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Mechanism of action of Jinghua Xiaozi Mixture in the treatment of Henoch Schonlein Purpura Nephritis revealed by Wnt/β-Catenin signaling pathway

Niqin Xiao[2,a,#], Caixia Zhao[1,b,#], Junyu Luo[2,c], Xianfeng Luo[2,d], Qiqi Chang[2], Jiao Xiong[2], Min Wang[2], Xi Li[2], Min Zhao[2], Zhifeng Wang[2,*], Ping He[1,*]

[1]The First Affiliated Hospital of Yunnan University of Chinese Medicine, Kunming, China
[2]Yunnan University of Chinese Medicine, Kunming, China
[a]xnq2022@126.com, [b]1076166634@qq.com, [c]2421922679@qq.com, [d]398030709@qq.com
[#]Authors contributed equally to this work
[*]Corresponding author

Abstract: *The experimental HSPN rat model was established by using the co-administration of bovine serum albumin (BSA), lipopolysaccharide (LPS), and carbon tetrachloride (CCl_4). Blood and proteinuria were detected in each group of rats. IgA deposition was detected by immunofluorescence, pathological changes of glomeruli and renal tubules under light microscope and changes of basement membrane, podocytes and mesangial area under electron microscope were observed, and whether to establish an effective HSPN rat model was comprehensively evaluated. Then the experimental rats were given different concentrations of JHXZ by gavage for 4 weeks, and western medicine piperazine ferulate tablets were used as positive control to observe the effects of JHXZ intervention on the pathological changes of renal tissue and the contents of urinary red blood cells and urinary protein in experimental rats. Finally, by measuring the protein expressions and mRNA levels of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7 and E-cadherin in renal tissues and mesangial cells, the correlation of Wnt/β-catenin signaling pathway in the pathogenesis of HSPN and its role in the treatment of HSPN diseases were evaluated.The results of the study showed that the results of urine testing, immunofluorescence, light microscope and electron microscope showed that there were increased red blood cells and proteins in urine, IgA deposition and mesangial proliferation, basement membrane proliferation, abnormal changes in podocyte morphology and quantity in all groups except the normal group, especially in the model group, which indicated that the HSPN rat model was successfully prepared. After high-dose JHXZ treatment, the IgA deposition in kidney, the proliferation degree of mesangial area and basement membrane, and the morphology and number of podocytes in HSPN rat model are close to normal, while the red blood cells, proteins in urine and IL-2, IL-4 and IFN-γ in blood are obviously decreased, and the effect is better with the increase of dose. The results of PCR and WB in vivo and in vitro were highly consistent. According to PCR and WB detection, it was found that high-dose JHXZ treatment could significantly reduce the gene and protein expression levels of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7 in model rats, and maintain the high expression of E-cadherin gene and protein. In addition, piperazine ferulate tablets are the first-line therapeutic drugs for HSPN, which was used as a positive control in this experiment. Our results show that compared with rats treated with piperazine ferulate tablets, the high-dose JHXZ group has better effects on improving the pathological changes of renal tissue, reducing urine red blood cells and reducing the expression of GSK-3β gene and protein. Our experiments proved that the activation of Wnt/β-catenin is involved in the pathogenesis of HSPN. JHXZ can regulate mesangial cells through Wnt/β-catenin to protect kidney.*

Keywords: *Jinghua Xiaozi mixture, Purpura nephritis, Signal pathway, Mechanism research*

1. Introduction

Henoch Schonlein Purpura Nephritis (HSPN) refers to the renal damage complicated by allergic purpura, which is the most common secondary glomerular disease in children [1]. Hematuria and/or proteinuria are the main clinical manifestations, and glomerular mesangial lesions are the main pathological changes [2, 3]. The incidence of HSPN is about 5%-62% of Henoch-Schonlein purpura, second only to nephrotic syndrome in children [4]. The prevalence of this disease is increasing year by year [5]. Under routine treatment, some patients can be cured, about 15% of children will develop renal

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

failure, and the prognosis of adult HSPN is even worse [6]. The course of HSPN is protracted and repeated [5], which brings heavy mental and economic burden to children and their parents, and affects the quality of life of children. The etiology and pathogenesis of HSPN are still unclear, and more and more studies tend to be systemic vasculitis mediated by immune factors, an autoimmune disease caused by abnormal glycosylated IgA1 immune complex, and other immune, complement, cytokine and other mechanisms involved. The mechanism of renal injury in henoch schonlein purpura is not completely clear, and its pathological features can be manifested as mesangial cell proliferation, extracellular matrix deposition, renal interstitial fibrosis, etc. [7].

Wnt/β-catenin signaling pathway is involved in the functional differentiation, proliferation, apoptosis and migration of cells, and plays an important role in the occurrence and development of tumors, organ fibrosis and embryo development [8-10]. Studies have shown that this signaling pathway plays an important role in the regeneration and repair of renal tubules and the occurrence of renal tumors during acute renal tubular injury [11],and it is closely related to the formation of renal fibrosis [10]. In normal mature kidney, Wnt signal is 'silented', and intracellular β-catenin and E-cadherin combine to form a complex, which prevents cells from migrating to maintain the integrity of tissue structure and avoid kidney damage [12]. Wnt signaling pathway includes Wnt protein family, Frizzled/low-density lipoprotein receptor-related protein, Fz/LRP), Dishevelled protein, β-catenin, colon adenomatous gene protein (APC), scaffold protein (axin/conductin), glycogen synthase kinase 3 (glycogen synthase kinase 3, GSK3), caseinkinasel (CKI) and T cell factor/lymphoid enhancer factor (TCF/LEF) [13, 14]. Among them, β -catenin is an important response factor in Wnt pathway, which mediates the transmission of various signals and the expression of downstream genes [9, 10]. Currently, there are 19 Wnt proteins reported [15] among them, Wnt4、Wnt3a and Wnt9b are the most important genes. When Wnt4 and Wnt9b genes are mutated, it will lead to renal dysplasia. Wnt3a serves as the activation Wnt/β-catenin-The inspiration of catenin can lead to the activation of pathways, and β-catenin plays an important role in the development and maturity of the posterior kidney [16]. After Wnt/β-catenin signaling pathway is activated, β-catenin activity increases and increases, and enters the nucleus from the cell membrane, thus activating the expression of downstream genes in the signaling pathway, such as the increased expression of Matrix Metalloproteinase (MMP-7), which has strong degradation ability. At the early stage of fibrosis, the expression of MMP-7 is also increased, which is a defense mechanism. With the aggravation of renal fibrosis, more and more cells are fibrosed, while the degradation ability of MMP-7 is weakened, and more and more MMP-7 will promote the damage of renal tubules [17]. Wnt/ β- The catenin signaling pathway can participate in the formation of renal units and play an important role in the development of the kidney. It may be involved in the apoptosis and proliferation of mesangial cells in the glomerulus, thereby participating in the pathogenesis of HSPN.

In recent years, much attention has been paid to the treatment. Western medicine mainly includes glucocorticoid, angiotensin converting enzyme inhibitor, immunosuppressant, angiotensin II receptor antagonist, anticoagulant therapy, plasma exchange, hemoperfusion, hemodialysis and symptomatic support therapy. Although glucocorticoid and immunosuppressant can reduce the damage of immune inflammation to glomerulus, they have poor curative effect and great adverse reactions [18]. And the repeated HSPN and the side effects such as hormone dependence and hormone resistance have never been effectively solved. At the same time, the side effects of these drugs lead to centripetal obesity, osteoporosis, growth disorder, three metabolic disorders and easy infection, which affect the quality of life of children. Traditional Chinese medicine (TCM) is based on the overall adjustment, which has the characteristics of flexibility of prescription and safety of drug effect and has great advantages for the treatment of children HSPN. JHXZ is based on Professor Liu Yimin's clinical medication experience in treating renal diseases, which is the "third and fifth batch of national famous old Chinese medicine academic guidance teachers and Yunnan famous Chinese medicine practitioners", and it has been used in clinic for more than several decades after inheritance and innovation, and nearly 10,000 cases of HSPN children have been observed, showing its good clinical efficacy. JHXZ uses Schizonepeta tenuifolia, Flos Lonicerae, Radix Rubiae, Radix Arnebiae, Herba Cirsii, Herba Leonuri, Herba et Gemma Agrimoniae, Radix Paeoniae Rubra, Red Melon, Orchid Ginseng, panax pseudo-ginseng, Verbena, Tripterygium hypoglaucum, Glycyrrhrizae Radix and other drugs,This formula has the effects of dispelling wind and blood stasis, cooling blood and calming the liver. In the early acute toxicity test of JHXZ in mice, the mice did not die. In addition, 60 children with Henoch schonlein purpura nephritis were divided into experimental group and control group according to the ratio of 1∶1 by simple randomized controlled trial. After being treated with JHXZ and piperazine ferulate tablets respectively, it was found that the therapeutic effect of the experimental group on reducing urinary red blood cells and urinary protein was better than that of the control group.

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

However, it is not clear whether there are other pathogenesis of this disease and whether JHXZ has other pathways or mechanisms. This experiment is to explore whether Wnt/β-catenin is involved in the pathogenesis of HSPN and whether JHXZ regulates mesangial cells through Wnt/β-catenin to protect the kidney.

2. Materials And Methods

2.1. In Vivo Experiment

2.1.1. Preparation and Grouping of Animal Models

Sixty healthy male SD rats with SPF grade were selected, 10 were randomly selected as blank group, and the remaining 50 rats were treated with BSA, LPS and CCl4 combined administration method to establish experimental rat IgA nephropathy model. Each rat was given immunogen BSA orally every other day at a dose of 400mg/kg for 12 weeks. CCl4 (0.5mL castor oil plus 0.1mL CCl4) was injected subcutaneously once a week for 9 weeks, and LPS was combined (LPS 0.05mg/rat was injected into the tail vein at the 6th and 8th week, respectively). The hematuria and proteinuria of rats in each group were detected, and the pathological changes of glomeruli and renal tubules under light microscope and the changes of basement membrane, podocyte and mesangial region under electron microscope were observed. After successful model construction, fifty IgA nephropathy model rats were numbered according to body weight and divided into 5 groups with 10 rats in each group according to random number table method. They were model group, JHXZ (high, medium and low) dose group and western medicine control group.

2.1.2. Drug Preparation and Intervention Methods

JHXZ is prepared by the Preparation Center of the First Affiliated Hospital of Yunnan University of Chinese Medicine. The process flow and quality standards are controllable and implemented with reference to relevant standards. JHXZ was fully dissolved and mixed with distilled water to prepare a solution with a concentration of 33%. After weighing, it was equivalently converted according to the dosage ratio of human and rats and was given orally at a dose of 3.5mL/kg. The high, medium and low dose groups were given gavage 3, 2 and 1 times a day for 4 weeks. The western medicine treatment group took piperazine ferulate tablets from the 13th week with a dose of 13.5mg/kg, three times a day. The blank group and model group were given the same amount of distilled water by gavage from the 13th week, and all rats were given standard diet and standard room temperature during the administration.

2.1.3. Urine Detection

At the end of the 16th week, the 24-hour urine of rats in each group was collected in the metabolic cage, and the 24-hour urine volume was recorded. The urine protein was detected by BCA method, and the number of urine red blood cells was detected by quantitative urine sediment counting method.

2.1.3.1. Urine Test

At the end of the 16th week, metabolic cages were used to collect 24 h urine from each group of rats, record the 24h urine volume, and detect quantitative urinary protein by BCA method and the number of urinary erythrocytes by quantitative urine sediment counting method.

2.1.3.2. Pathomorphological Changes of Renal Tissue

At the end of the experiment, the kidney of the rat was removed, half of the left kidney was frozen immediately for IgA immunofluorescence detection(Zeiss Germany,Observer.A1), and the other half was fixed with 10% neutral formaldehyde, dehydrated, transparent, waxed, embedded in paraffin, sliced, pushed, baked, moulted, watered, stained by HE, and sealed with neutral gum. Pathological changes of renal tissue were observed under light microscope(Hitachi HT7700). Fixation with 2.5% glutaraldehyde was used to observe the morphology and quantity of podocyte and mesangial cells in renal tissue.

2.1.3.3. The Concentration of Inflammatory Factors in Peripheral Blood was Detected by ELISA

After anesthesia, about 200μl of blood was taken from the eyeball of the rats, centrifugation was performed, serum was taken, and the reaction was terminated after coating, enzyme-labeled antibody and substrate solution were added according to the instructions of the ELISA kit(NEOBIOSCIENCE). Finally, the absorbance OD values of IL-2 (NEOBIOSCIENCE,ERC001.2), IL-4, (NEOBIOSCIENCE,ERC001.96), IFN-γ (NEOBIOSCIENCE,ERC101g.96)and other inflammatory factors were measured at 450n.

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

2.1.3.4. Real-time PCR was Used to Detect the Expression Level of Signal Molecule mRNA

Total cell RNA was extracted from the isolated kidney tissue using TRIzol reagent (Life,Cat.no.15596-018), and cDNA was synthesized by reverse transcription, and the cDNA was used as the template for Real-time PCR reaction(PCR instrument:Applied Biosystems,2720)to detect the amplified Ct values of mRNA of related molecules in Wnt/-β-catenin signaling pathway, with GAPDH as the internal reference. The mRNA expression and changes of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7 and E-cadherin were obtained by calculating 2-△△Ct.

2.1.3.5. Western Blot was Used to Determine the Expression of Signal Molecule Protein

Kidney tissue protein was extracted, and after protein denaturation, electrophoresis, membrane transfer and incubation of corresponding antibody, Image-Pro Plus 6.0 software was used for development. The expression and change of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7 and E-cadherin in renal tissues of each group were detected by means of average optical density(Antibody manufacturer:Affinity).

2.2. In Vitro Experiment

2.2.1. Preparation of Drug-containing Serum

Fifty SD female rats with SPF grade were fed adaptively for 1 week, numbered according to body weight, and divided into 5 groups with 10 rats per group according to random number table method. They were divided into blank drug-containing serum group, JHXZ (high, medium and low) dose drug-containing serum group, and western drug-containing serum group. According to the dosage ratio of human and rats, the rats in JHXZ high-dose drug-containing serum group were given 10.5mL/(kg·d), respectively. The rats in JHXZ medium dose drug-containing serum group were given 7mL/(kg·d), the rats in JHXZ low dose drug-containing serum group were given 3.5mL/(kg·d), and the rats in Western drug-containing serum group were given the corresponding drugs by intragastric administration respectively. The blank serum group was given equal volume of normal saline intragastric once a day for a total of 7 days, and the diet was normal. Two hours after the last administration, blood was taken from the abdominal aorta (6mL each). The blood was placed at 4℃ for 4h and centrifuged at 3000r/min for 30min. The upper serum was taken and inactivated at 56℃ for 30min, and the serum in the same group was mixed and filtered through 0.45μm and 0.22μm disposable filter membrane for bacteria removal, 5mL per tube was divided and stored at -20℃ for later use.

2.2.2. Isolation and Culture of Gomerular Mesangial Cells

Six SPF-grade SD rats were selected, 1 was randomly selected as the blank control group, and the remaining 5 rats were randomly divided into model group, JHXZ (high, medium, low) dose group and western medicine control group according to the previous modeling method. After the modeling was successful, the rats were immersed in 75% ethanol for 20s, the kidney tissue was extracted by aseptic clipping, the kidney cortex was separated, and the kidney tissue was cut into 1mm×1mm tissue blocks, and 0.25% trypsin 5mL was pre-digested at 37℃ for 20min. The digestive fluid was discarded, and the kidney tissue blocks were transferred into a centrifuge tube containing 5mL of 0.1% type I collagenase and digested in a constant temperature water bath at 37℃ for 30min. The digestive fluid was absorbed, centrifuged at 1000r/min for 15min, the supernatant was discarded, and appropriate amount of RPMI-1640 was added to prepare cell suspension. The remaining kidney tissue blocks were digested by repeated oscillations twice, and the digestive fluid obtained each time was collected and centrifuged to make cell suspension. The cell suspension obtained for 3 times was mixed and inoculated in a 25cm culture bottle with 5% CO2 at 37℃. After 3 days, the liquid was changed for the first time, and then the liquid was changed every 3 days. After cell fusion, the cells were digested with 0.25% trypsin, and the cells were passed 1:1 and inoculated in another 25cm culture bottle for further culture. The third-generation cells were selected for the experiment.

2.2.3. Addition of Drug-containing Serum

Taking healthy adherent human mesangial cells in logarithmic growth period, the cells were digested by egg trypsin and blown with sterile rubber dropper, and made into single cell suspension, which was counted by fine counting plate and made into a density of 4×10^4cell/mL. Each group was evenly inoculated into 12-well plates, and 200μL of culture solution was added to each hole, and the holes around the cells were filled with Phosphate Buffer(Beijing Solarbio Science & Technology Co.,Ltd.NO.P1003) with the same volume. The culture plate was cultured in a cell incubator, and when the cells grew to 80% fusion, they were cultured in serum-free medium for 24 hours, so that the cells were in the GO phase.

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

The blank control group and model group were given 10% blank drug-containing serum group rat serum +RPMI-1640 culture medium, and the other four groups were given 10% rat serum +RPMI-1640 culture medium, which were cultured for 6 days in succession, and the medium was changed once every 3 days.

2.2.4. Real-time PCR was Used to Detect the mRNA Expression Level of Signal Molecules

After 6 days of continuous culture, the total RNA of glomerular mesangial cells in each group was extracted with TRIzol reagent, and the cDNA was synthesized by reverse transcription, and the Real-time PCR reaction was carried out with this cDNA as a template, and the Ct value of Wnt/β-catenin signaling pathway related molecular mRNA was amplified, with GAPDH as an internal reference. The expression and changes of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7, E-cadherin and other mRNA were obtained by calculating 2-△△△ CT.

2.2.5. Western Blot was Used to Determine the Expression of Signal Molecule Protein

After 6 days of continuous culture, the total protein of glomerular mesangial cells in each group was extracted. After protein denaturation, electrophoresis, membrane transfer and corresponding antibody incubation and development, Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7, E-cadherin and so on in renal tissues of each group were detected with the average optical density.

3. Statistics

Process and analyze data using SPSS 26.0 software. If the data follows a normal distribution, it is expressed as mean±standard deviation, and the differences are compared using two independent sample t-tests or one-way ANOVA. If the data is not normally distributed, it is expressed as the median (IQR, interquartile range) and non parametric tests are used. If $p<0.05$, it is considered statistically significant.

4. Results

4.1. In Vivo Testing

4.1.1. Urine Detection

A large amount of hematuria and proteinuria appeared in the urine of the model group, while the red blood cells and proteins in the urine of rats treated with high-dose JHXZ and western medicine significantly decreased, and with the increase of JHXZ dose, the red blood cells and proteins in the urine gradually decreased. The effect of high dose of JHXZ on reducing red blood cells in urine is better (Figure 1).

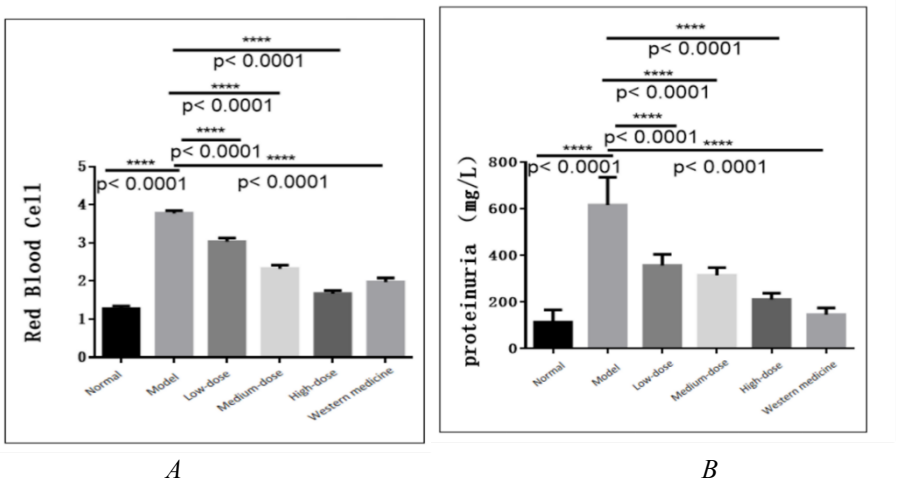


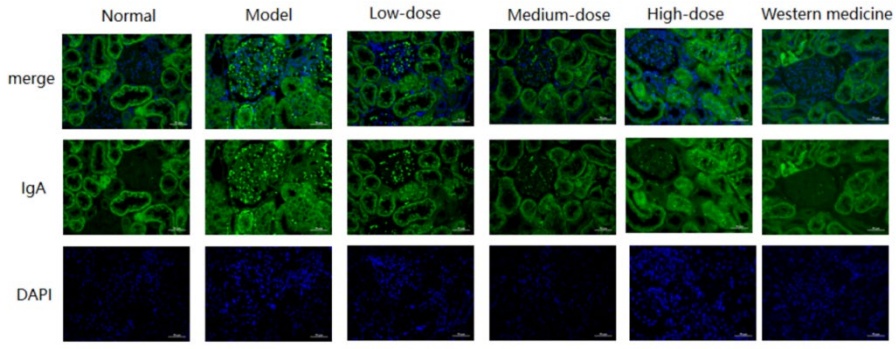
*Figure 1: Urine tests in each group (A) erythrocyte test in urine; (B) protein test in urine. All data show significant differences, ****p < 0.0001.*

4.1.2. Renal Histopathology

After staining with DAPI and merge staining, it can be found that there is significant deposition of IgA complex in the model group, while there is a significant difference in the treatment group. The renal

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

immunofluorescence complex is significantly reduced after treatment with high-dose JHXZ. Moreover, the deposition of IgA complex after JHXZ treatment decreased with the increase of drug dose. After HE staining, mesangial cells proliferated obviously in the model group, but the degree of mesangial cell proliferation was significantly reduced after high-dose JHXZ treatment, and the mesangial cell proliferation decreased with the increase of drug dose after JHXZ treatment. Compared with rats treated with piperazine ferulate tablets, high-dose JHXZ has a better effect on improving mesangial cell proliferation. 2.5% glutaraldehyde fixation was used for electron microscope ultrastructure analysis, and it was observed that podocytes in the model group had mild edema, uniform intracellular matrix, visible tight junction (TJ) and acceptable intercellular space. The nucleus (N) is irregular, the nuclear membrane is complete, the perinuclear space is not obviously widened, and chromatin is a little marginal. Mitochondria (M) are slightly swollen, uniform in size, complete in membrane, dissolved and diluted in matrix, and cristae are broken and reduced. The basement membrane (BM) is complete and continuous, with local thickening (shown by red arrow), and the electron density of the matrix is uniform, and no obvious electron dense matter is deposited. The number of foot processes (FP) is small, and most of them are fused and widened. However, the podocyte edema was slightly relieved after high-dose JHXZ treatment, and the mitochondria swelling was reduced, the basal thickness was uniform, the number of podocytes was abundant, and the structure of podocytes after JHXZ treatment became more normal with the increase of drug dose. Compared with rats treated with piperazine ferulate tablets, high-dose JHXZ has a better effect on improving the pathological changes of renal tissue under light microscope (Figure 2).



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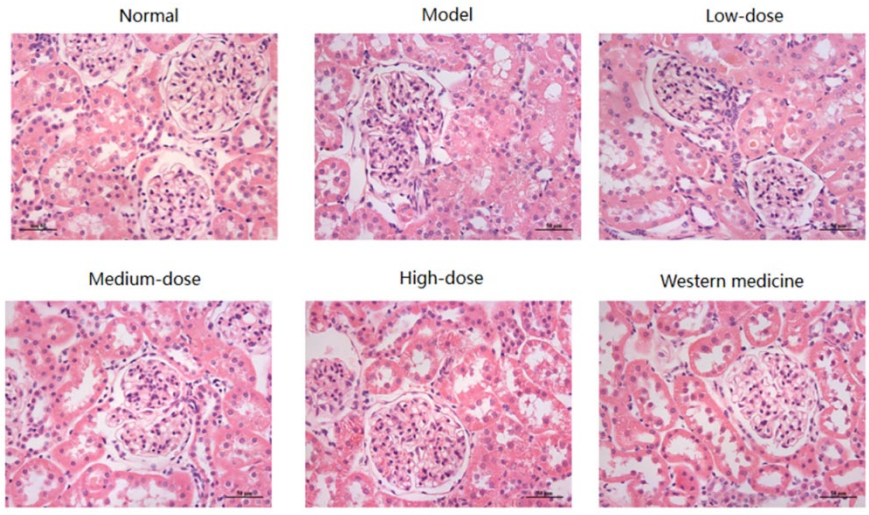

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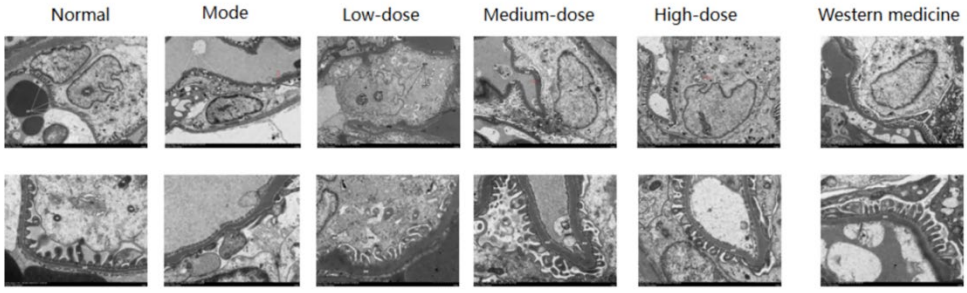
ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801



C

Figure 2: Histopathological and morphological manifestations of kidney (A) renal IgA complex deposition manifestations in each group after merge and DAPI staining; (B) thylakoid cell manifestations in each group after HE staining (C) foot cell manifestations in each group observed after fixation by 2.5% glutaraldehyde for ultrastructural analysis by electron microscopy.

4.1.3. ELISA for Detection of IL-2, IL-4, IFN-γ

IL-2, IL-4 and IFN-γ in the model group were significantly increased by ELISA, while IL-2, IL-4 and IFN-γ were significantly decreased after high-dose JHXZ and western medicine treatment, and IL-2, IL-4 and IFN-γ were gradually decreased with the increase of JHXZ dose (Figure 3).

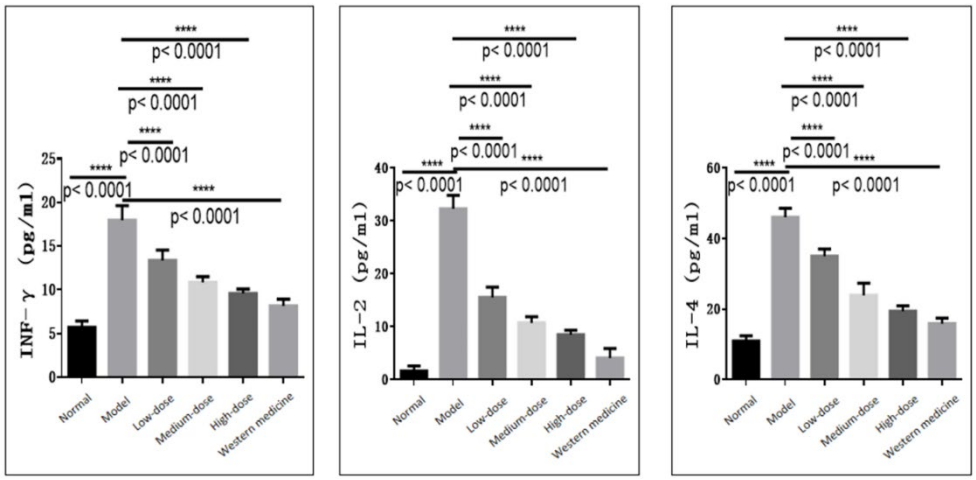


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*Figure 3: ELISA for IFN-γ, IL-2, IL-4 expression levels (A) ELISA for IFN-γ expression levels; (B) ELISA for IL-2 expression levels; (C) ELISA for IL-4 expression levels. All data showed significant differences, ****p < 0.0001.*

4.1.4. PCR was Used to Detect the Expression of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7 And E-cadherin Genes

It was found by PCR that the expression of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4 and MMP-7 genes in the normal group were all at a low level, while E-cadherin was at a high level, while the untreated model group was on the contrary. E-cadherin was at a low level, and the other genes were expressed at a high level. In this study, it was found that the expression levels of Wnt3a, Wnt4, Wnt9b, β-catenin, TCF-4 and MMP-7 genes in the high-dose Chinese medicine group and the western medicine group could significantly decrease, and the high expression of E-cadherin gene could be maintained. And the gene expression level after JHXZ treatment was more inclined to normal expression with the increase of dose. For the GSK-3β gene, no significant difference was demonstrated between the model group and each treatment group, but in terms of the overall trend, JHXZHJ reduced the expression of the GSK-3β gene and was more effective than the western drug group (Figure 4).

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

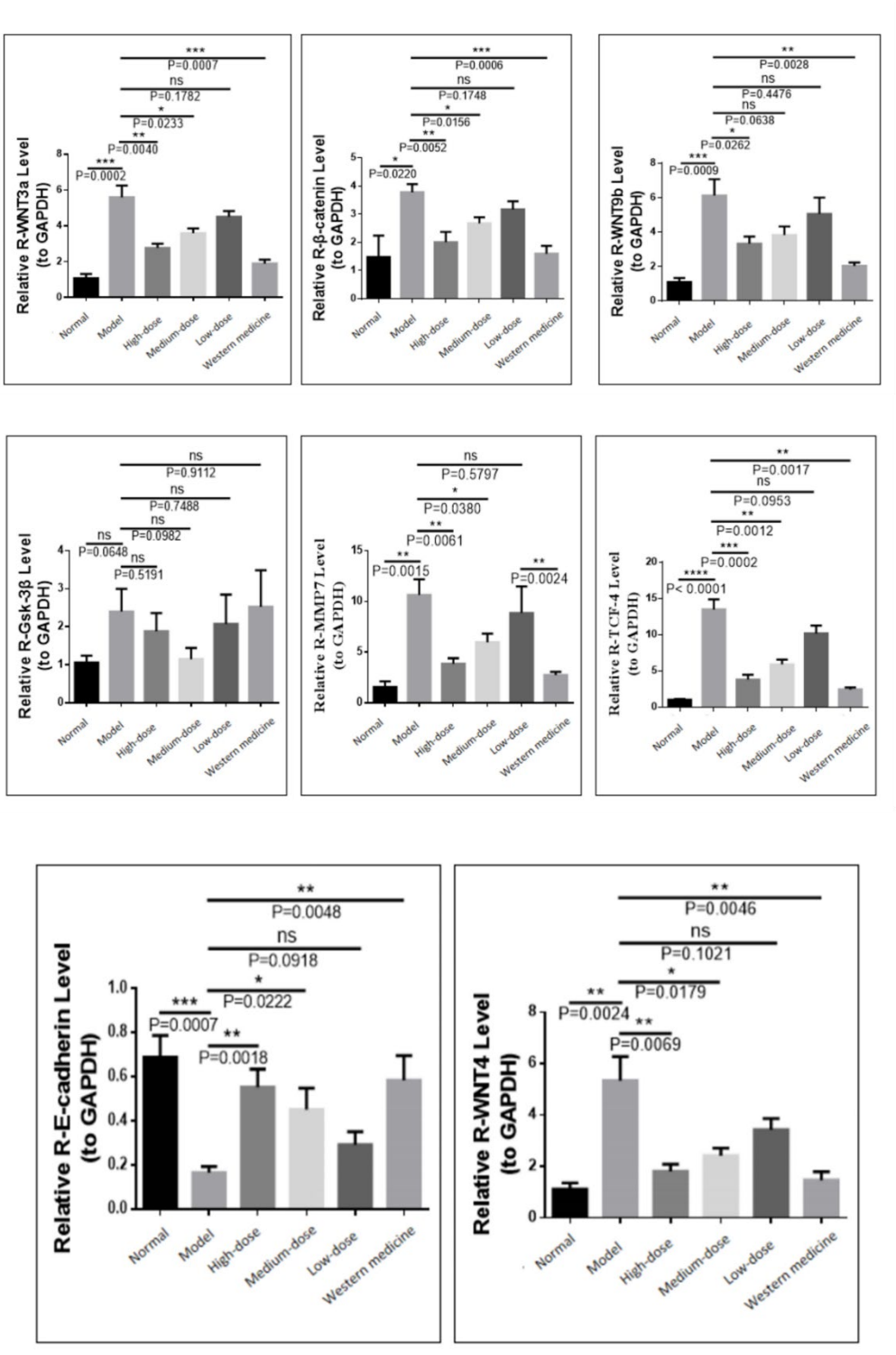


*Figure 4: PCR detection of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7, E-cadherin gene expression. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns for no significant difference.*

4.1.5. WB was Used to Detect the Expression of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7 And E-cadherin Proteins

According to WB detection results, it was found that the expressions of Wnt3a, Wnt4, Wnt9b, β-catenin, TCF-4 and MMP-7 in normal group were all at a low level, while E-cadherin was at a high level, whereas in untreated model group, the expression of E-cadherin was at a low level, and the other proteins were expressed at a low level. In this study, it was found that the high-dose group of traditional Chinese medicine and the western medicine group could significantly reduce the protein expression levels of Wnt3a, Wnt4, Wnt9b, β-catenin, TCF-4 and MMP-7, and maintain the high expression of E-cadherin,

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

and the protein expression level after JHXZ treatment tended to be normal with the increase of dose. For GSK-3β protein, the effect of traditional Chinese medicine group is better than that of western medicine group (Figure 5).

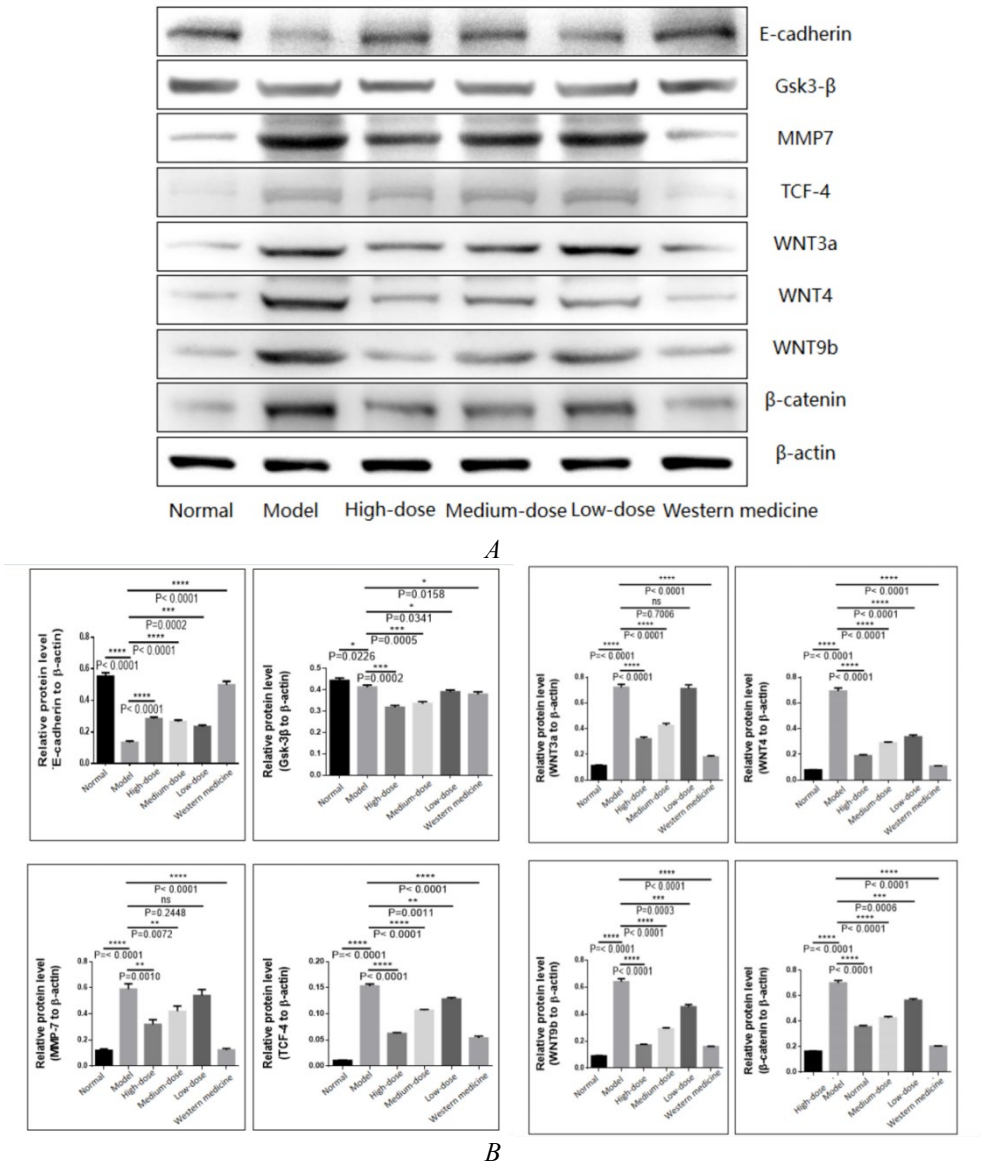


*Figure 5: WB detection of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7, and E-cadherin protein expression (A) WB protein blotting; (B) WB gray value statistic, all of which exhibited significant differences, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

4.2. In Vitro Experiment

4.2.1. PCR was Used to Detect the Expression of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7 And E-cadherin Genes

According to the PCR detection results, the expression of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4 and MMP-7 genes in the normal group was at a low level, while the expression of E-cadherin was at a high level, while the expression of the untreated model group was on the contrary. E-cadherin was at a low level, and the other genes were expressed at a high level. In this study, it was found that the expression levels of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4 and MMP-7 genes in the high-dose Chinese medicine group and the western medicine group could significantly decrease, and the high expression of E-cadherin gene could be maintained. And the gene expression level after JHXZ treatment was more inclined to normal expression with the increase of dose. For GSK-3β gene, the effect of the Chinese medicine group was better than that of the western medicine group (Figure 6).

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

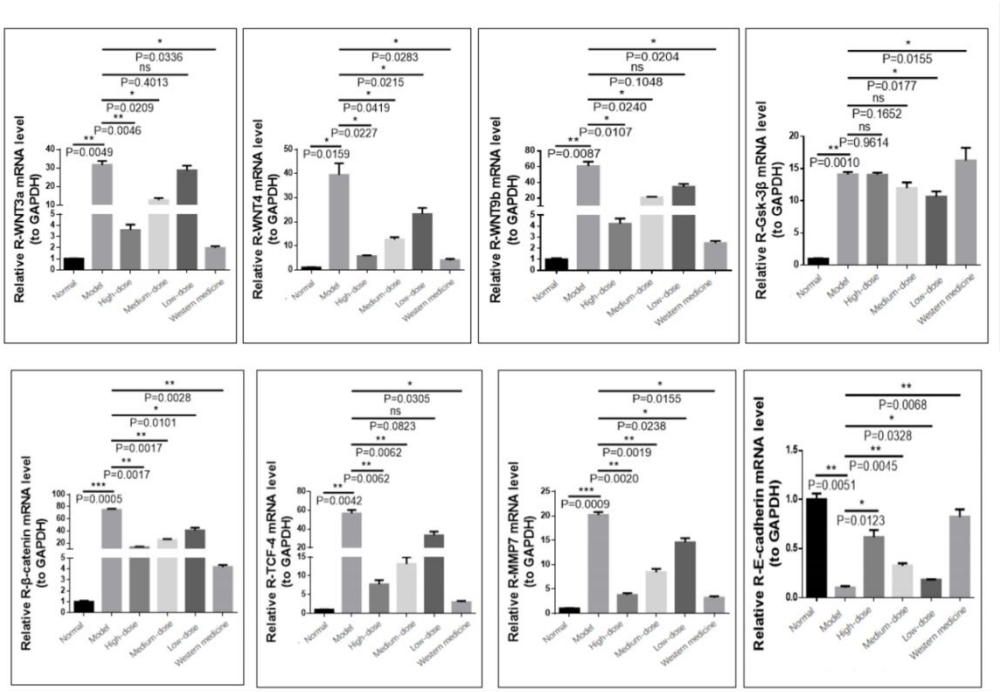
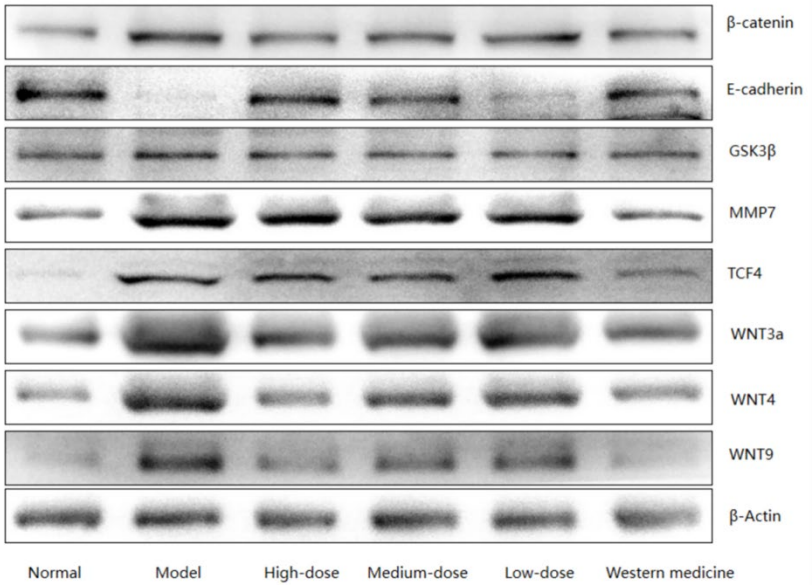


*Figure 6: PCR detection of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7, E-cadherin gene expression. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns for no significant difference.*

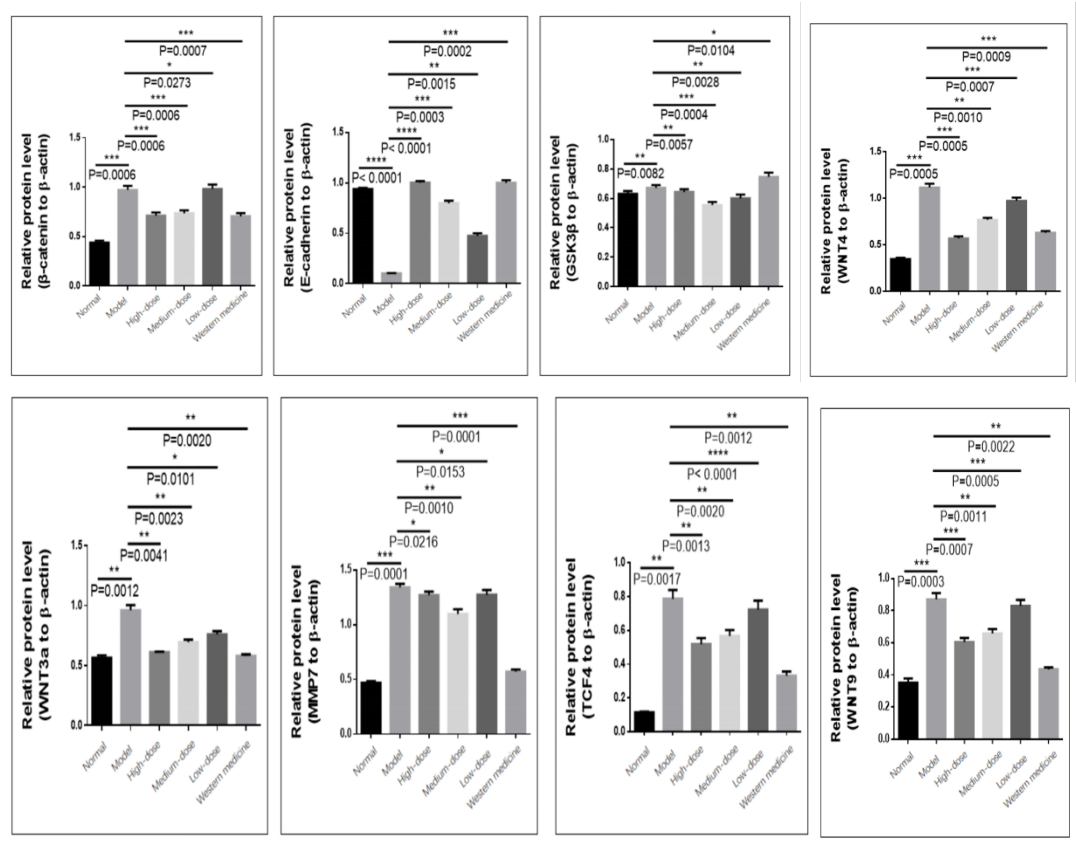
4.2.2. WB was Used to Detect the Expression of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7 And E-cadherin Proteins

According to the results of WB detection, it was found that the expression of Wnt3a, Wnt4, Wnt9b, β-catenin, TCF-4 and MMP-7 proteins in the normal group were all at a low level, while E-cadherin was at a high level, while the untreated model group was on the contrary. E-cadherin was at a low level, and other proteins were expressed at a high level. In this study, it was found that the expression levels of Wnt3a, Wnt4, Wnt9b, β-catenin and TCF-4 in the high-dose Chinese medicine group and the western medicine group could significantly decrease, and the high expression levels of E-cadherin protein could be maintained, and the protein expression levels after JHXZ treatment tended to normal expression with the increase of dose. As for GSK-3β protein, the effect of the Chinese medicine group was better than that of the western medicine group. For MMP-7 protein, the Chinese medicine medium dose group was more dominant, but overall, the Chinese medicine group could down-regulate its protein expression level (Figure 7).



A

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801



B

*Figure 7: WB detection of Wnt3a, Wnt4, Wnt9b, GSK-3β, β-catenin, TCF-4, MMP-7, and E-cadherin protein expression (A) WB protein blotting; (B) WB gray value statistic, all of which exhibited significant differences, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

5. Discussion

Anaphylactoid purpura is a systemic, systemic small vessel inflammatory disease induced by an immunoglobulin IgA, most involved in the skin, joints, gastrointestinal tract, and kidneys. Anaphylactoid purpura is most common in children, but it can also occur in adults. Studies have shown that 94% of children and 89% of adults have a tendency to self-heal, and the prognosis is good, therefore, supportive therapy is the main treatment strategy for Henoch purpura [18]. However, the most important factor affecting the prognosis of patients with anaphylactoid purpura is whether the kidney is involved, and the severity of kidney involvement. Studies have found that 20% of children with HSPN develop chronic kidney disease, and the proportion of children with newly diagnosed HSPN whose disease severity is moderate or higher can go on to develop chronic kidney disease [18]. Therefore, active treatment of HSPN and delay the development of the disease into end-stage renal disease are the key points and difficulties in the treatment of HSPN. Western medicine mainly focuses on immunosuppressants such as glucocorticoids and cyclophosphamides in treatment, combined with anticoagulants and renin-angiotensin system blockers, etc., but the adverse reactions easily caused include Cushing's syndrome, secondary diabetes, as well as cardiohepatotoxicity and bone marrow suppression caused by cyclophosphamide [19], which greatly limits the clinical application of these drugs. While traditional Chinese medicine in the treatment of purpura nephritis has shown its unique advantages, and the clinical effect is exact. JHXZ takes "expunging wind and dispersing stasis, cooling blood and flattening liver" as the treatment principle, has been used in clinic for more than decades, the treatment of wind-heat syndrome of children with purpura nephritis has significant effect, no obvious adverse reactions were found, and has good clinical application value.

HSPN and IgA nephropathy are two common glomerular diseases. Studies have found that both of them are systemic immune complex diseases mediated by IgA. Renal pathology showed mesangial hyperplasia and deposition of immune complex dominated by IgA in the mesangial region [20]. Clinical urine tests showed proteinuria and hematuria in different degrees. Due to the many clinical and

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

pathological similarities between the two renal diseases, some have suggested that they are essentially variations of the same clinicopathological entity of the same disease or different stages of the development of the same disease. Studies have found [18, 21] that IgA plays a decisive role in the pathogenesis of anaphylactoid purpura. Fundamentally speaking, purpura nephritis is IgA nephropathy with a rash. Davin JC [22] believed that the abnormal glycosylation of IgA1 played an important role in the pathogenesis of purpura nephritis, which undoubtedly emphasized the consistency of the immunopathogenesis of purpura nephritis and IgA nephropathy.

In view of the many similarities in pathological and clinical manifestations of the two renal diseases, this study established an experimental HSPN rat model by using the combined administration of BSA, LPS and CCl4. After 4 weeks of intervention with different concentrations of JHXZ, the results showed that JHXZ had a significant therapeutic effect on HSPN. Immunofluorescence results indicated that IgA deposition existed in all groups except the normal group, and IgA deposition was obvious in the mesangial region of rats in the model group, indicating that the HSPN rat model was successfully prepared. Compared with the model group, the fluorescence intensity of each drug administration group was weakened, and there were differences in all groups, among which, the middle and high concentration groups and the western drug group were significantly different from the model group. At the same time, compared with the normal group, the mesangial hyperplasia and tubule swelling were evident in the model group under light microscope, accompanied by segmental glomerular sclerosis, glomerular vascular loop collapse, and perivascular inflammatory cell infiltration were observed. Under electron microscope, podocyte edema, irregular cell nuclei, mitochondrial swelling, matrix dissolution and thinning, ridge fracture and reduction, local thickening of basement membrane, small number of foot processes, fusion and widening were observed in the model group. The above results were consistent with the pathological manifestations of HSPN. After JHXZ intervention, mesangial hyperplasia, basement membrane widening, cell edema and mitochondrial swelling of rats were improved, suggesting that JHXZ has therapeutic effect on HSPN, and the high dose group is the most obvious. In addition, piperazine feruloate tablet is the first-line treatment for HSPN, and it was used as a positive control for HSPN in this study. Our results showed that JHXZ high-dose group was more effective in improving renal histopathologic changes and reducing urinary erythrocytes than rats treated with piperazine ferulate tablets.

HSPN children are stimulated by antigen, which can promote the proliferation and differentiation of Th cells into Th1 cells. TH1 cells are activated to secrete the widely bioactive cytokine IL-2 [23]. Meanwhile, IL-2 can promote the adhesion of lymphocytes and macrophages to the vascular endothelium, leading to immune injury [24]. Animal experiments have shown that IL-2 is significantly elevated in the established HSP rabbit model [25, 26]. Jiaxin Xu et al. [27] found that the serum IL-2 of children in the HSPN group was higher than that in the control group. The results of this experimental model group showed an increase in IL-2, which is consistent with other experimental results.Indicating that the increase of IL-2 was involved in the pathogenesis of HSPN.

IFN-γ is a kind of glycoprotein secreted by the main Th1 cells, and it is an activating factor of mononuclear macrophages, which can promote the lethality of macrophages to antigens. IFN-γ can promote the expression of histocompatibility antigen complex on cell surface, initiate the immune response, and enhance the immune function. Studies have found that the serum level of IFN-γ in HSPN is increased [28]. Taomin Bai et al. [29] found that the increase of serum IFN-γ was related to kidney injury. And the results of the model group in this experiment show that IFN- γ Rise,which further indicated that the increase of IFN-γ was involved in the pathogenesis of HSPN.

IL-4 is produced by Th2 cells and is a cytokine for cell inflammation. IL-4 can make T lymphocytes prolifate and grow, and (or) stimulate B lymphocytes to produce IgE and IgG1, participate in allergic reaction, activate IL-4/STAT6 signaling pathway, and promote IgA1 secretion. Inhibitive Fcy receptor IIb is preferentially expressed on the surface of IL-4 cells, which activates the balance of inhibitive Fcy receptor [30] and promotes the binding of Fcy receptor to IgE, resulting in allergic reactions. HSPN is an allergic reaction, which may be caused by IL-4 promoting the proliferation and growth of mast cells, secreting a large number of IgE receptors and binding with IgE, generating circulating immune complex and causing damage to the glomerulus. Studies have found that IL-4 in HSPN serum is higher than normal. In this experiment, it was also found that IL-4 increased,suggesting that the increase of IL-4 is involved in the pathogenesis of HSPN [31].

Therefore, the increase of IL-2, IFN-γ and IL-4 will lead to the occurrence of HSPN, while in this study, it was found that IL-2, IFN-γ and IL-4 all showed a downward trend after treatment with JHXZ. Therefore, HSPN may inhibit the expression of IL-2, IFN-γ and IL-4, thus affecting the balance of Th1 and Th2. Thereby delaying the occurrence and development of HSPN and alleviating kidney damage. At

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

the same time, the experimental study found that JHXZ had no obvious side effects on liver and kidney function within 4 weeks of use and had obvious curative effect.

Wnt/β-catenin signaling plays an important role in organ fibrosis and embryo development. Studies have shown that this signaling pathway is closely related to tubular regeneration and repair and the formation of renal fibrosis during acute tubular injury [32, 33].

The Wnt signaling pathway has been divided into three pathways, which are called Wnt /β-catenin signaling pathway, JNK-mediated planar cell polarity pathway, and calcium ion mediated pathway. Different Wnt proteins mediate different pathways to the Wnt signaling pathway. Currently reported Wnt proteins mainly include 19 kinds, such as Wnt1, Wnt3a, Wnt4, Wnt5a, Wnt9b, Wnt8, Wnt11 and so on, among them, Wnt3a, Wnt4, and Wnt9b are the most important. The main components of Wnt signaling pathway include Wnt protein family Fz/LRP, Dishevelled protein, β-catenin, APC, axin, GSK3, CKI and TCF [34, 35]. As the downstream gene of Wnt /β-catenin signaling pathway, MMP-7 will promote the damage of renal tubules with the aggravating of renal fibrosis [17].

Wnt3a is the upstream protein that mediates the activation of Wnt/β-catenin signaling pathway, and it is also a secretory membrane protein. After the cells exclude Wnt3a protein in the way of autocrine or parasecretory, Wnt3a signaling protein binds to the cell membrane of effector cells, which serves as the revelation of the activation of this pathway. Causing activation of downstream intracellular signaling, leading to activation of the pathway [36]. Studies have shown that Wnt4 gene mutation will lead to kidney dysplasia, Wnt9b is the induction signal of kidney development, when Wnt9b gene mutation or deletion can make the differentiation of renal mesenchymal cells stagnant, will also lead to kidney dysplasia. Xiu Li and other studies show that [37], compared with normal children, Wnt4 is highly expressed in the cytoplasm of renal tubule cells in the kidney tissues of HSPN children.

β-catenin is the core of Wnt/β-catenin signaling pathway, and it is also the sign of Wnt/β-catenin signaling pathway, and its expression is the biggest difference between Wnt/β-catenin signaling pathway and the other two signaling pathways. When the Wnt signaling pathway is silent, there is no expression of β-catenin protein in the cytoplasm, or only a low concentration of expression, the reason for this phenomenon is that the β-catenin protein generated in the cell will be degraded by the APC complex, and when the upstream Wnt signaling protein is secreted, Moreover, it binds with Frizzled receptor protein on the cell membrane. Under the action of intracellular Dsh protein, the decomposition process of intracellular β-catenin protein is inhibited, causing the accumulation of β-catenin protein in the cytoplasm and activation of downstream protein, thus activating the whole signal pathway [38].

GSK-3β is a silk/threonine kinase that regulates a variety of cellular responses, such as cell growth, differentiation, programmed death and inflammatory responses, and is a key enzyme in the Wnt signal transduction pathway [39]. In the Wnt signaling pathway, GSK-3β primarily degrades the β-catenin complex by phosphorylating the amino-terminal threonine of β-catenin, leading to β-catenin accumulation and nuclear transfer, thereby initiating the Wnt signaling pathway.

TCF-4 is a member of the TCF family, and its gene is Transcription factor 7-like 2 (TCF7-L2), which is an important signaling molecule in Wnt signaling pathway. It is shown that when Wnt protein is bound with Frizzled transmembrane protein (FZ) receptor and low-density lipoprotein receptor-related protein on the cell membrane surface, β-catenin can accumulate in large quantities in the cytoplasm and enter the nucleus. The incoming β-catenin binds with TCF-4 to form β-Catenin-TCF-4 complex, which activates a series of target genes downstream of Wnt signaling pathway [39].

E-cadherin is a transmembrane glycoprotein that joins epithelial cells together at adhesion junctions, maintaining their normal morphology and polarity. Beta-catenin reacts with E-cadherin on the cell surface to form a stable complex and bind to actin. In normal epithelial tissue where E-cadherin is highly expressed, β-catenin is sequestered on the cell membrane, preventing its release into the cytoplasm and into the nucleus [40], preventing β-catenin from binding to members of the DNA-binding protein family Lymphoenhancer factor (LEF)/TC in the nucleus. It can be seen that changes in the expression level of E-cadherin have regulatory effects on the Wnt/β-catenin pathway.

MMP family is a family of zinc ion dependent proteolytic enzymes, which can effectively degrade extracellular matrix and play an important role in tumor growth and metastasis. Mmp-7 is the smallest member of MMP family, which can degrade various extracellular matrix components. Such as laminin, fibronectin, elastin, type IV collagen, etc. [41]. When the Wnt/β-catenin signaling pathway is activated, the activity of β-catenin increases and increases, entering the nucleus from the cell membrane, and then activating the expression of downstream genes in the signaling pathway, such as the increased expression of MMP-7, which has a strong ability to degrade the extracellular matrix, and can degrade the

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

extracellular matrix. In the early stage of fibrosis, ECM synthesis is increased, while the expression of MMP-7 is also increased. The increase of MMP-7 in the early stage is a defense mechanism for renal fibrosis. With the aggravation of renal fibrosis, there are more and more fibrotic cells, while the degradation ability of MMP-7 is weakened, resulting in the reduction of ECM degradation, and more and more MMP-7 will promote the damage of renal tubules. Promoting the development of cell migration, resulting in the destruction of the basement membrane of the renal tubules, further aggravating the progression of renal fibrosis [42].

In the results of this study, Wnt3a/β-catenin signaling pathway of Wnt3a, Wnt4, Wnt9b, GSK-3β, TCF-4, MMP-7 and β-catenin genes and proteins were highly expressed in both in vivo and in vitro model groups. And the expression of E-cadherin gene and protein was significantly decreased. Therefore, we inferred that Wnt3a/β-catenin signaling pathway was abnormally expressed in HSPN kidney tissue. After JHXZ intervention, the expression levels of Wnt3a, Wnt4, Wnt9b, GSK-3β, TCF-4, MMP-7 and β-catenin genes and proteins tended to be normal, while the expression levels of E-cadherin genes and proteins increased. Therefore, we hypothesized that the activation of Wnt/β-catenin pathway is involved in the pathogenesis of HSPN, and JHXZ regulates renal mesangial cells through Wnt/β-catenin pathway to protect the kidney.

6. Conclusion

The limitation of the present experimental study is that the components of traditional Chinese medicine are complex with multiple targets and pathways, and although the components of JHXZHJ have been identified, further research is needed to elucidate the pharmacokinetic study of the components that enter the bloodstream, and at the same time, there may be participation in the pathogenesis of HSPN through other pathways. After the present study, it was found that JHXZHJ may regulate renal mesangial cells through the Wnt/β-catenin pathway, so as to achieve the effect of treating HSPN, and the formula has been used in the clinic for more than ten years, and the observation of nearly 10,000 cases of HSPN children has shown its good clinical efficacy, therefore, the present experiment elucidates one of the important signaling pathways for the treatment of HSPN with JHXZHJ, and provides a certain experimental reference value for the future research. Therefore, this experiment has elucidated one of the important signaling pathways for JHXZHJ treatment of HSPN, and also provided a certain experimental reference value for future research, and if it can be confirmed by clinical sample testing in the future research of JHXZHJ treatment of HSPN, it will greatly improve the credibility, so further research and exploration are needed in the future.

Data availability statement

The original contributions presented in the study are publicly available.

Ethical review

The study was prospectively reviewed and approved by the Ethics Committee of Laboratory Animal Welfare and Ethics of Yunnan University of Traditional Chinese Medicine.The animal experiments in this study were conducted according to the 2006 Guideline of the Chinese Ministry of Science and Technology for the Care and Use of Laboratory Animals.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Niqin Xiao: Conceptualization, Writing - original draft, Complete the experiment,Writing – review & editing. Caixia Zhao: Complete the experiment,Writing-review & editing. Junyu Luo: Complete the experiment,Writing-review & editing. Xianfeng Luo: Writing - review & editing. Qiqi Chang: Writing - review & editing. Jiao Xiong: Writing - review & editing. Min Wang: Complete the experiment, Writing – review & editing. Xi Li: Complete the experiment, Writing – review & editing. Min Zhao:Complete

ISSN 2618-1584 Vol. 6, Issue 8: 1-16, DOI: 10.25236/FMSR.2024.060801

the experiment, Writing – review & editing. Zhifeng Wang: Supervision, Writing. Ping He: Writing – review & editing, Project administration, Funding acquisition.

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