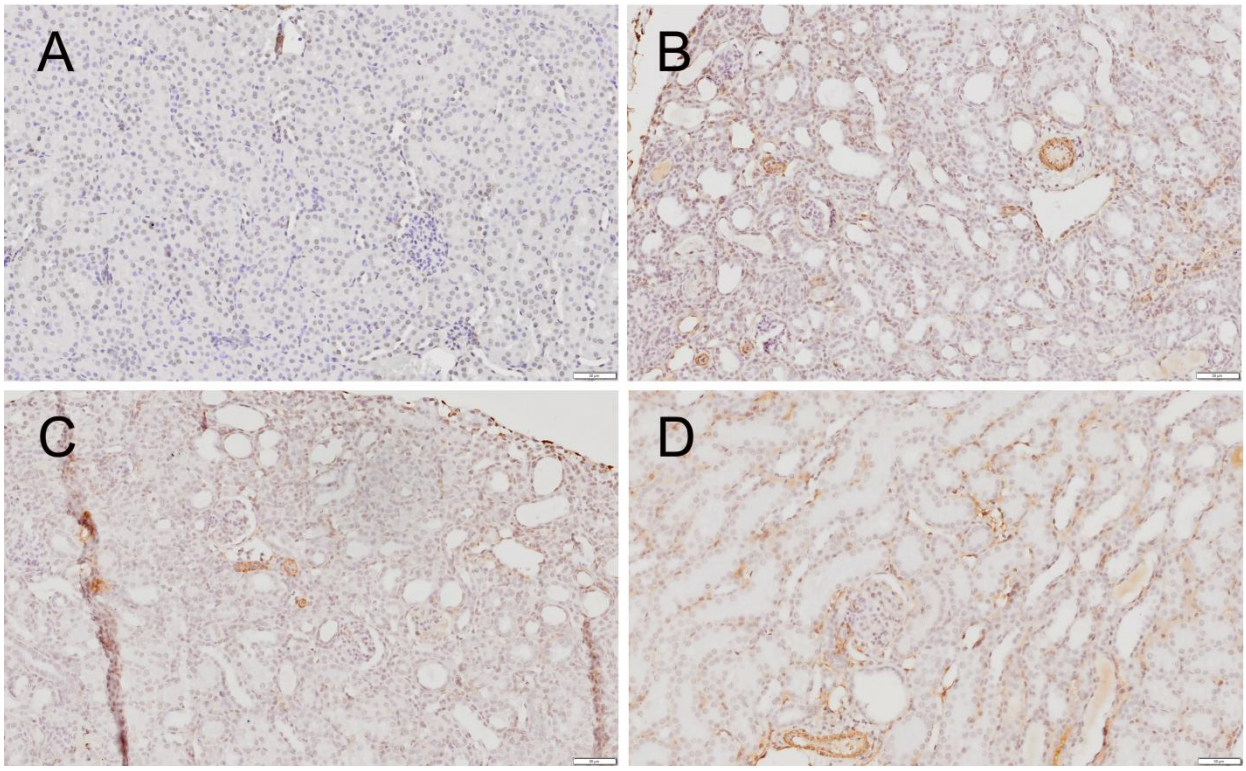

Figure 2 Expression of α-SMA in renal tissues of each group by immunohistochemistry. Compared with the sham group, the model group could be observed high expression of α-SMA and the deposition of myofibroblasts. Compared with the model group, less brown collagen was observed in the irbesartan and Yishenbupi groups, and brown collagen deposition was least in the Yishenbupi group. UUO, unilateral ureteral occlusion.

Compared with the sham group, a large amount of brown collagen was expressed in the renal interstitium and around blood vessels in the model group, suggesting high expression of α-SMA and the deposition of myofibroblasts. Compared with the model group, less brown collagen was observed in the irbesartan and Yishenbupi groups. Moreover, brown collagen deposition was least in the Yishenbupi group, which indicates that both irbesartan and Yishenbupi prescription can inhibit the expression of α-SMA in RIF, and the Yishenbupi prescription can inhibit the generation of myofibroblasts and delay the progress of RIF more effectively, as shown in Figure 2.

The western blot revealed that compared with the sham group, the expressions of renal tubulointerstitial fibrosis markers, including α-SMA, fibronectin, and vimentin, were significantly increased in the model group ($P < 0.01$). Compared with the model group, the relative expressions of α-SMA, fibronectin, and vimentin were significantly decreased in the irbesartan and Yishenbupi groups, and the differences were statistically significant ($P < 0.01$). Compared with the irbesartan group, the expressions of α-SMA, fibronectin, and vimentin decreased significantly in the Yishenbupi group. The differences were statistically significant ($P < 0.05$), as shown in Figure 3.

Discussion

α-SMA is the main marker of fully mature myofibroblasts. During EMT, the expression of α-SMA and vimentin increases, which is strongly associated with renal fibrosis [9-10]. The increased expression of α-SMA in kidney disease [11-12] can promote the progress of renal fibrosis and serve as an indicator for detecting renal fibrosis. Vimentin is a marker protein for ECM deposition and EMT transformation [13], it is widely distributed in mesenchymal cells, such as myofibroblasts, and its expression is correlated with the activation of renal tubular epithelial cells [14]. Under normal conditions, vimentin is present in small amounts in the glomeruli, arterioles, and mesenchymal cells. Under pathological conditions, vimentin can be expressed on renal tubular epithelial cells to provide a scaffold for the formation of collagen fibers and the deposition of ECM [15]. The fibronectin is an ECM protein that binds to and disperses on renal tubular epithelial cells and renal interstitial myofibroblasts, integrates transferrin receptors, and forms the necessary connections between the structural components of ECM [16]. Studies on kidney injury caused by ischemia have found that during renal fibrosis, fibronectin accumulation indicates changes in activity and progress. Active cells in the glomeruli can

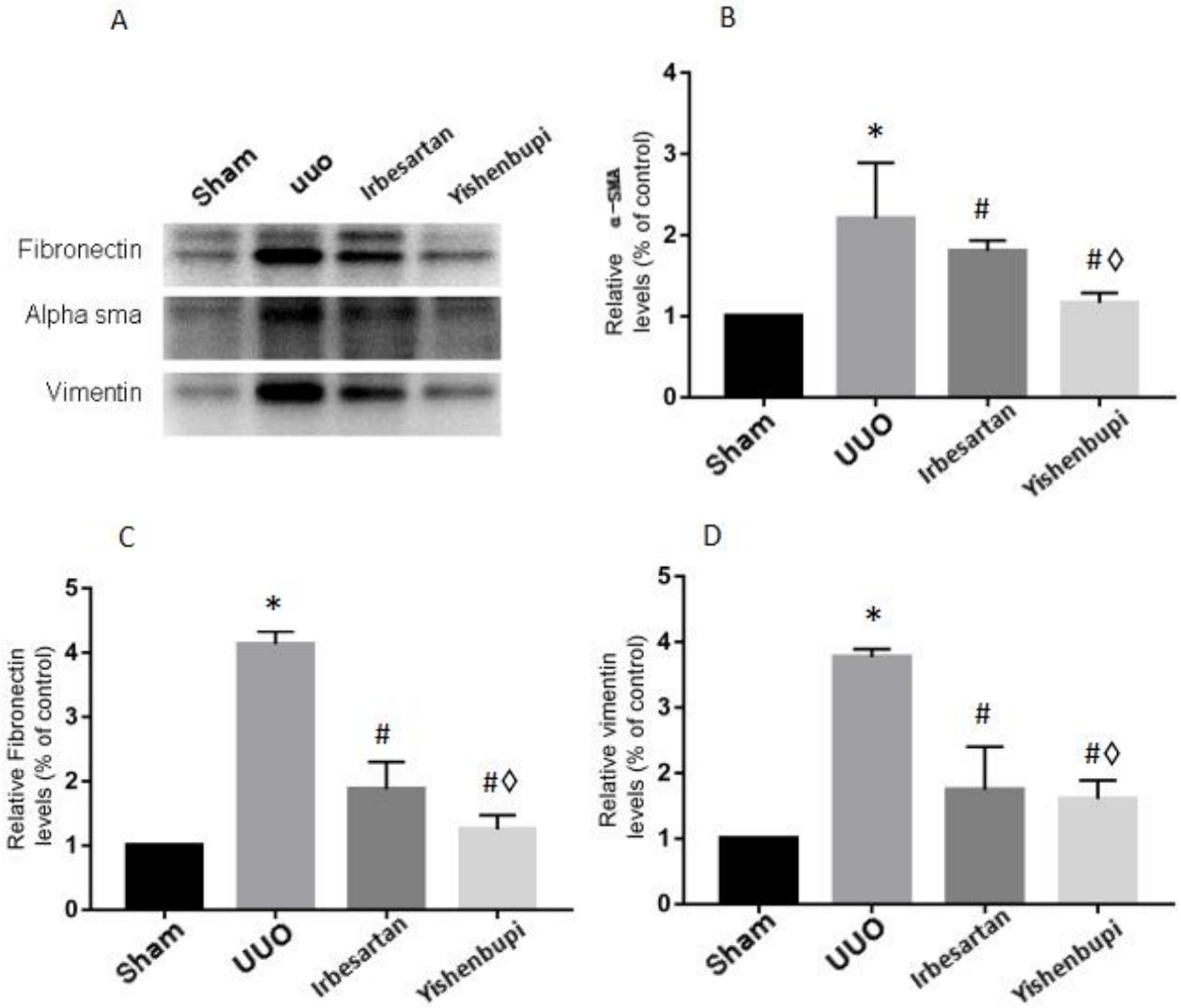

Figure 3 Expression of renal fibrosis-associated α-SMA, fibronectin, and vimentin in each group by western blot. Note: **A** shows the expression of α-SMA, fibronectin, and vimentin in rat kidneys of each group detected by western blot. **B**, **C**, and **D** show the relative quantitative data for α-SMA, fibronectin, and vimentin, respectively. Compared with the sham group (n = 12), *P < 0.01; compared with UUO model group (n = 12), #P < 0.01; irbesartan group (n = 12) was compared with the Yishenbupi group (n = 12), ◇P < 0.05. UUO, unilateral ureteral occlusion.

promote the secretion of ECM and speed the progress of renal fibrosis [17].

The UUO rat model can induce EMT to transform epithelial cells into myofibroblasts, thereby promoting renal fibrosis [18]. After being transformed into myofibroblasts, the renal tubular epithelial cells move to the renal tubular epithelial matrix and produce ECM proteins through the EMT. Our study showed that in the model group, a large amount of brown collagen was expressed in the renal interstitium and around blood vessels by immunohistochemical analysis; renal tubular epithelial atrophy, different degrees of renal interstitial edema, fibrous hyperplasia, and a massive infiltration of inflammatory cells was observed; some glomeruli were less visible and developed sclerosis, which is consistent with the pathology of RIF [19]. After the intervention, pathological conditions were improved to different degrees in both the irbesartan and Yishenbupi groups. Glomerulosclerosis, renal interstitial inflammatory cell infiltration, and fibrous hyperplasia were significantly reduced. The Yishenbupi group showed better efficacy than the irbesartan group. Compared with the sham group, the expression of vimentin, fibronectin, and α-SMA increased in the kidney tissues of UUO rats and increased most in the model group. Compared with the model group, the expression of α-SMA, fibronectin, and vimentin decreased significantly in each intervention group. The Yishenbupi group performed better than the irbesartan group in reducing

TMR
doi: 10.12032/TMRIM202004013

fibrosis-associated components, such as α-SMA, fibronectin, and vimentin. The Yishenbupi prescription showed excellent results in improving the pathological changes of RIF, possibly by reducing the transformation of EMT and deposition of ECM and down-regulating the expression of renal interstitial fibrosis-associated α-SMA, fibronectin, and vimentin, thereby alleviating the progress of RIF and protecting renal function. Studies have confirmed that the compound in the Fulingpi (*Cortex Poriae Cocos*) has an antagonistic effect on renal fibrosis by inhibiting the renin-angiotensin system, Wnt/β-catenin signaling pathway, and TGF-β/Smad signaling pathway [20]. Gancao (*Radix Glycyrrhizae*) extract has an antagonistic effect on renal fibrosis by inhibiting the expression of cytokine TGF-β1 [21]. Notoginsenoside activates SIRT1, inhibits the TGF-β1/Smad pathway, and reduces EMT, thus protecting the renal tubular epithelial cells [22]. Cuscutae Semen extract can reduce the expression of α-SMA, CTG, and Col-I in kidney tissue and effectively alleviate the progress of RIF in rats [23]. Yiyiren (*Semen Coicis*) oil injection can inhibit the TGF-β1/Smad signal transduction pathway, down-regulate the expression of α-SMA and TGF-β1 in renal interstitial tissue, and reduce UUO-induced renal tubular fibrosis [24].

Conclusion

In conclusion, the Yishenbupi prescription reduced the expression of renal fibrosis-associated proteins, such as α-SMA, fibronectin, and vimentin, thus improving renal interstitial fibrosis and delaying the progress of RIF in UUO rats. The Yishenbupi prescription is an effective compound preparation for inhibiting the progress of RIF, which is regulated by multiple targets. Thus, it lays a foundation for further investigation on the target and mechanism of TCM in anti-RIF.

References

1. Shen M. Effects of CUEDC2 in renal interstitial fibrosis and its mechanism. Southern Medical University, 2016.
2. Li R, Guo Y, Zhang Y, et al. Salidroside ameliorates renal interstitial fibrosis by inhibiting the TLR4/NF-κB and MAPK signaling pathways. Int J Mol Sci 2019, 20: 1103.
3. Liu Y. New insights into epithelial-mesenchymal transition in kidney fibrosis. J Am Soc Nephrol 2010, 21: 212–222.
4. Paolillo M, Schinelli S. Extracellular matrix alterations in metastatic processes. Int J Mol Sci 2019, 20: 4947.
5. He PF, Gao M, Zhang Y, et al. An experimental study on applying TCM compound prescriptions in anti-renal fibrosis. Yunnan J Trad Chin Med Mater Med 2019, 40: 86–88.
6. Xie YX, Long CL, Tao ZH, et al. Effect of spleen-invigorating and kidney-tonifying prescription on microinflammation, oxidative stress, and nutritional status in patients with stage III chronic kidney disease. Chin J Integr Tradi West Nephrol 2016, 17: 54–56.
7. Xie LP, Xie YX, Shi W, et al. Effect of invigorating-spleen and tonifying-kidney prescription on the renal function and TGF-β1 level in patients with stage III chronic kidney disease. Liaoning J Trad Chin Med 2016, 43: 965–967.
8. Moriyama T, Kawada N, Ando A, et al. Up-regulation of HSP47 in the mouse kidneys with unilateral ureteral obstruction. Kidney Int 1998, 54: 110–119.
9. Xie SB, Wang WM, Chen N. Progression of renal tubulointerstitial fibrosis and expression of α-SMA, TGF-β-1 and VDR in rat UUO models. J Shanghai Jiaotong Univ (Med Sci) 2010, 30: 752–757+796.
10. Sun XH, Cai JQ, Huang LH, et al. The relation of tubular vimentin expression and chronic allograft nephropathy. Mod Med J Chin 2011, 13: 1–2.
11. Suo Y, Gu YY, Wang S. Effects of α-SMA on proliferation and apoptosis of renal tubular epithelial cells induced by high glucose. Shandong Med J 2017, 57: 14–17.
12. Liu Y. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism,and therapeutic intervention. J Am SocNephrol 2004, 15: 1–12.
13. Battaglia RA, Delic S, Herrmann H, et al. Vimentin on the move: new developments in cell migration. F1000Res 2018, 7: F1000.
14. Ma QY, Li RP, Tie XL, et al. Effects of Qufeng Tongluo prescription on renal interstitial fibrosis-associated protein α-SMA and vimentin expression of UUO rats. Chin J Chin Med 2019, 34: 1665–1669.
15. Tang YH, Zhu CL. An experimental study on expression of vimentin in rat renal tubulointerstitial fibrosis and its significance. J Guiyang Med Coll 2010, 35: 160–163+167.
16. Zollinger AJ, Smith ML. Ibronectin, the extracellular glue. Matrix Biol 2017, 60–61: 27–37.
17. Zuk A, Matlin KS. Induction of a laminin isoform andα3β1-inte-grin in renal ischemic injury and repair in vivo. Am J Physiol Renal Physiol 2002, 283: 971–984.
18. Iwano M, Plieth D, Danoff TM, et al. Evidence that fifibroblasts derive from epithelium during tissue fifibrosis, J Clin Invest 2002, 110: 341–350.
19. Wang HY. Nephrology. 3rd ed. Beijing: People's Medical Publishing House, 2008.
20. Wang M. Effective constituents and mechanism of the surface layer of poria cocos against renal

fibrosis. Northwest University, 2019.
21. Liu WJ, Nan Y, Lu YM, et al. Protective effect of glycyrrhetic acid on early and middle stages of renal fibrosis in mice with kk-ay diabetes. Yunnan J Trad Chin Med Mater Med 2020, 41: 72–78.
22. Pan J. Effect of notoginsenoside on EMT of rat renal tubular epithelial cells induced by high glucose/TGF-β_1. Zhejiang Chinese Medical University, 2019.
23. Xu YP, Xiang Z, Pan Y, et al. Effect of Cuscutae Semen extract on renal interstitial fibrosis in rats. Chin Trad Pat Med 2013, 35: 2103–2108.
24. Hu Y, Lin FL, Zou QL, et al. Effect of Coicis Semen oil on renal interstitial fibrosis in rats with unilaterai urethral obstruction. Chin J Chin Mater Med 2013, 38: 1982–1986.