

## Original Article

# Impact of Wenyang-Huoxue-Lishui formula on cytoskeletal structures and related proteins of immortalized podocytes in mice with puromycin aminonucleoside (PAN)-induced injury

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**Abstract:** In this study, we aimed to determine the impact of Wenyang-Huoxue-Lishui formula (WHL) on cytoskeletal structures and related proteins of immortalized podocytes in mice with puromycin aminonucleoside (PAN)-induced injury. Podocyte injury was induced by puromycin treatment, followed by intervention with traditional Chinese medicine (TCM)-containing serum. The mRNA and protein expression levels of cathepsin L (CatL) and synaptopodin were detected. The protein expression of membrane-associated guanylate kinase with inverted orientation (MAGI)-2 as well as Ras homolog gene family, member A (RhoA) was detected. The distribution of dendrin and changes in fluorescence distribution of cytoskeletal microfilaments and intermediate filaments were also observed. After the intervention, the mRNA and protein expression of cathepsin L were downregulated ( $P < 0.01$ ), while the mRNA expression of synaptopodin remained unchanged ( $P > 0.05$ ). The protein expression of MAGI-2, RhoA, and synaptopodin was upregulated ( $P < 0.01$ ). The ratio of nuclear translocation of dendrin decreased ( $P < 0.01$ ), but was not restored to the normal level ( $P < 0.01$ ). The cytoskeletal structures were restored in each treatment group. WHL reduced the nuclear translocation of dendrin and decreased cytoplasmic CatL levels after induction of podocyte injury, thereby reducing synaptopodin degradation, increasing RhoA expression, improving F-actin and intermediate filament remodeling, stabilizing cytoskeletal structure, and reducing foot process effacement, and subsequently reducing proteinuria.

**Keywords:** Wenyang-Huoxue-Lishui formula, podocytes, dendrin, microfilament, intermediate filament

## Introduction

Albuminuria is the major manifestation of primary nephrotic syndrome, and sustained high-level albuminuria is an important risk factor that contributes to progressive renal damage and eventual loss of renal function [1]. Therefore, identifying ways to attenuate the development of albuminuria in primary nephrotic syndrome can effectively reduce renal risk factors and inhibit progressive renal damage. Albuminuria in primary nephrotic syndrome is associated with the dysfunction of glomerular filtration barrier. Podocytes form the final filtration barrier to the glomerular filtration apparatus, which covers the outer aspect of capillary loops. Alterations in the morphology of podocytes

will lead to abnormality in the glomerular filtration barrier, resulting in proteinuria and progressive renal damage. In the setting of nephrotic syndrome, the elaborate structure of podocytes alters, which is termed as podocyte foot process effacement. The characteristics of podocyte process effacement are retraction, widening, and shortening of the processes of each podocyte, followed by the loss of interdigitating foot process pattern among individual cells [2]. When podocytes are injured, podocyte cytoskeleton reorganization is the common final pathway that causes foot process effacement and ultimately results in proteinuria [3].

Actin cytoskeleton is abundant in highly dynamic foot processes. Microtubules and vimentin-

type intermediate filaments are located in the cell body and primary processes, and they provide mechanical and structural integrity to podocytes [2]. It is well known that the actin cytoskeleton interacts with intermediate filaments and microtubules [4]. Microtubules and vimentin-type intermediate filaments do not form a part of the structures of foot process. This raises the possibility of dynamic remodeling of actin network in the setting of nephrotic syndrome, accompanied by simultaneous reorganization of microtubules and intermediate filaments.

Over the last decades, an increasing number of proteins involved in maintaining the podocyte cytoskeleton has been identified. Among these proteins, membrane-associated guanylate kinase with inverted orientation (MAGI)-2 is exclusively expressed in podocytes and interacts with the slit diaphragm (SD) protein nephrin to form and maintain the normal structure and function of glomerular filter. MAGI-2 deficiency in mice is presented with diffuse podocyte foot process effacement and progressive podocyte loss [5, 6]. In normal state, dendrin is located in the SD complex. However, in injured podocytes, dendrin is redistributed to the nucleus. Dendrin is a transcription factor of cathepsin L (CatL), which can induce the expression of cytoplasmic CatL [7]. Elevated cytoplasmic CatL level is associated with podocyte injury, which is presented by the reorganization of podocyte cytoskeleton and podocyte process effacement. High-level cytoplasmic CatL mediates podocyte injury by hydrolyzing its substrate synaptopodin, which can induce stress fiber formation by competitively blocking smurf1-mediated ubiquitination of Ras homolog gene family, member A (RhoA). Small GTPases such as RhoA play a critical role in regulating actin network and downregulating RhoA via loss of stress fiber and podocyte process, which is a hallmark of effaced podocytes [8].

Glucocorticoids and immunosuppressants are used to treat nephrotic syndrome via inhibition of immune response and inflammation. Over the past 40 years, the clinical remission rate and prognosis of nephrotic syndrome have markedly improved with the development and improvement of diagnostic techniques, new immunosuppressive agents, and treatment methods. However, the use of glucocorticoids and

immunosuppressants are limited in clinics owing to their toxicities and side effects.

Based on the theory of traditional Chinese medicine (TCM), we developed Wenyang-Huoxue-Lishui (WHL) formula containing 11 TCM herbs to treat nephrotic syndrome in clinical settings and obtained certain curative effects. In our previous studies, we observed that WHL can downregulate CatL and upregulate synaptopodin in adriamycin rat models, thus leading to the stabilization of actin skeleton, improvement of podocyte effacement, and reduction of proteinuria. Based on these findings, we hypothesized that the beneficial therapeutic effects of WHL against nephrotic syndrome are mediated, at least in part, by a direct effect on podocyte-related proteins, which in turn contributes to the stabilization of podocyte cytoskeleton. To test this hypothesis, we designed studies to investigate whether the protective effects of WHL are associated with mediating the expression or distribution of MAGI-2/dendrin/CatL/synaptopodin/RhoA in puromycin aminonucleoside (PAN)-induced podocyte injury and to determine the treatment effects of WHL toward the stability of podocyte cytoskeleton.

### Materials and methods

#### *Animals*

Twenty, six-week-old, male Sprague Dawley rats of specific pathogen free (SPF) grade (200±20 g) were purchased from the Center of Experimental Animals of Hubei Academy of Medical Sciences (SCXK 2014-0004, Wuhan, China). The rats were housed in a temperature- and humidity-controlled environment with free access to tap water and standard rat chow. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol had been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Hubei University of Chinese Medicine.

#### *Composition of WHL*

WHL consists of 10 g of radix aconiti lateralis preparata, 5 g of cortex cinnamomi, 30 g of Astragalus membranaceus, 6 g of ephedra, 10 g of deerhorn glue, 10 g of Grifola, 10 g of

*Ligusticum wallichii*, 5 g of safflower, 10 g of *Leonurus japonicus*, 3 g of hirudo, and 10 g of the root of bidentate achyranthes. The components of WHL were commercially produced by China Resources Sanjiu Medicinal & Pharmaceutical Co., Ltd., as dry powder (Shenzhen, China). The dry powder form of each component of WHL was mixed together and dissolved three times with distilled water. The final concentration was made to 200%, followed by autoclaving and then storing in aliquots at 4°C until use.

### *Preparation of WHL-containing serum*

Fifteen Sprague Dawley rats were intragastrically administered WHL twice daily for 7 days (4.667 g/kg per day). Simultaneously, the remaining 5 rats were administered normal saline. Two hours after the last administration, blood was collected from each rat via the carotid artery. The blood samples were placed at room temperature for 2 h, and then centrifuged at 3,000 rpm for 10 min to separate the drug-containing serum. The serum of rats in the same group was pooled, filtered through 0.22-µm filters, inactivated at 56°C for 30 min, and stored at -20°C before use.

### *Cell culture*

The murine podocyte line was kindly gifted by Prof. Guo-hua Ding (Renmin Hospital of Wuhan University). The podocytes were cultured in 10% fetal bovine serum (FBS)-containing RPMI1640 medium at 37°C, 5% CO<sub>2</sub>, and 100% relative humidity in an incubator. The podocyte culture was expanded by allowing to grow in a medium containing 10 U/mL mouse interferon-γ (IFN-γ) (PEPRO Tech, NJ, USA) at 33°C. The cells were subcultured every 48 h and harvested from the subconfluent culture (60-70%) using 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA). Podocyte differentiation was induced by culturing at 33°C without IFN-γ for 10 to 14 days. The morphology of podocytes in culture was carefully examined by phase-contrast microscopy.

### *Cell treatment*

Near-confluent podocytes were incubated with serum-free medium for 24 h to arrest and synchronize cell growth. Then, the podocytes were treated with puromycin aminonucleoside (PAN)

(Saint Louis, MO, USA) to a final concentration of 45 mg/L for 12 h. After pretreatment, the culture medium was changed to fresh medium, and the podocytes were cultured with Dulbecco's Modified Eagle's medium (DMEM) supplemented with dexamethasone (1 µmol/L) or two different concentrations of WHL-containing serum for 24 h. Meanwhile, the normal control was cultured with fresh serum-free medium for 24 h.

### *Methyl thiazolyl tetrazolium (MTT) assay*

Podocytes in the logarithmic growth stage were seeded in 96-well plates (1×10<sup>3</sup>/well) and incubated at 37°C for 14 days, followed by incubation in serum-free RPMI1640 medium for 24 h to synchronize the cells. The medium was aspirated, and medium containing 10% and 20% rat blank/WHL serum, 10% FBS serum, puromycin (5 mg/mL puromycin stock solution diluted with complete podocyte medium to prepare a final concentration of 45 mmol/L), or dexamethasone (diluted with complete medium to prepare a final concentration of 1 µmol/L) was added, respectively. The culture plates were placed in a 5% CO<sub>2</sub> incubator (12 to 48 h, interval 12 h) at 37°C. The test cells in the 96-well plates were then placed in 20 µL of MTT solution (5 mg/mL) for incubation at 37°C with 5% CO<sub>2</sub> for another 4 h. After removing the supernatant, 150 µL of dimethyl sulfoxide (DMSO) was added into each well and shaken for 10 min to dissolve the crystals sufficiently. The optical density (OD) value of each well was measured at 490 nm. Each experiment well was set up two repeated wells, and the experiment was performed three times independently, together with the blank control group (containing only MTT culture medium). The other experimental steps were the same. The OD value of each group represented the viability of podocytes, and the higher the OD value, the better the viability of podocytes.

### *Quantitative real-time polymerase chain reaction (PCR)*

Quantitative real-time PCR analysis was performed to quantitate the expression of CatL and synaptopodin mRNA in podocytes. Total RNA was extracted from podocytes by using Trizol (Invitrogen Corporation, California, USA). cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Th-

**Table 1.** The Primer Sequences for CatL, synaptopodin,  $\beta$ -actin in Quantitative Real-Time PCR

Gene	Sequences (5'-3')	Length (bp)	Annealing temperature (°C)
$\beta$ -actin	GTGACGTTGACATCCGTAAAGA	287	60
	GTAACAGTCCGCCTAGAAGCAC		60
CatL	CTACCGCCACCAGAAGCACA	144	60
	CTAAACGCCCAACAAGACCC		60
Synaptopodin	ACCAGCCAGATAGAGCAAAGC	262	60
	GGGGAGACCTAACCCGAGAA		60

ermo). Quantitative PCR was performed using FastStart Universal SYBR Green Master (Roche) and specific primers for CatL and synaptopodin (primer sequences are shown in **Table 1**) (Invitrogen Biotechnology Co., LTD). The mRNA expression of CatL and synaptopodin was normalized to the mRNA level of  $\beta$ -actin. Fold expression changes were determined using the comparative computed tomography (CT) method for relative quantification by calculating  $2^{-\Delta\Delta CT}$ . The PCR conditions were as follows: 95°C for 5 min and 40 cycles of 95°C for 15 min, annealing at 60°C for 20 s, and 72°C for 45 s.

#### Western blotting

Western blot assay was performed to measure the protein expression of MAGI-2, CatL, synaptopodin, and RhoA in podocytes. The total proteins were extracted using RIPA Lysis Buffer [containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM sodium pyrophosphate, 25 mM  $\beta$ -glycerophosphate, 1 mM EDTA, 1 mM  $\text{Na}_3\text{VO}_4$ , and 0.5  $\mu\text{g}/\text{mL}$  leupeptin) for 5 min. Then, the lysate was collected into a centrifuge tube and placed on ice for 30 minutes, followed by centrifugation at 12,000 $\times$  g for 10 min at 4°C.

The protein concentrations were determined by the BCA protein assay (Pierce). Equal amounts of protein (30  $\mu\text{g}$ ) were loaded onto a 12% SDS-acrylamide gel. After electrophoresis, the proteins were transferred onto nitrocellulose membranes. The membranes were then blocked with 4% bovine serum albumin (BSA)-Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) for 2 h at room temperature, followed by incubation with different antibodies of  $\beta$ -actin, MAGI-2, CatL, synaptopodin, and RhoA and

subsequent incubation with a secondary antibody conjugated with horseradish peroxidase (HRP). Western blot assay was visualized by using an enhanced electrochemiluminescence (ECL) sensor and exposed to an X-ray film. Quantitative data were obtained using alphaEaseFC software (Alpha Innotech). The ratio of the interested protein to  $\beta$ -actin was shown.

#### Immunofluorescence staining

The podocytes on glass coverslips were washed three times with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde for 30 min. After washing three times with PBS, blocking was performed for 30 min at room temperature. The cells were then incubated with the primary antibodies overnight at 4°C. The next day, after washing with PBS again, the cells were incubated with secondary antibodies at 30°C in a humidified chamber for 30 min and then washed three times with PBS. The sections were mounted with anti-fade Fluorescence Mounting Medium with DAPI (ZSGB, Beijing, China) and placed under confocal laser scanning microscopy (LEICA TCS SP2SE, Germany) for observation and image acquisition.

#### Double staining for phalloidin and intermediate filament

The podocytes grown on glass coverslips and treated as described above were incubated with FITC-conjugated phalloidin, and rabbit antibodies against vimentin were added and incubated overnight at 4°C. The images were acquired by confocal laser scanning microscopy as described above.

#### Statistical analysis

All data were analyzed with SPSS21.0 software and were presented as mean  $\pm$  standard error ( $\bar{x} \pm s$ ). Differences among multiple groups were analyzed using Kruskal-Wallis test. The Student-Newman-Keuls post hoc test was applied to compare data among groups when normality (and homogeneity of variance) assumptions were satisfied; otherwise, the equivalent non-parametric test was used. A  $P < 0.05$  was considered statistically significant.



**Table 2.** Comparison of MTT results among different groups ( $\bar{x} \pm s$ )

Group	Time (h)		
	12	24	48
A: Blank control	0.557 $\pm$ 0.052	0.518 $\pm$ 0.078	0.488 $\pm$ 0.072
B: Puromycin group	0.375 $\pm$ 0.048*	0.149 $\pm$ 0.042*	0.023 $\pm$ 0.010*
C: 10% blank serum group	0.552 $\pm$ 0.065	0.515 $\pm$ 0.058	0.479 $\pm$ 0.055
D: 20% blank serum group	0.549 $\pm$ 0.062	0.513 $\pm$ 0.075	0.477 $\pm$ 0.035
E: 10% WHL-containing group	0.544 $\pm$ 0.058	0.499 $\pm$ 0.067	0.396 $\pm$ 0.055
F: 20% WHL-containing group	0.548 $\pm$ 0.045	0.512 $\pm$ 0.066	0.480 $\pm$ 0.072
G: Dexamethasone group	0.546 $\pm$ 0.075	0.503 $\pm$ 0.028	0.420 $\pm$ 0.042

Note: Compared with group A, \*P<0.01.

## Results

### MTT

After 12 h, the viability of puromycin group (45 mg/L) was significantly lower than that of the blank control group (P<0.01). After 24 h, the viability of puromycin group (45 mg/L) reduced further. After 48 h, almost no MTT absorption by the podocytes was observed in the puromycin group (45 mg/L). There was no significant difference in the viability of podocytes among dexamethasone, 10% and 20% rat WHL-free serum, and 10% and 20% WHL-containing serum groups when compared with the 10% FBS group (Table 2, P>0.05).

### Detection of mRNA expression of CatL and synaptopodin in podocytes

The mRNA expression of CatL was significantly upregulated after PAN treatment compared with the control group (CON) (P<0.01). The mRNA expression of CatL in the dexamethasone (DEX), 10% TCM-serum-treatment (Group 1), and 20% TCM-serum-treatment (Group 2) groups reduced when compared with the model group (MOD) (P<0.01); however, it did not reach the normal level when compared with Group CON (P<0.01). There was no statistical significance in the mRNA expression of CatL in Group DEX when compared with Group 1 or Group 2, respectively (P>0.05). The serum level of CatL mRNA in Group 2 was lower than that in Group 1 (P<0.01), and the expression of CatL mRNA following TCM-serum treatment alone was not affected when compared with Group CON (P>0.05; Figure 1).

There was no statistical significance in the expression of synaptopodin mRNA in podocytes

between different groups (P>0.05; Figure 1).

### Protein expression of MAGI-2, CatL, synaptopodin, and RhoA

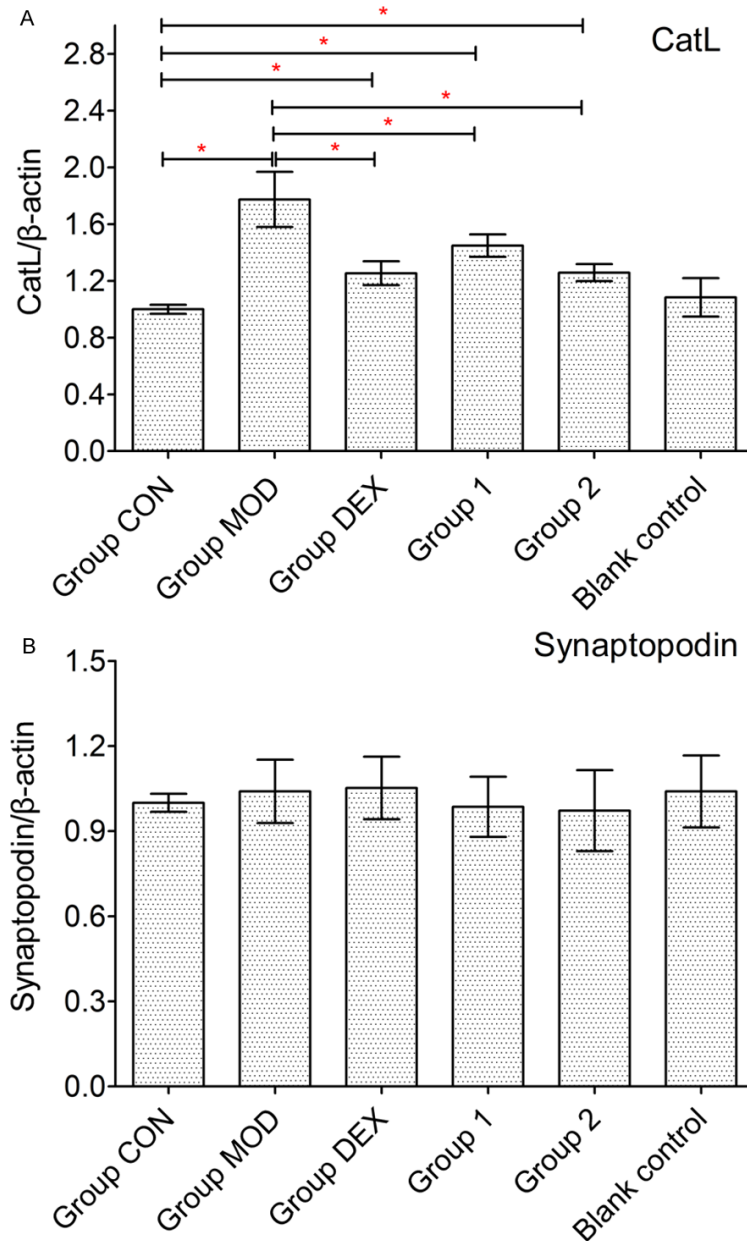
The protein expression of MAGI-2, RhoA, and synaptopodin was significantly downregulated after PAN treatment compared with Group CON (P<0.01).

The expression of CatL protein was significantly upregulated after PAN treatment compared with Group CON (P<0.01). The expression of MAGI-2, RhoA, and synaptopodin increased in Group DEX, Group 1, and Group 2 (P<0.01), while the expression of CatL protein decreased (P<0.01) compared with Group MOD. Group DEX exhibited better effects in upregulating the protein expression of MAGI-2, synaptopodin, and RhoA than Group 1 (P<0.01); however, there was no statistical significance in the expression of CatL protein compared with Group 1 (P>0.05). There was no statistical significance in the protein expression of MAGI-2, RhoA, CatL, and synaptopodin between Group DEX and Group 2 (P>0.05). The protein expression of MAGI-2, RhoA, and synaptopodin in Group 2 was higher than that in Group 1 (P<0.01), but the expression of CatL protein was lower than that in Group 1 (P<0.01). The protein expression of MAGI-2, RhoA, CatL, and synaptopodin was not affected by TCM-serum-treatment alone when compared with Group CON (P>0.01; Figure 2).

### Comparison of nuclear translocation of dendrin in podocytes

Immunofluorescence staining was performed to detect the percentage of dendrin in DAPI. Nuclear translocation of dendrin was determined by double staining of dendrin protein and DAPI. Co-localization of the red fluorescent-labeled dendrin protein and the blue fluorescent-labeled DAPI indicates the nuclear translocation of dendrin. The results revealed that the above ratio increased in Group MOD compared with Group CON (P<0.01). The above ratio decreased in Group DEX, Group 1, and Group 2 compared with Group MOD (P<0.01),

## Effect of WHL on podocytes in a PAN-induced injury mouse model



**Figure 1.** Expressions of CatL and synaptopodin mRNA in immortalized podocytes of each group. The expression of CatL and synaptopodin mRNA were measured by the quantitative real-time PCR after podocytes were treated with PAN in the present of dexamethasone or WHL-containing serum at different concentration for 24 h. Quantification shown in the bar graph. Mean  $\pm$  SEM at least 5 independent experiments. A. Quantitative real-time PCR of CatL mRNA expression. B. Quantitative real-time PCR of synaptopodin mRNA. Note: Group CON: the control group; Group MOD: the model group; Group DEX: the Dexamethasone group; Group 1: 10% WHL-containing group; Group 2: 20% WHL-containing group; Blank control: 20% blank serum group. \*denotes  $P < 0.01$ .

but did not reach the normal level relative to Group CON ( $P > 0.05$ ). There was no significant difference in the above ratio between Group

DEX and Group 1 or Group 2, respectively ( $P > 0.05$ ), but the ratio in Group 2 was lower than that in Group 1 ( $P < 0.01$ ) (Figures 3, 4).

### Observation of cytoskeletal microfilaments and intermediate filaments

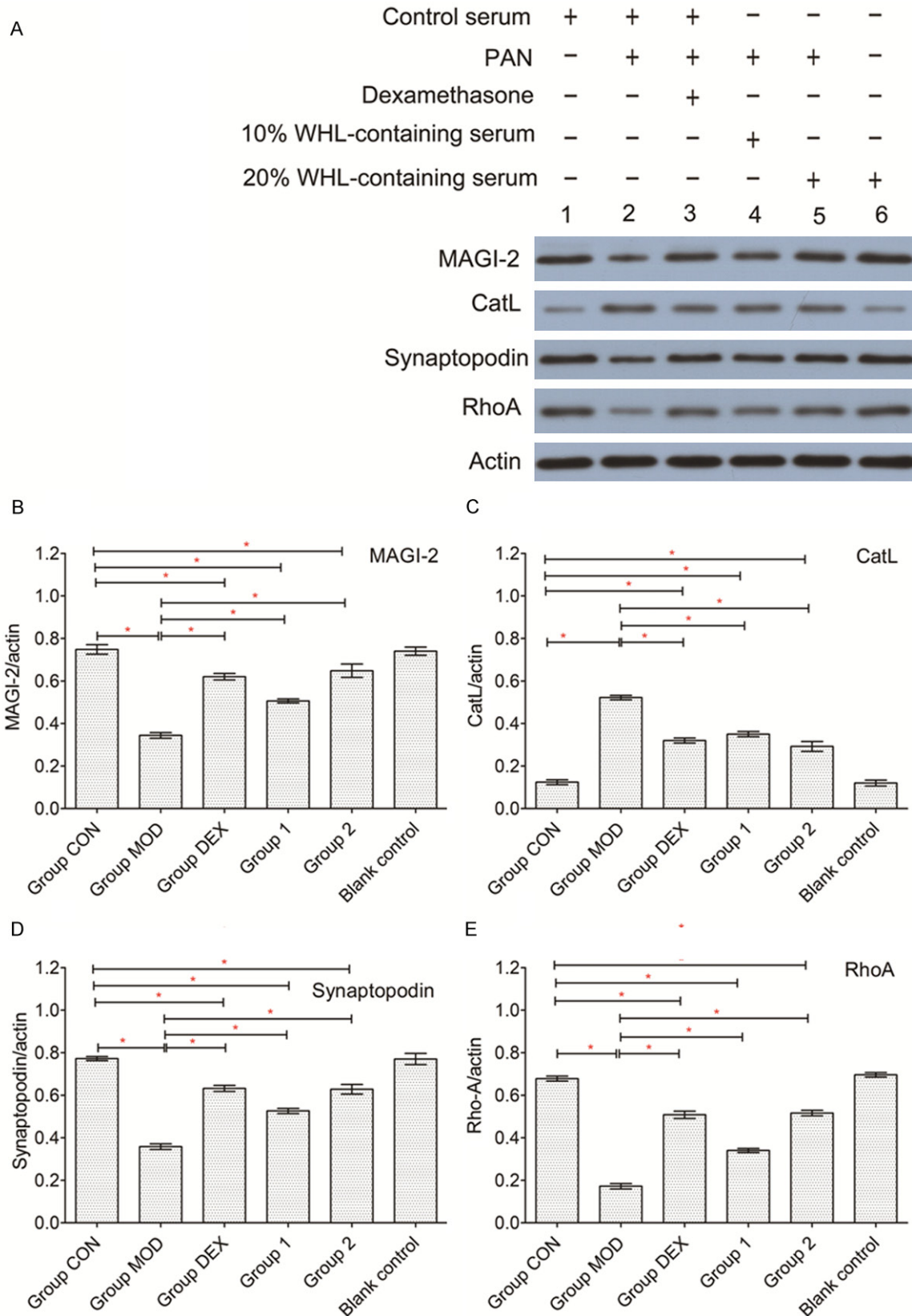
The cytoskeletal microfilaments and intermediate filaments in normal podocytes exhibit clear structures, orderly arrangement, and stretched pseudopodia. After PAN-induced injury to podocytes, the cell body became smaller, and the structure in Group MOD changed: the pseudopodia retracted and became shorter; the intercellular connection disappeared; the cells became round or oval; the cytoskeletal microfilaments and intermediate filaments were unarranged; and the structure was unclear. The above changes were restored in Group DEX, Group 1, and Group 2 compared with Group MOD. The cytoskeletal microfilaments and intermediate filaments were re-arranged in a slightly ordered fashion. The above conditions were better in Group DEX and Group 2 than in Group 1 (Figure 5).

### Discussion

Our previous *in vivo* experiments revealed that WHL can improve foot process effacement and reduce the occurrence of proteinuria by reducing the expression of CatL, while increasing the expression of the substrate of CatL (synaptopodin). In the *in vitro* experiment, we further confirmed that WHL can increase

MAGI-2 level, inhibit nuclear translocation of dendrin and transcription of CatL mRNA, reduce CatL expression, reduce synaptopodin

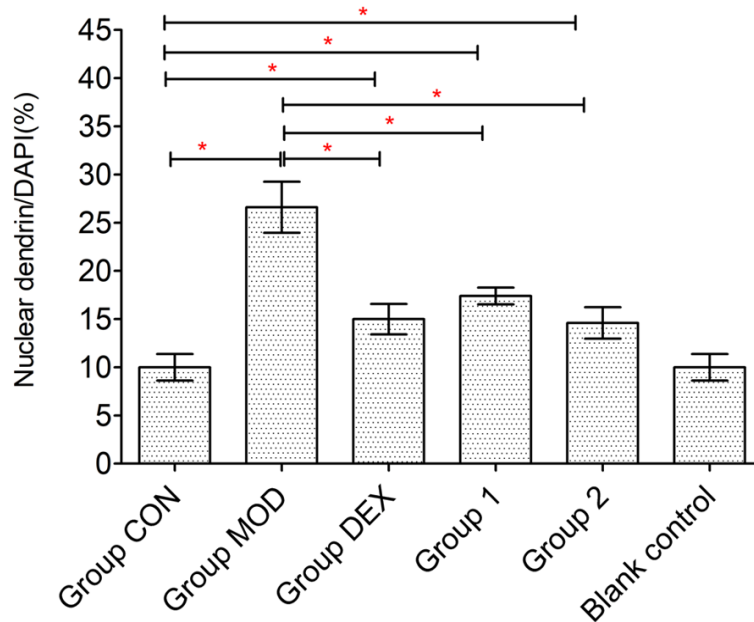
# Effect of WHL on podocytes in a PAN-induced injury mouse model



**Figure 2.** Expressions of MAGI-2, CatL, synaptopodin, and RhoA protein in immortalized podocytes of each group. The expressions of MAGI-2, CatL, synaptopodin, and RhoA protein in podocytes of each group were analyzed by western blotting. Quantification shown in the bar graph. Mean  $\pm$  SEM at least 5 independent experiments. A. West-

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ern blot analysis of MAGI-2, CatL, synaptopodin, and RhoA protein expression. B. MAGI-2 protein levels normalized with actin. C. CatL protein levels normalized with actin. D. Synaptopodin protein levels normalized with actin. E. RhoA protein levels normalized with actin. Note: Group CON: the control group; Group MOD: the model group; Group DEX: the Dexamethasone group; Group 1: 10% WHL-containing group; Group 2: 20% WHL-containing group; Blank control: 20% blank serum group. \*denotes  $P < 0.01$ .



**Figure 3.** Expressions of Dendrin in DAPI. Double-positive cells of dendrin and nuclei were counted. A total of 20 fields of vision per section at 400 magnification were observed. The ratios of nuclear relocation of dendrin were shown in the bar graph. Note: Group CON: the control group; Group MOD: the model group; Group DEX: the Dexamethasone group; Group 1: 10% WHL-containing group; Group 2: 20% WHL-containing group; Blank control: 20% blank serum group. \*Denotes  $P < 0.01$ .

degradation, and increase RhoA protein expression, thereby improving the morphology of podocytes [9].

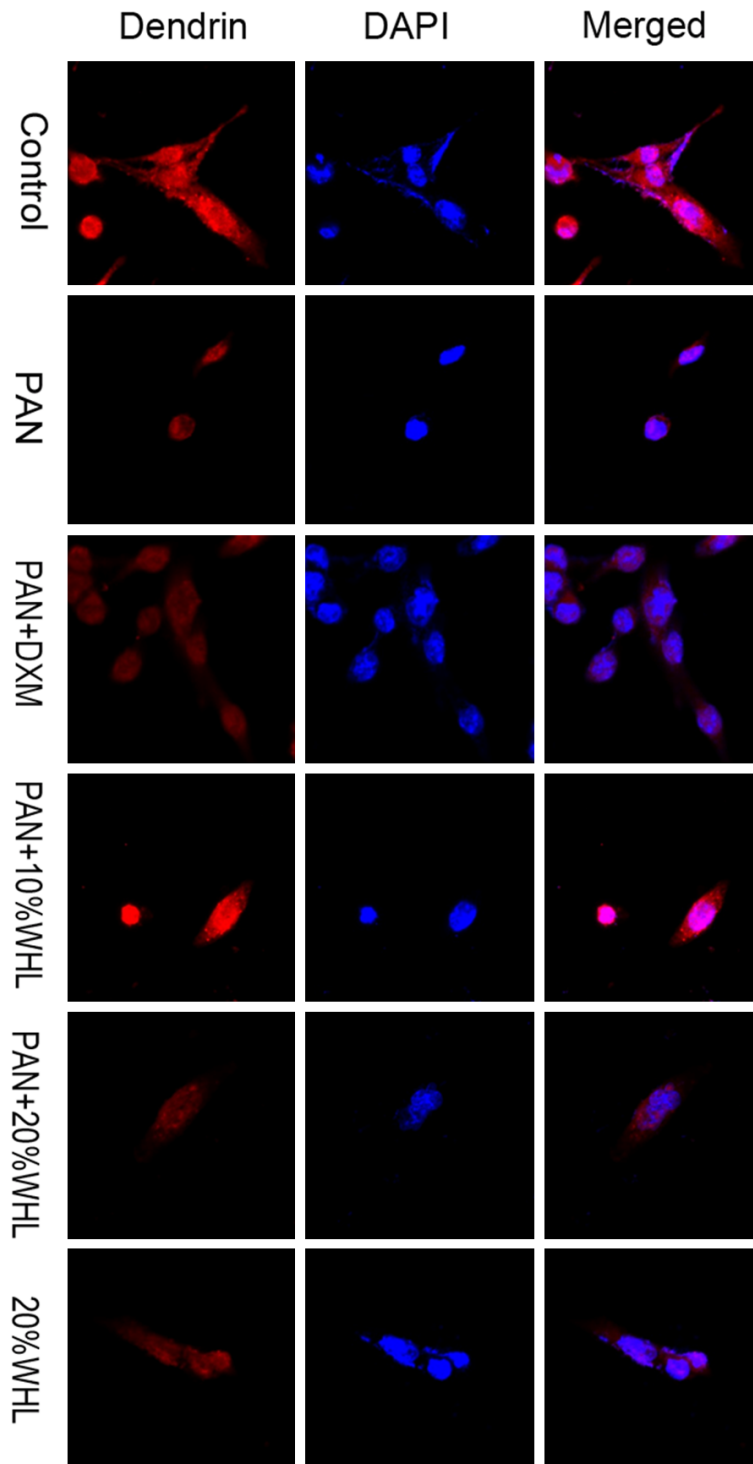
In clinical practice, Western medicine mainly uses hormones and symptomatic support therapies for the treatment of nephrotic syndrome. However, it leads to disease relapse, resistance, and side effects, which are associated with hormone therapy. TCM does not cause these problems and shows better effects against nephrotic syndrome. According to Chinese medicine, nephrotic syndrome mainly occurs in weak physical conditions of the lungs, spleen, or kidneys, which is caused by pathogenic wind invasion. Accumulation of dampness toxins can further damage the Yang of the lungs, spleen, and kidneys. Therefore, Yang deficiency of the spleen and kidneys is an intrinsic condition of this disease, and invasion of pathogenic wind

and accumulation of dampness toxins are the main external incentives. Water dampness and static blood are not only pathological products, but also pathogenic factors, and exist throughout the whole course of this disease. They result in recurrent, persistent, and refractory disease. Therefore, the treatment of nephrotic syndrome by Chinese medicine should warm the Yang qi, activate blood, and alleviate water retention. Based on this theory, we developed WHL to effectively reduce proteinuria in clinical settings and reduce the recurrence of nephrotic syndrome.

Proteinuria is one of the main symptoms of nephrotic syndrome. The severity of proteinuria is closely related to the progress of renal disease, which is related to the destruction of the structure and function

of glomerular filtration barrier. Podocytes are one of the components of the glomerular filtration barrier, and abnormalities in podocyte cytoskeleton will lead to foot process effacement, which is closely associated with proteinuria [10]. Cytoskeleton is generally composed of microtubules, microfilaments, and intermediate filaments. The podocyte cytoskeleton mainly comprises F-actin. Under pathological conditions, actins, which are highly ordered and parallelly connected, become disordered, shortened, and branched, thereby leading to secondary proteinuria [10]. Once the damage factors are removed, these morphological changes can be reversed [10]. The intermediate filaments have a tension-bearing role, structural role in maintaining cellular shape and rigidity, and supporting role in specific types of cells. Intermediate filaments and microfilaments are closely related. The actins (constituting the



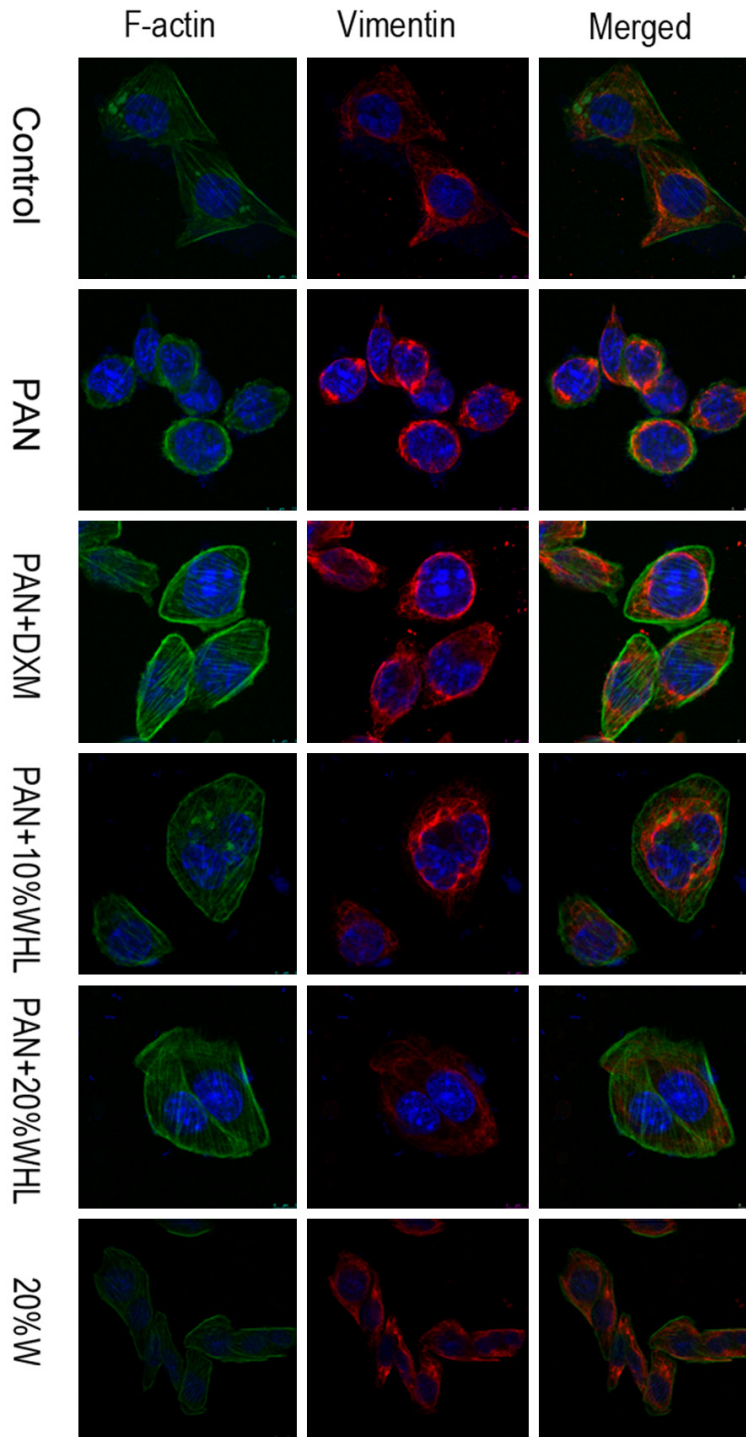


**Figure 4.** Fluorescence expressions of Dendrin in immortalized podocytes of each group. To investigate the localization of dendrin in podocytes treated by PAN with or without dexamethasone or WHL-containing serum, immunofluorescent staining analyses were performed. The colocalization of dendrin and the nuclei indicated the nuclear import of dendrin in cultured podocytes. In the control group, dendrin was predominately located in cytoplasm. After PAN treated, there were many clear dendrin-positive nuclei. However, the nuclear relocation of dendrin was partially restored by dexamethasone or two different concentration of WHL-containing serum. The blank serum had no

influence on the nuclear import of dendrin. Note: Dendrin is labeled with red fluorescence, and DAPI blue fluorescence is used to label the nuclei ( $\times 1000$ ).

microfilaments) and the tail of vimentin (constituting the intermediate filaments) directly connect with each other closely through a dense network [11, 12]. In this experiment, the results of fluorescent staining showed that the cytoskeletal structure was disordered after PAN damage, and DEX and TCM-containing serum can stabilize podocyte structure, which can help improve foot process effacement and reduce proteinuria. Consistent with our results, certain studies also reported that DEX can stabilize PAN-damaged cytoskeleton and actin filaments [13, 14], which is independent of its immunosuppressive effects.

Controlling and stabilizing actin skeletal proteins is important for maintaining glomerular filtration. Over the past 10 years, many podocyte actin-related proteins have been found. MAGI-2 is a podocyte actin-related protein, which is expressed only in renal podocytes and is involved in foot process effacement [14, 15]. MAGI-2 can connect cell adhesion molecules, receptors, and signal molecules with the cytoskeleton to maintain cell structure, thus playing key roles in maintaining glomerular filtration barrier integrity and in podocyte survival. Studies have shown that MAGI-2 gene mutation or deficiency can lead to structural damage of podocytes, foot process effacement, and serious podocyte loss, which in turn result in the



**Figure 5.** Distribution of cytoplasmic microfilaments and intermediate filaments in immortalized podocytes of each group. In the control group, podocytes indicated parallel actin filaments and intermediate filaments in ordered arrangement. The podocytes also exhibited pseudopodia. In the model group, the highly ordered microfilaments and intermediate filaments filament bundles in podocyte become disordered and short under PAN treatment. Also, PAN stimulated cell contraction, resulting in smaller cell size. This cytoskeleton derangement and cell contraction was partially reversed by dexamethasone or two different concentration of WHL-containing serum. The blank serum had no influence on the structure of microfilaments and

intermediate filaments as well as the podocytes morphology. Note: F-actin is labeled with green fluorescence; vimentin is labeled with red fluorescence; and DAPI blue fluorescence is used to label the nuclei ( $\times 1000$ ).

development of proteinuria and eventually, kidney disease or renal failure [5, 6, 16]. Nuclear translocation of dendrin occurred in the renal glomeruli of MAGI-2-knockout mice, which caused abnormal expression of its downstream proteins [5]

Dendrin protein is a SD protein complex, which is closely associated with the actin cytoskeleton [17-19]. Dendrin can be repositioned from SD to the nuclei of damaged podocytes in animal models, such as PAN-induced podocyte injury model. In clinical studies of focal segmental glomerulosclerosis, membranous nephropathy, and lupus nephropathy, it has been found that dendrin is located in the podocyte nuclei, indicating that dendrin is a new marker of glomerular podocyte injury in the nuclear translocation of podocytes [19, 20]. Dendrin is a transcription factor, and its nuclear translocation can regulate the expression of CatL, which in turn increases the expression of cytoplasmic Cat-L.

The proteolytic pathway of CatL is involved in the regulation of foot process proteins [18] and plays an important role in the development of multiple reasons caused proteinuria [21]. The increase in cytoplasmic proteolytic activity of CatL can alter the podocyte actin cytoskeleton from parallel ordered contractile protein

to dense and disordered network structure, causing podocyte structural abnormalities. In animal proteinuria models such as lipopolysaccharide mouse model, PAN mouse model, or streptozotocin-induced diabetic mouse model, the expression of CatL in the kidneys increased, and all these animal models exhibited foot process effacement [22, 23]. Moreover, in *in vitro* experiments with lipopolysaccharide-/puromycin-treated podocytes, the expression of cytoplasmic CatL increased [22]. The knock-down of CatL gene can protect from foot process effacement and proteinuria caused by lipopolysaccharides [22, 23]. Similarly, CatL inhibitors can reduce proteinuria in experimental rats [22]. Clinical studies have also demonstrated a significant increase in CatL expression in podocytes with small lesions, focal segmental glomerulosclerosis, membranous nephropathy, and diabetic nephropathy [21, 22]. The renal biopsy results of patients with membranous nephropathy, focal segmental glomerulosclerosis, and diabetic nephropathy revealed 2-fold or higher expression of CatL mRNA [22]. The expression of CatL protein is also increased in diabetic podocytes [22], and recent studies have shown that CatL plays a key role in the development of proteinuria and kidney damage in early diabetic nephropathy [22, 23]. Consistent with our previous studies [24], it was found that WHL can decrease the expression of CatL in PAN-damaged podocytes and remodel the skeletal protein F-actin, thus helping to stabilize the podocyte actin skeleton, improve foot process effacement, and reduce proteinuria.

Synaptopodin is a CatL substrate [7, 22, 25-27]. Being an important regulator of the morphology and function of podocyte foot processes, synaptopodin can be hydrolyzed by CatL, thus playing an important role in maintaining the structural integrity of cytoskeleton and regulating podocyte migration. The expression of synaptopodin is closely related to the severity of kidney diseases, and its expression is reduced in glomerular diseases such as focal segmental glomerulosclerosis. Increased expression of cytoplasmic CatL leads to the reduction of synaptopodin and disrupts the formation of SD, thereby leading to foot process effacement and rearrangement of actin cytoskeleton, which result in podocyte injury, proteinuria, or even end-stage renal disease [7].

RhoA is an important protein that can dynamically regulate the cytoskeleton. It belongs to the Rho protein family and is a molecular switch that can control the transduction of cell signals from the plasma membrane to the cytoskeleton, including cytoskeleton kinetics [28]. The basic expression level of RhoA is conducive to maintaining the integrity of the glomerular filtration barrier. In lipopolysaccharide- and doxorubicin-treated podocytes, the expression and activity of RhoA are decreased, accompanied by the loss of podocyte stress fibers [29]. RhoA-knockout or small interfering RNA knockdown can also lead to the loss of podocyte stress fibers [29]. Synaptopodin is the upstream regulator of RhoA [28], and it can inhibit RhoA proteasome degradation and regulate podocyte migration. Synaptopodin can upregulate RhoA and stabilize protein levels to induce stress fibers. The above activities are related to competitively inhibiting the E3 ubiquitin ligase Smurf1 and reducing the ubiquitination of RhoA [28]. Silencing synaptopodin gene causes a decrease in the expression of RhoA protein [28], inhibition of podocyte skeletal rearrangement, and formation of stress fibers.

In our experiments, the level of synaptopodin significantly decreased after induction of podocyte injury by PAN, and the expression of RhoA protein was decreased. However, synaptopodin mRNA expression did not change significantly, suggesting that the change in synaptopodin did not occur at the level of gene transcription. A possible reason for this could be that when MAGI-2 declines, nuclear translocation of dendrin occurs, which results in increased expression of the downstream CatL mRNA, increased CatL level, and enhancement of enzymatic hydrolysis of synaptopodin, thereby leading to structural disorders of podocyte actin and intermediate filaments, as well as morphological changes of podocytes. WHL can increase MAGI-2 level, inhibit nuclear translocation of dendrin and CatL mRNA transcription, decrease CatL expression, decrease synaptopodin degradation, increase RhoA protein expression, and improve podocyte morphology. In agreement with our results, Ransom found a decrease in RhoA activity in PAN-injured mouse podocytes, but glucocorticoids increased RhoA activity and improved the cytoskeletal stability of podocytes [13].

In conclusion, WHL can improve the expression and distribution of podocyte-associated proteins, restore the morphology and structure of cytoskeletal microfilaments and intermediate filaments, and improve foot process effacement, thus reducing proteinuria and improving nephrotic syndrome.

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## Disclosure of conflict of interest

None.

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