Effects of Wuling Powder Mediating Notch Pathway on Mice with Nephrotic Syndrome

Luotong JING, Yihan LI, Honglanxi LI, Wenyan ZHANG, Lin QIN, Ning LIANG*

Guangxi University of Chinese Medicine, Nanning 530000, China

Abstract [**Objectives**] This study was conducted to investigate the renal protective effects of Wuling Powder on mice with nephrotic syndrome (NS) based on Notch pathway. [**Methods**] Sixty KM mice were randomly divided into normal group, model group, prednisone acetate positive group, high-dose Wuling Powder group, medium-dose Wuling Power group and low-dose Wuling Power group, with 10 mice in each group. Three days after prophylactic administration, a comprehensive nephropathy model was prepared by injecting 1 mg/ml doxorubicin hydrochloride solution (7.5 mg/kg) into the tail vein. After successful modeling, prednisone acetate and Wuling SAN were given high, medium and low doses for intervention for 28 d, respectively. After that, urinary protein and creatinine contents of mice in each group were detected, and pathological damage of renal tissue was observed by HE and Masson staining. The mRNA levels of Notch1, Jagged1 and Hes1 in mouse kidney tissues were detected by RT-PCR, and the expression levels of Notch1, Jagged1 and Hes1 proteins were detected by Western blot. [**Results**] Wuling Powder could effectively reduce the contents of urine protein ($P<0.01$) and Scr ($P<0.01$) in NS mice, and alleviate the pathological injury of kidney. Compared with the model group, the prednisone acetate group and various Wuling Powder groups could down-regulate the expressions of Notch1, Jagged1 and Hes1 mRNA in the kidney tissue of mice ($P<0.01$), and the expression of Notch1 protein in the renal tissue of mice decreased ($P<0.01$). The contents of Hes1 in the prednisone acetate group and the high- and medium-dose Wuling Powder groups significantly decreased ($P<0.05$). [**Conclusions**] Wuling Powder could protect the kidneys in mice with NS through Notch pathway.

Key words Wuling Powder; Nephrotic syndrome; Mice; Notch pathway

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Nephrotic syndrome (NS) is the main cause of chronic renal failure in the world[1]. It is a glomerular disease caused by the damage of the structure and function of glomerular filtration barrier, which leads to a large number of protein loss in plasma. Clinically, it often manifests as a series of symptoms related to the damage of glomerular filtration barrier, such as low albumin, massive proteinuria, edema and hyperlipidemia[2]. Modern medical treatments for NS mainly include glucocorticoid, alkylating agent, cyclosporine and other immunosuppressants, but all of them have problems such as large dosage and many toxic and side effects, which lead to unsatisfactory clinical treatment of NS. A large number of clinical and experimental data show that Chinese medicine intervention therapy can delay the symptoms of proteinuria in DN patients in many ways, and it is conducive to improving renal function and has unique advantages such as safety, effectiveness and multiple effects[3-4]. Wuling Powder is composed of five Chinese herbs: Oriental Waterplantain Rhizome, Poria, polyporus, Cinnamomi Ramulus and Rhizoma Atractylodis Macrocephalae. Studies have shown that Wuling Powder has a bidirectional regulatory function on water metabolism in the urinary system, as it reduces renal pathological status and improves renal function, exerting renal protective effects[5-6].

In this study, a mouse model of nephrotic syndrome was established *in vivo* to evaluate the pharmacodynamics of Wuling Powder in treating NS, and to explore the therapeutic and renal protective effects of Wuling Powder on NS mice from an overall perspective, so as to provide theoretical reference for clinical treatment.

Materials and Methods

Experimental animals

Sixty SPF KM mice, half male and half female, weighing 20 – 22 g, were purchased from the Experimental Animal Center of Guangxi Medical University with the license number SYXK Gui 2019-0001.

Drugs and reagents

Five Chinese herbs consisting Wuling Powder: polyporus (batch number: 20210301) 90 g, Poria (batch number: 20210801-1) 90 g, Rhizoma Atractylodis Macrocephalae (batch number: 20210902) 90 g, Oriental Waterplantain Rhizome (batch number: 20210705) 150 g, and Cinnamomi Ramulus (batch number: A07583) 60 g, the medicinal decoction pieces of which were purchased from Hunan Yao Sheng Tang Chinese Medicine Technology Co., Ltd.; doxorubicin hydrochloride (Beijing Solebao Life Sciences Co., Ltd., batch number: 1015p021); urine protein quantitative test kit and creatinine determination kit (Nanjing Jiancheng Bioengineering Institute, batch numbers: 20211227 and 2021126, respectively); reverse transcription kit

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Luotong JING (1997 –), master, devoted to research about pharmacodynamic evaluation and application research of traditional Chinese medicine.

* Corresponding author. Ning LIANG, associate professor, devoted to research and development of new immunomodulatory drugs for traditional Chinese medicine and ethnic medicine.

and Hes1 antibody (CST, 11988s); β-actin and goat anti-rabbit IgG secondary antibody (Beijing BIOSS Biotechnology Co., Ltd., batch number: BS-0061R and BS-0295G-HRP, respectively).

Experimental instruments

Real-time fluorescence quantitative PCR instrument (ROCHE, Switzerland); freezing centrifuge (Thermo Fisher Scientific, USA); high-speed freezing centrifuge (Thermo Fisher); full-wavelength microplate reader (Thermo scientific); analytical balance (METTLER TOLEDO).

Model establishment and drug administration in groups

After 7 d of adaptive feeding, 60 KM mice were randomly divided into a normal group, a model group, a prednisone acetate positive group (1 mg/ml, 8.7 g/kg), a high-dose Wuling Powder group (14.4 g/kg), a medium-dose Wuling Powder group (7.2 g/kg) and a low-dose Wuling Powder group (3.6 g/kg), with 10 mice in each group. According to the literature[7-8] and previous experimental results, mice of the nephrotic syndrome model were prepared by injecting 1 mg/ml adriamycin hydrochloride solution (7.5 mg/kg) into the tail vein 3 d after preventive administration, and mice in the normal group were injected with 0.9% normal saline in the same way. At 7 d after injection of doxorubicin hydrochloride, the 24 h urine protein content of the model mice was statistically significant compared with that of the normal group ($P<0.05$). Mice in each group were given drugs by gavage according to their weight, and the normal group and model group were given 0.9% normal saline once a day for 28 d.

Specimen collection

After 28 d of drug intervention, mice in each group were put into metabolic cages, and 24 h urine of mice was collected, and the collected urine volume was recorded. The urine was centrifuged to get a supernatant, and the urine protein content was detected according to the instructions of urine protein kits. The mice in each group were fasted for 12 h, and after 1 h of intragastric administration, the eyeballs were removed to take blood. After the blood collection was completed, the mice in each group were killed by cervical dislocation, and the kidneys were quickly separated. The left kidney was fixed in 4% paraformaldehyde for pathological staining, and the right kidney was placed in a freezing tube, which was put in liquid nitrogen for quick freezing, and then transferred to a refrigerator at −80 ℃ for storage.

Observation of pathological changes of renal tissue in mice

The renal tissue fixed in 4% paraformaldehyde solution was cut to an appropriate section size, and subjected to gradient dehydration and paraffin embedding, and the paraffin block was cut into pieces 4 μm in thickness, which were placed on glass slides, baked, stained with HE and Masson. The prepared sections were observed for pathological morphology and structure of the renal tissue under a microscope.

Detection of renal tissue-related proteins by RT-PCR

An appropriate amount of mouse kidney tissue was extracted for total RNA strictly using RNA extraction kits, and RNA concentration and purity were determined using a micro nucleic acid protein analyzer. cDNA was synthesized according to the instructions of reverse transcription kits, and PCR was performed using instructions of kits. With GAPDH as the internal reference gene, the relative expression level of mRNA was calculated by the $2^{-\Delta\Delta CT}$ method.

Table 1 Primers sequences

Primer	Molecule name	Sequence
Notch1	Mus-F-notch1	ACTTGTCAGATGTGGCCTCG
	Mus-R-notch1	ATTCAAGTGGCTGATGCCCA
Jagged1	Mus-F-jagged1	GGGCTCTTTGCCTTCTGGAAC
	Mus-R-jagged1	ATGCACGACTGGAAAACAACA
Hes1	Mus-F-hes1	GCGGAATCCCCTGTCTACCT
	Mus-R-hes1	GTCTTAGGGCTACTTAGTGATCGG
β-actin	Mus-F-β-actin	CTACCTCATGAAGATCCTGACC
	Mus-R-β-actin	CACAGCTTCTCTTTGATGTCAC

Western blot detection

Appropriate mouse kidney tissue was added with RIPA protein lysis buffer and magnetic grinding beads, and ground using a tissue grinder at low temperature and high speed. After full grinding, the sample was centrifuged using a high-speed centrifuge at 4 ℃ and 12 000 r/min for 10 min. The supernatant was collected and determined for the total protein concentration according to BCA protein detection kits, and a protein loading buffer was then added. Next, 40 μg of protein sample was taken from each group for SDS gel electrophoresis. At the end of electrophoresis, the gel was taken for PVDF membrane transfer, and after blocking and membrane washing, incubation was performed with the primary antibody overnight at 4 ℃. Next, the membrane was washed and incubated with the secondary antibody solution at room temperature for 1 h. After washing the membrane, color development and saving were performed. With β-action as an internal reference, the results were analyzed using the Image J system.

Statistical analysis

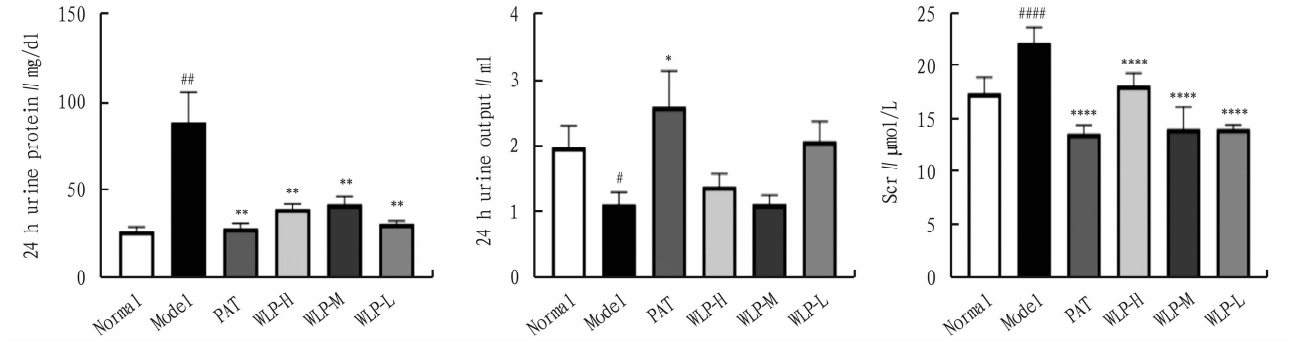
All data were statistically processed by SPSS 21.0 software, and the results conforming to normal distribution were expressed in $\bar{x} \pm s$. One-way ANOVA was used for comparison among groups, and *LSD* test was used for pairwise comparison between groups, with $P<0.05$ indicating a statistically significant difference.

Results and Analysis

Comparison on effects of Wuling Powder on 24 h urine volume and urine protein level in NS mice

Compared with the normal group, mice in the model group showed a decrease in 24 h urine output and an increase in urine protein and creatinine levels ($P<0.05$ or $P<0.01$). Compared with the model group, the mice in the prednisone acetate group showed an increase in 24 h urine output ($P<0.05$), while the prednisone acetate group and the high-, medium- and low-dose groups of Wuling Powder showed a significant decrease in urine protein content ($P<0.01$) and a significant decrease in creatinine content ($P<0.01$). Compared with the prednisone acetate group, the mice in the medium-dose group of Wuling Powder had

a decrease in 24 h urine output ($P<0.05$), while the high-dose group and medium-dose group of Wuling Powder had an increase in urine protein content ($P<0.05$), and the high-dose group of Wuling Powder had an increase in creatinine content ($P<0.05$).



Compared with the normal group, $^{\#\#}P<0.01$, $^{\#\#\#\#}P<0.000\ 1$; compared with the model group, $^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.01$.

Fig. 1 Effects of Wuling Powder on 24 h urine volume and urine protein and creatinine levels in NS mice ($\bar{x}\pm s$, $n=8$)

Effects of Wuling Powder on renal pathological changes in NS mice

HE staining showed that the normal control group showed a complete glomerular structure, no abnormal changes in matrix and mesangium, no obvious thickening of basement membrane and no obvious infiltration of inflammatory cells in the interstitial tissue. Compared with the control group, mice in the model group showed pathological changes such as glomerular hypertrophy, renal tubular dilation, narrowing of capillary lumen, significant thickening of capillary basement membrane, and infiltration of inflammatory cells in the mesenchyme. Compared with the model group, the pathological changes of renal tissue in the prednisone acetate and various Wuling Powder groups were improved to different degrees, that is, the renal pathological situation was alleviated. Through Masson staining, pathological damage to the kidneys, thickening of the glomerular basement membrane, proliferation of tubulointerstitial fibrous tissue, tubular collapse, vacuolar degeneration and extensive interstitial fibrosis were further observed in the model group. Compared with the model group, various treatment groups showed varying degrees of improvement, and renal tissue damage was significantly alleviated.

Effects of Wuling Powder on Notch1, Jagged1 and Hes1 mRNA levels in NS mice

Compared with the blank group, the expression levels of Notch1, Jagged1 and Hes1 mRNA in the model group all increased ($P<0.01$). Compared with the model group, the prednisone acetate group and various Wuling Powder groups showed decreased expression levels of Notch1, Jagged1 and Hes1 mRNA in the kidney tissue of mice ($P<0.01$).

Effects of Wuling Powder on expression of Notch1, Hes1 and Jagged1 proteins in NS mice

Compared with the blank group, the model group exhibited increased expression levels of Notch1, Hes1 and Jagged1 proteins ($P<0.05$). Compared with the model group, the prednisone acetate group and various Wuling Powder groups exhibited significantly-decreased expression level of Notch1 protein in the kidney tissue of mice ($P<0.01$), and the contents of Hes1 in the prednisone acetate group and high- and middle-dose Wuling powder groups decreased significantly ($P<0.05$), and the contents of Jagged1 in the prednisone acetate group and high-dose Wuling powder group decreased significantly ($P<0.05$).

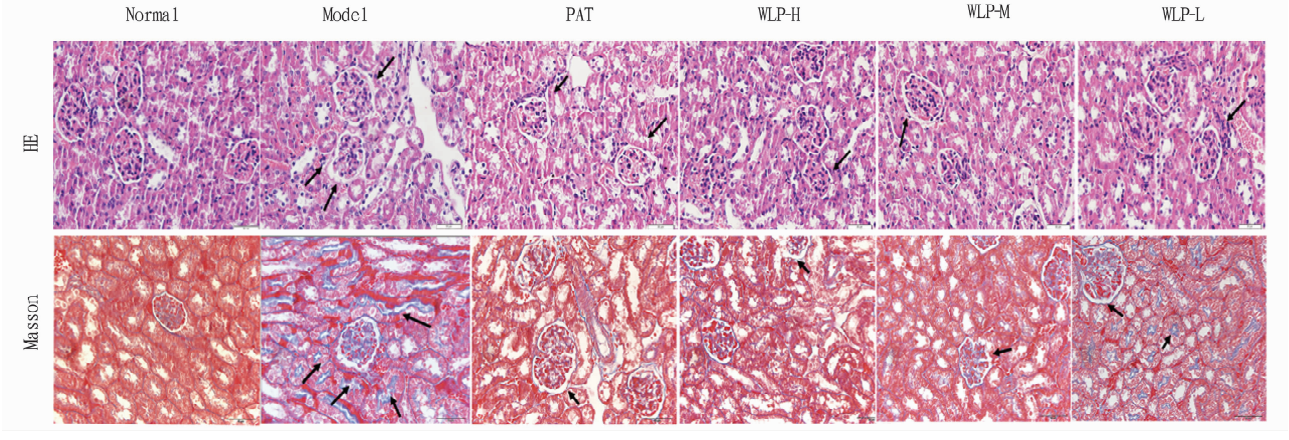
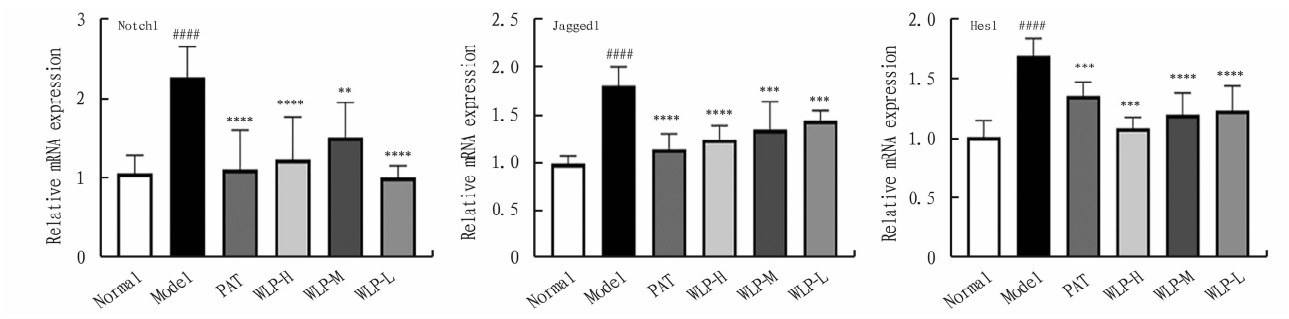
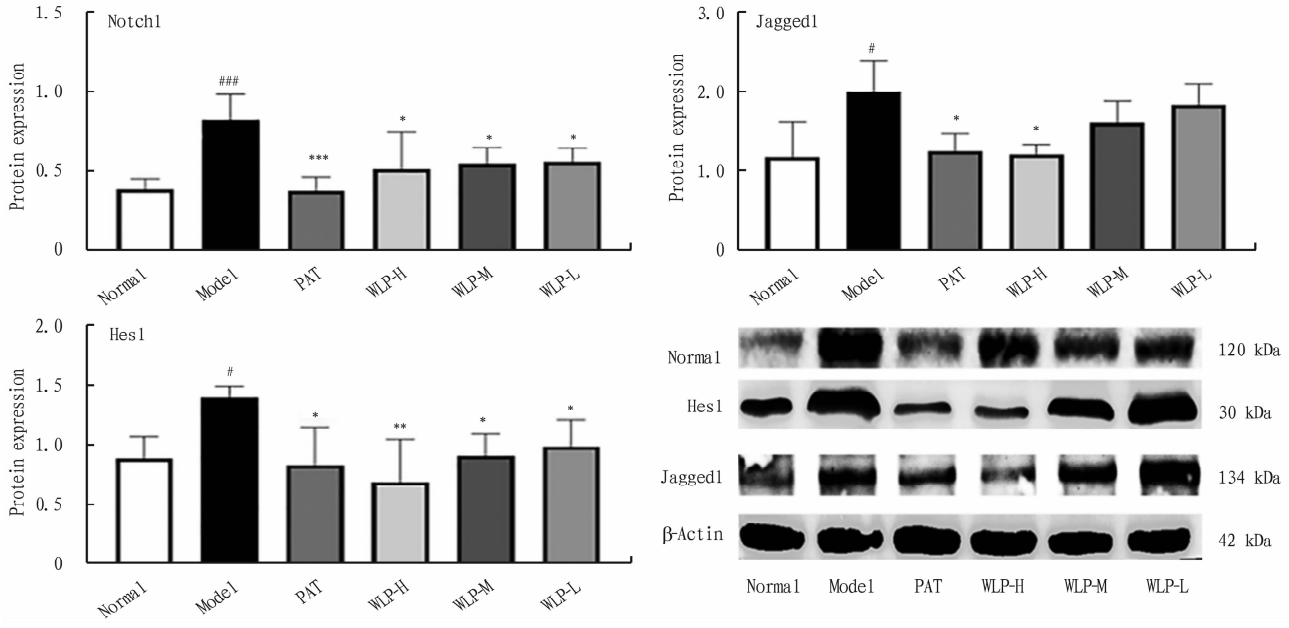


Fig. 2 Effects of Wuling Powder on renal pathological changes in NS mice (HE, Masson, ×400)



Compared with the normal group, $^{\#\#\#\#}P<0.000\ 1$; compared with the model group, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.000\ 1$.

Fig. 3 Expression of Notch1, Jagged1 and Hes1 mRNA



Compared with the normal group, $^{\#}P<0.05$, $^{\#\#\#}P<0.001$; and compared with the model group, $^{*}P<0.01$, $^{**}P<0.01$, $^{***}P<0.001$.

Fig. 4 Effects of Wuling Powder on expression of Notch1, Hes1 and Jagged1 proteins in NS mice

Conclusions and Discussion

Nephrotic syndrome belongs to the category of "edema", "consumptive disease" and "turbid urine" in traditional Chinese medicine, and often shows the characteristic of mixed deficiency and excess, frequent recurrence and difficult cure in clinic[9-10]. Wuling Powder is a classic ancient formula derived from Zhang Zhongjing's *Treatise on Febrile Diseases*, composed of five Chinese herbs: Oriental Waterplantain Rhizome, Poria, polyporus, Cinnamomi Ramulus and Rhizoma Atractylodis Macrocephalae. This prescription has the effects of warming yang and transforming qi, removing dampness and promoting water circulation, and is often used to treat various diseases such as Taiyang disease, cholera, cough with phlegm and gonorrhea. Nowadays, it is often used in clinic to treat chronic urinary retention, chronic diarrhea, nephrotic syndrome with yang deficiency, chronic glomerulonephritis with yang deficiency and water and dampness retention[11-14]. Studies have shown that Wuling Powder has a definite curative effect on NS, and can effectively reduce the level of urine protein, relieve edema symptoms and kidney damage[15-16], proving that Wuling Powder is a classic ancient prescription for NS treatment with great development potential.

Notch signaling pathway is a highly conservative signal transduction system, which plays a key role in the process of glomerulosclerosis and tubulointerstitial fibrosis and participates in the occurrence of renal diseases[23-24]. In this study, adriamycin was used to induce a mouse model of nephrotic syndrome and the pharmacodynamics of Wuling Powder in treating NS was observed. The experimental results showed that the model group exhibited a lot of urine protein and obvious NS pathological damage, and Wuling Powder could reduce the urine protein and creatinine contents of NS mice and effectively improve the renal function and renal pathological damage of NS mice after intervention. Compared with the control group, the expression of Notch1, Jagged1, and Hes1 mRNA in the model group was upregulated, and after treatment with different doses of Wuling Powder, the expression was downregulated; and the expression of Notch1, Hes, and Jagged1 proteins was significantly upregulated in the kidneys of NS mice, while Wuling Powder could significantly reduce their expression. The above results suggested that Wuling Powder could exert its effects in the NS mouse model by blocking the Notch signaling pathway, reducing

urine protein and alleviating renal tissue damage.

In summary, this study preliminarily confirmed that Wuling Powder had a protective effect on the kidneys of NS mice, and alleviated renal pathological damage. Its mechanism might be related to the inhibition of the Notch pathway, providing a theory for later development and utilization of Wuling Powder and drug development.

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