Tropical Journal of Pharmaceutical Research February 2024; 23 (2): 279-289
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v23i2.7

Original Research Article

Effect of Jiawei Tangzhiqing granules on JAK2/STAT3 signal pathway and Th17/Treg ratio in diabetic nephropathy mice

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Sent for review: 18 September 2023 *Revised accepted: 2 February 2024*

Abstract

Purpose: *To investigate the effect of Jiawei Tangzhiqing granules on JAK2/STAT3 signaling pathway and Th17/Treg ratio in diabetic nephropathy (DN) mice.*
Methods: *Selected 60 male SPF C57BL/6N mice, divided into control group and DN model group; the latter was further further split into model control, Western medicine group, and three Jiawei Tangzhiqing granule groups (high, medium, and low doses). Treatments were administered orally for 12 weeks. Key health indicators and renal tissue pathology were analyzed. Th17 and Treg levels in CD4+ T cells were quantified, and JAK2 and STAT3 protein expression in renal tissues was determined via Western blot.*
Results: *Model group showed a significant decrease in body weight and increases in 24-h urine volume, food, and water consumption compared to the control group. Th17 cell count increased, and Treg cell count decreased, leading to a higher Th17: Treg ratio. Conversely, Jiawei Tangzhiqing granules reduced this effect dose-dependently, with the highest dose being more effective than irbesartan. JAK2 and STAT3 protein expressions, elevated in model group, were significantly reduced in the granule-treated groups.*
Conclusion: *Jiawei Tangzhiqing granules alleviate renal damage in DN by suppressing JAK2/STAT3 signaling pathway and correcting the Th17: Treg ratio imbalance. These findings suggest a potential therapeutic role for these granules in managing DN. There is a need to find out if there is a correlation between Th17/Treg balance and JAK2/STAT3 signaling pathway.*

Keywords: *Jiawei Tangzhiqing granules, Diabetic nephropathy, Th17/Treg, JAK2/STAT3 signal pathway*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

In recent times, there has been a marked rise in diabetes cases in China. A range of 20 to 40 % of individuals with diabetes develop diabetic nephropathy (DN) [1]. Currently, there is no effective treatment for DN. Thus, once a large amount of proteinuria appears, the disease progresses rapidly, thereby seriously endangering national health and causing a heavy economic burden at the national and family

levels. Research indicates that immune dysfunction serves as an independent risk factor for diabetic nephropathy (DN). The immune system's activation triggers inflammatory responses, which exacerbate the onset and progression of DN. Th17 and Treg, two critical subtypes of T lymphocytes, play a significant role in this context. The balance between Th17 and Treg is essential for regulating immune responses and controlling inflammation [2,3]. The JAK/STAT signal pathway has a wide range of biological regulatory functions. It participates in cell growth, proliferation and apoptosis, and it regulates the differentiation of helper T lymphocytes such as Th1 and Th17, as well as Treg [4,5].

Jiawei Tangzhiqing granules, an innovative empirical formulation, were developed by Professor Wang Xu. This development draws inspiration from the academic philosophies of renowned Traditional Chinese Medicine master Zhou Zhongying. The formulation is the culmination of Professor Wang's extensive clinical experience garnered over many years. The formulation tonifies the spleen and kidney, resolves phlegm and disperses stasis. An application for a national patent has been made in respect of the granules. The formula consists of *Astragalus*, *Codonopsis*, *Polygonatum sibiricum*, Goji berry, Bombyx Batryticatus, *Sinomenium acutum* and *Lycopus lucidus*. *Jiawei Tangzhiqing* granules has produced good therapeutic effects on diabetes and its complications, especially DN. This study was aimed at investigating the mechanism of action of *Jiawei Tangzhiqing* granules through determining the effect of formulation on JAK2/STAT3 signal pathway and Th17/Treg balance in a mouse model of DN.

EXPERIMENTAL

Animals

For this study, 60 male SPF C57BL/6N mice weighing between 20 and 23 grams was selected. These animals were obtained from Zhejiang Vitonlihua Experimental Animal Technology Co. Ltd. (license no. SCXK (Zhejiang) 2021-0006). The housing conditions for the mice included a controlled environment with a consistent cycle of 12 h of light followed by 12 h of darkness. Ambient temperatures were regulated to stay within 18 to 25 ^{o}C, and humidity levels were maintained at 40 – 50 %. The mice had free access to food and water. One week was allowed for the mice to acclimatize to the laboratory environment before initiating treatment.

Drugs and reagents

The prescription for modified sugar-lipid clearing granule was as follows: 15 g of *Astragalus membranaceus, 15 g* of *Trichosanthes kirilowii* Maxim, and 10 g of each of the following: *Codonopsis pilosula*, *Polygonatum sibiricum*, *Lycium barbarum*, *Bombyx batryticatus*, *Lysimachia christinae*, and *Salvia miltiorrhiza*. The experiment utilized traditional Chinese herbs acquired from Nantong Affiliated Hospital, associated with Nanjing University of Traditional Chinese Medicine. Verification of these herbs was conducted by the hospital's pharmacy department's Deputy Chief Pharmacist. The Irbesartan tablets, 75 mg in dosage, utilized for this study bore the batch number H20030016 and were supplied by Zhejiang Huahai Pharmaceutical Co Ltd. Additionally, the urinary albumin, blood urea nitrogen, and creatinine assay kits were obtained from Nanjing Jiancheng Bioengineering Institute. Kit for JAK2 was purchased from Abcam, UK, while STAT3 Kit was product of Proteintech, USA. Antibody for GAPDH was purchased from Suzhou Siwu Bai Biotechnology Co. Ltd. The ELISA kit for IL-17, CD25 antibody, CD4 antibody, and FoxP3 antibody were bought from eBioscience, USA.

Main instruments

Blood biochemical analyzer was provided by Hitachi Corporation. Flow cytometer, ELISA reader and cell culture incubator were purchased from Thermo Fisher Scientific. Centrifuge and pipette were products of Eppendorf. Wet transfer system and SDS-PAGE electrophoresis apparatus were products of Bio-Rad Laboratories, USA, while laser confocal microscope was bought from Zeiss, Germany.

Ethical statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health [6], and the protocol was approved by the Committee on the Ethics of Animal Experiments of the First Clinical Medical College of Nanjing University of Chinese Medicine (approval no. TCM(2022)004-2).

Procedures

Establishment of animal model, grouping, and drug administration

Sixty male SPF C57BL/6N mice were acclimatized until they exhibited normalized blood glucose levels and showed no proteinuria

indications. Ten mice were allocated randomly to sham group, while the others were prepared for DN model development. These mice were subjected to a fasting period of 12 h before receiving streptozotocin intraperitoneal injection, at a dosage of 80 mg/kg body weight. After 72 h of being fed normally, their fasting blood glucose (FBG) levels were assessed through blood samples collected from the tail vein. The mice, which had been adaptively raised to maintain stable blood glucose levels without exhibiting proteinuria, were utilized in this experiment. A group of ten mice was selected at random to form sham group. The remaining were designated for the creation of DN model. The process involved fasting the mice for 12 h, followed by an intraperitoneal streptozotocin injection, dosed at 80 mg/kg. Post a regular feeding period of 72 h, FBG measurements were taken from blood extracted from the tail vein. A DN diabetes model was deemed successful if FBG reached or exceeded 16.7 mmol/L. After 8 weeks, urine samples from the mice were collected for quantitative urinary protein analysis. A urinary protein level of 30 mg/L or more indicated successful DN model establishment. The DN model mice were then divided into five groups of ten each: the Western medicine group received 25 mg/kg of irbesartan orally, prepared in distilled water at a concentration of 2.5 mg/mL, with an oral volume of 10 mL/kg; the traditional Chinese medicine (TCM) group received a TCM liquid in high, medium, and low doses of 5.2, 2.6, and 1.3 g/kg respectively, also at an oral volume of 10 mL/kg; and the model and sham groups received an equivalent volume of physiological saline instead of medication. All groups were administered their respective treatments daily at a set time, continuing for 12 weeks. Each week, the mice's body weight, urine output, food, and water consumption were recorded.

Assay of biochemical indices

After 12 weeks of treatment, 24-h urine samples were collected from each group of mice on the day before the mice were euthanized. The urine samples were assayed for microalbumin and protein levels. Blood was collected via eye enucleation under anesthesia, and serum was obtained after centrifugation. Automated biochemical analyzers were used to determine serum levels of indicators such as blood glucose, blood lipids, creatinine and blood urea nitrogen.

Renal histopathological examination

In this study, mice renal tissues were harvested under anesthesia induced by chloral hydrate. These samples were then promptly fixed in paraformaldehyde, followed by embedding in paraffin, and sectioned into thin slices. For histological analysis, these sections were stained with Hematoxylin and Eosin (H & E), Masson's trichrome, and Periodic Acid-Schiff (PAS). Observations of the stained kidney tissue sections were made using a microscope to identify any histopathological alterations. Additionally, images of these observations were taken for subsequent analysis and documentation.

Determination of Th17 and Treg

Peripheral blood mononuclear cells were isolated from peripheral blood using a mouse peripheral blood lymphocyte separation kit, and the cell concentration was adjusted to 1 x 10^6 cells/mL. An appropriate cell suspension was placed in a flow cytometry tube and divided equally into two parts which were labeled with Th17 and Treg markers. After staining at room temperature in the dark for 30 min., the cells were washed, centrifuged, resuspended, and then analyzed for Th17 and Treg using a flow cytometer.

Western blot assay

Following a 12-week treatment period, the mice's renal tissues were surgically removed under anesthesia. To extract proteins from these samples, RIPA lysis buffer was employed, and their concentrations were determined using the BCA technique. These proteins underwent separation through SDS-polyacrylamide gel electrophoresis and were later transferred to a nitrocellulose membrane. This membrane was then incubated with primary antibodies against JAK2 and STAT3, at a dilution ratio of 1:2000, and kept at 4 °C overnight. Post incubation, the membrane was washed and subsequently incubated with HRP-conjugated secondary antibodies at a dilution of 1:5000 for 2 h at room temperature. Visualization of the protein bands was achieved with the aid of an ECL kit, followed by an analysis of their grayscale intensities.

Statistical analysis

The data was analyzed using SPSS software, version 23.0. Mean and standard deviation (SD) were employed for descriptive statistical representation. To compare different groups, the study applied One-way Analysis of Variance (ANOVA). For pairwise comparisons among groups, the SNK test was utilized. A p-value less than 0.05 was regarded as statistically significant.

RESULTS

General conditions of mice

After a treatment period of 12 weeks, the mice in model group exhibited a reduction in average body weight and significant increases in urine output, feed consumption, and water intake over 24 h ($p < 0.05$), in contrast to sham group. Conversely, in the irbesartan group and the group treated with high doses of traditional Chinese medicine, there were observed increases in average body weights and reductions in the parameters of 24-h urine output, feed consumption, and water intake, relative to model group (Figure 1).

Changes in blood glucose and lipid levels

After a treatment duration of 12 weeks, data indicated increased fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), total cholesterol (CHO1), and low-density lipoprotein (LDL) levels in model group when contrasted with sham group, demonstrating statistical relevance ($p < 0.05$). On the other hand, significant declines in FBG, HbA1c, CHO1, and LDL were observed in both the valsartan and the high-dose traditional Chinese medicine groups relative to model group ($p < 0.05$; Figure 2).

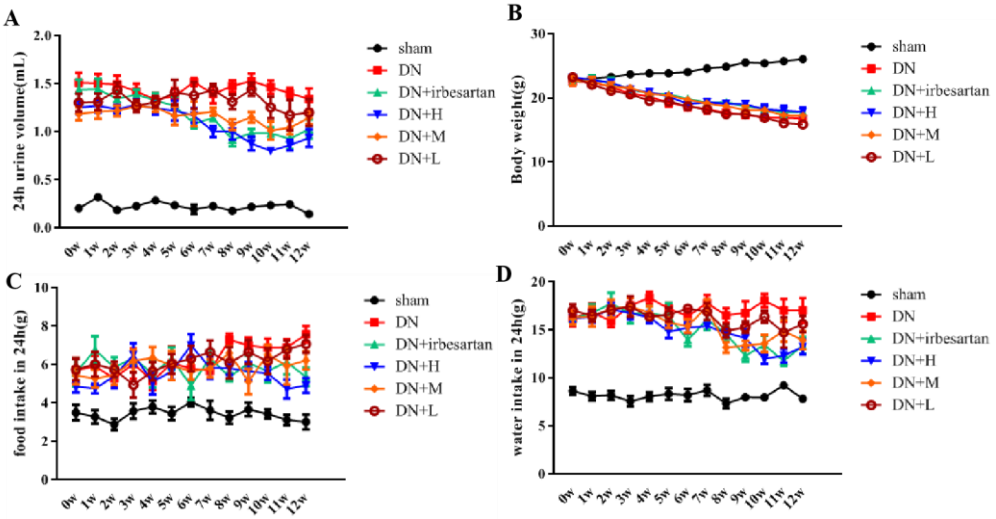

Figure 1: Body weight, 24 h urine output, feed intake and water intake of mice in each group

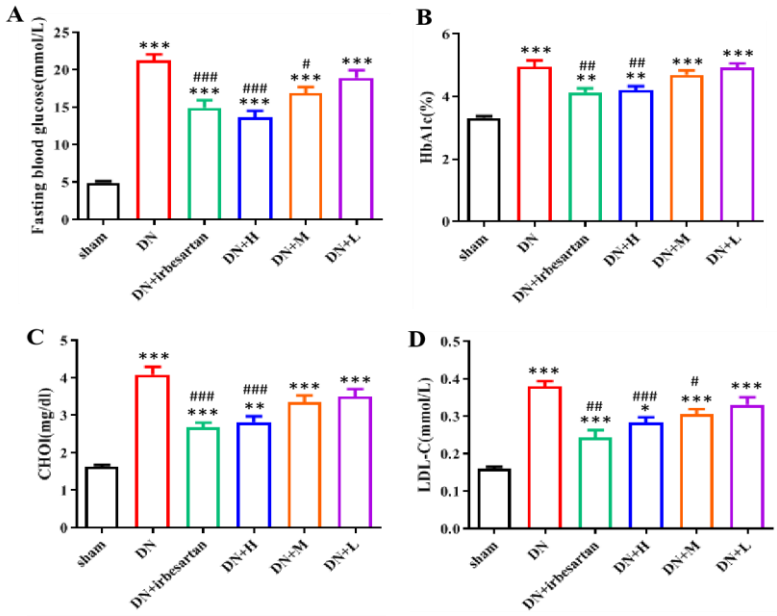

Figure 2: Changes in blood glucose and blood lipids in each group of mice. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. sham group. $^{\#}P < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. model group

Changes in renal function and urinary protein levels

After a 12-week treatment period, mice in model group demonstrated significantly higher CREA, BUN levels than those in sham group. On the other hand, these indices were significantly decreased in both the Irbesartan and the high-dose TCM groups, in comparison to model group (Figure 3).

After H & E staining, kidney tissue of sham group showed normal morphological structure. In contrast, the kidney tissue of model group showed severe pathological changes such as vacuolization of mesangial cells, extensive swelling and rupture of renal tubular lumens, and thickening of the basement membrane. The kidney tissue morphology in the telmisartan group resembled that observed in the high-dose group, characterized by only minor lesions, including slight swelling and occasional rupture of renal tubular lumens, along with mild glomerular congestion. In contrast, the low-dose group displayed more severe kidney damage, such as pronounced vacuolization in mesangial cells and extensive swelling and rupture of renal tubular lumens, though less severe than in model group. The kidney morphology in the medium-dose group fell between the low-dose and high-dose groups, exhibiting mild vacuolization of mesangial cells and minor swelling and rupture in some renal tubular lumens (Figure 4).

In Masson's trichrome-stained sections, a marked escalation in collagen fiber deposition was observed in the renal tissues of model group when juxtaposed with sham group. In contrast, reductions in collagen fiber amounts were noted in both telmisartan and the high-dose TCM groups compared to model group. Notably, the decrease was more significant in the high-dose TCM group, as shown in Figure 5. Utilizing Periodic Acid-Schiff (PAS) staining, it was observed that sham group maintained a normal glomerular architecture, including a clear basement membrane. Model group, however, showed thickened basement membranes and increased staining in the mesangial areas. Reduction in mesangial area staining and near-normalization of basement membrane structures were evident in both the telmisartan and high-dose TCM groups. Notably, the high-dose TCM group demonstrated a more evident reduction in staining compared to the telmisartan group. Meanwhile, the low-dose TCM group showed heightened staining relative to model group. The mesangial area staining intensity in the medium-dose TCM group was intermediate, positioned between the high and low-dose groups (Figure 6).

After a treatment duration of 12 weeks, there was a significant increase in Th17 cell levels and a significant reduction in Treg cell levels in model group, compared with sham group ($p < 0.05$). This change resulted in a higher Th17/Treg cell ratio. In contrast, both the valsartan group and the high-dose TCM group showed a significant decrease in Th17 cells and an elevation in Treg cells relative to model group, which led to a decreased Th17/Treg ratio (Figure 7 and Figure 8).

After a treatment course of 12 weeks, there was a significant increase in the levels of JAK2 and STAT3 proteins within the renal tissues of the mice in model group, in contrast to sham group. However, in the renal tissues of the TCM high-dose group mice, these protein levels significantly decreased compared to model group. Figure 9 illustrates these findings.

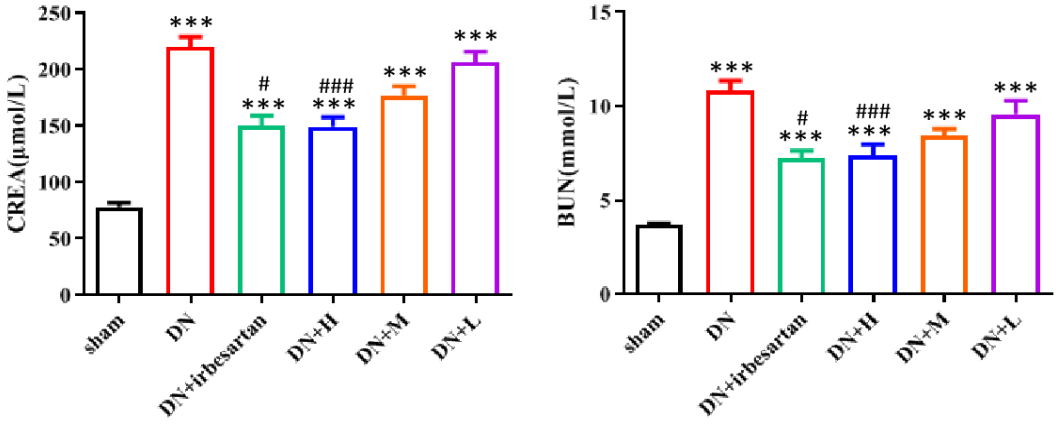

Figure 3: Renal function and urinary protein of mice in each group
*$P < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. sham. $^{\#}P < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. model group

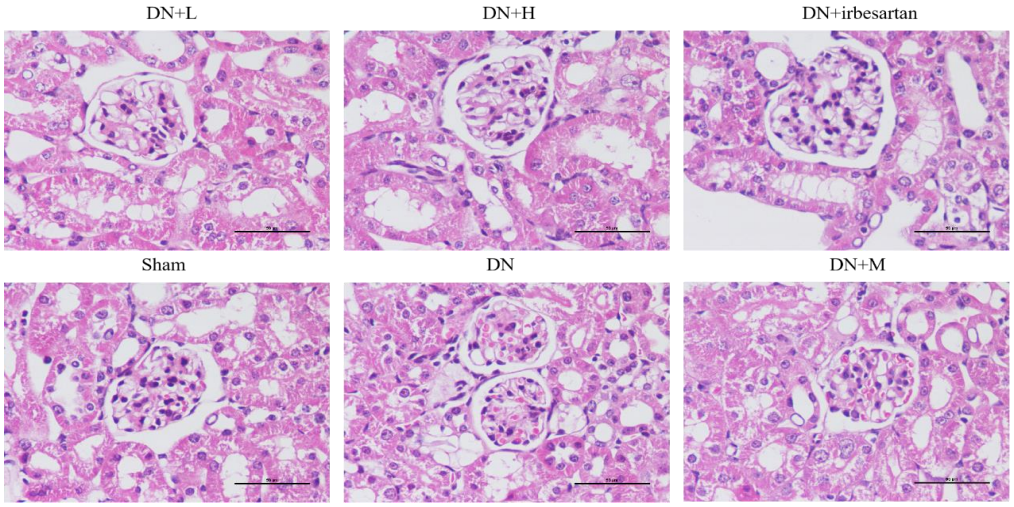

Figure 4: Results of H&E staining of kidney sections from the various groups (×400)

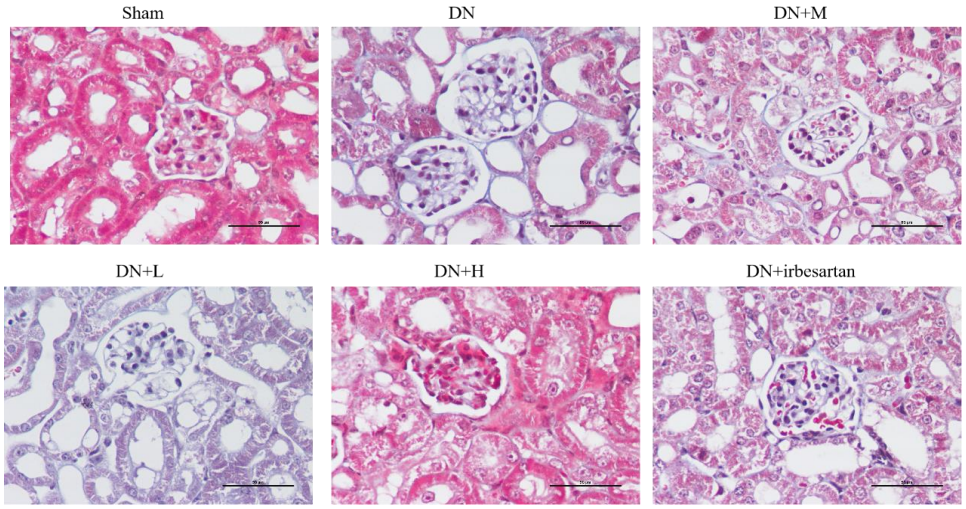

Figure 5: Results of Mason staining of kidney sections from the groups (×400)

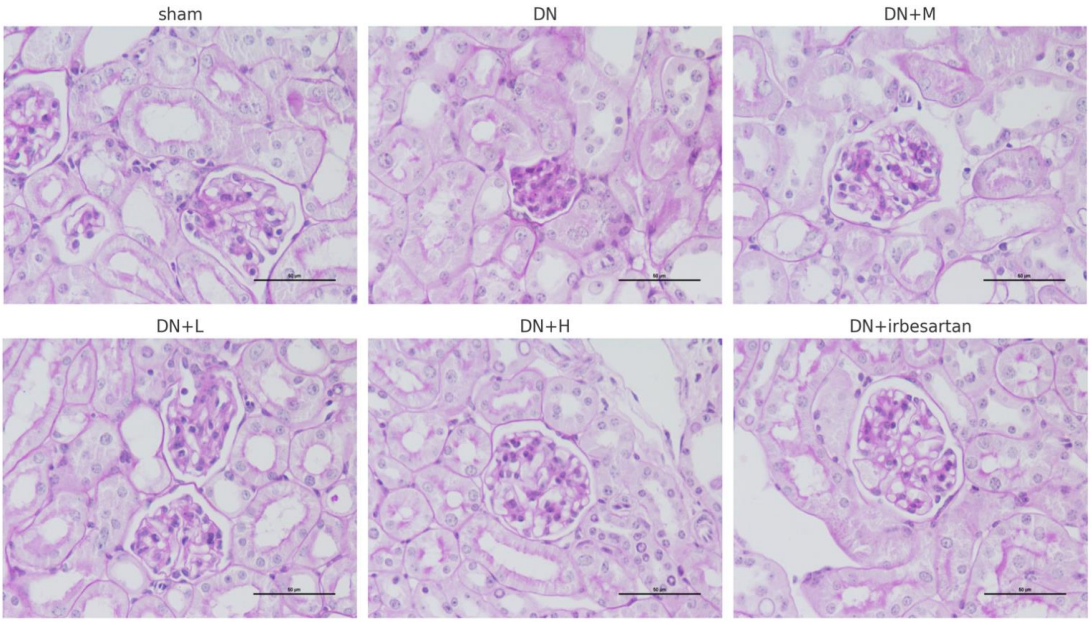

Figure 6: Results of PAS staining of kidney sections from the groups (×400)

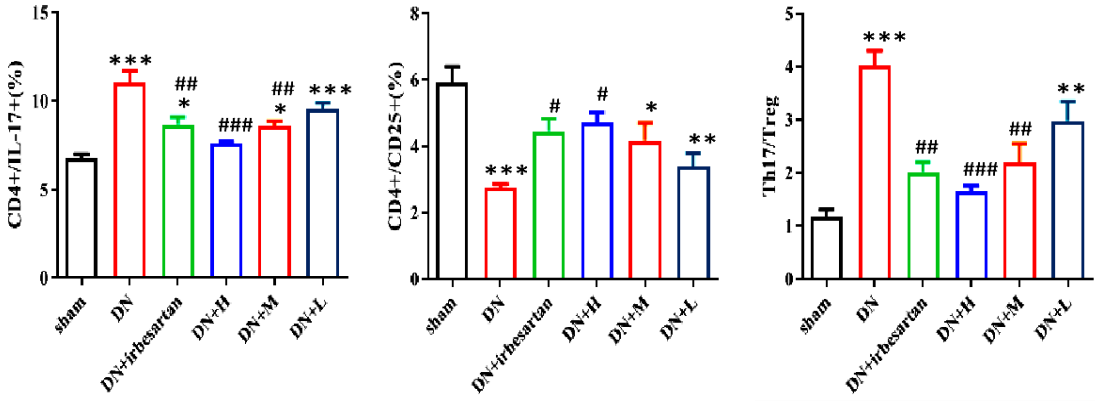

Figure 7: Comparative Analysis of Th17 to Treg Cell Ratios Across Different Study Groups.
*P < 0.05, **p < 0.01, ***p < 0.001, vs. sham group. $^{\#}P$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001, vs. model group

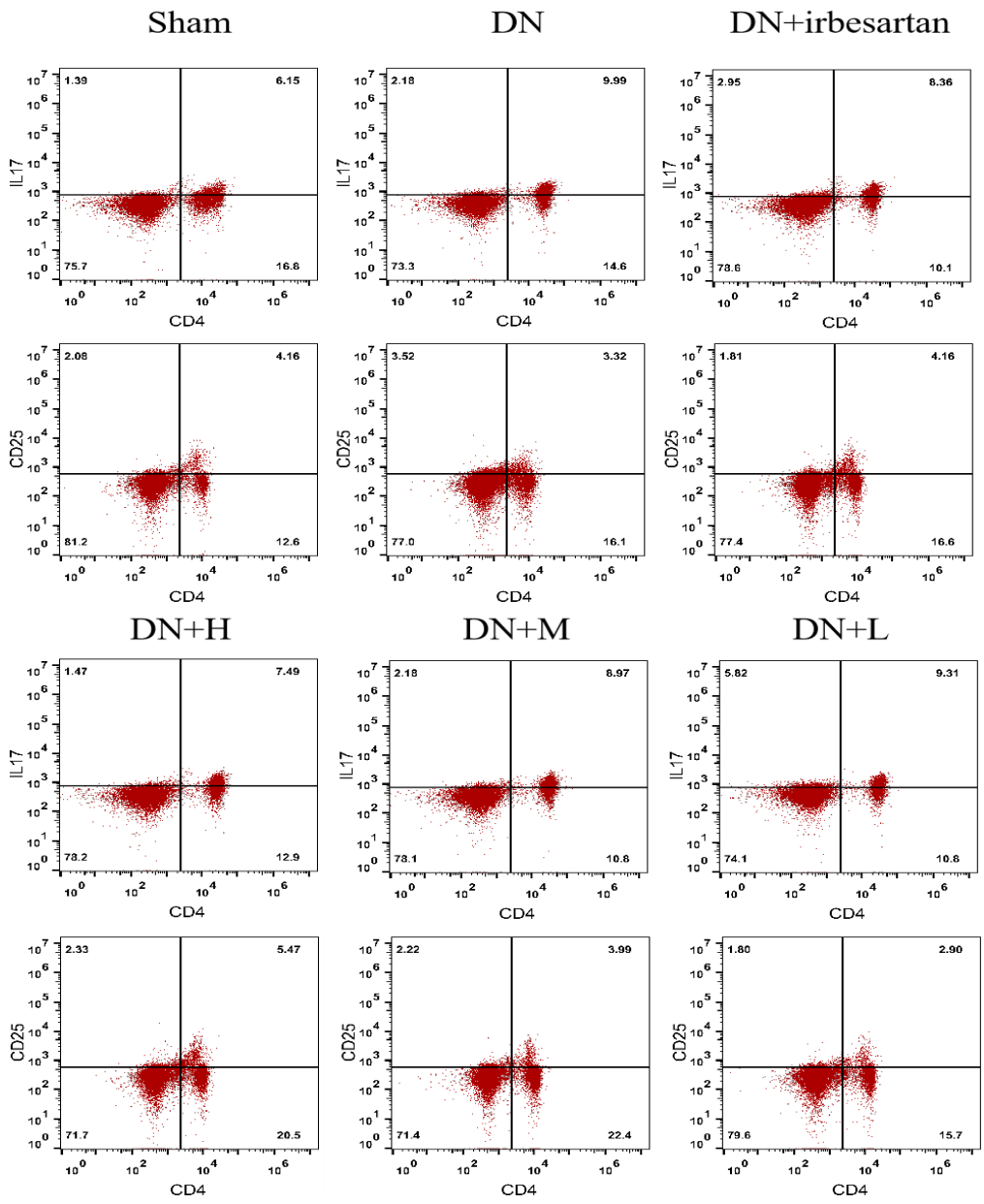

Figure 8: Levels of antibodies on the surface of microglia

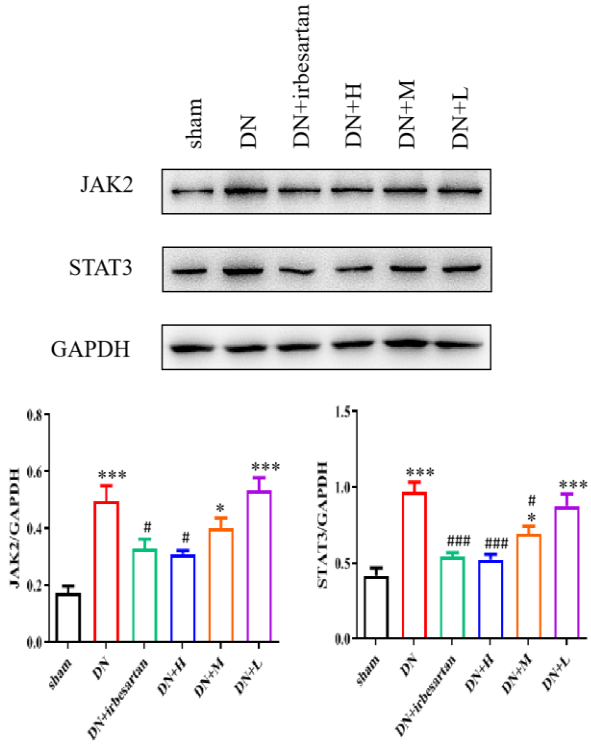

Figure 9: Protein expression levels of JAK2 and STAT3 in the groups. *P < 0.05, ***p < 0.001, vs. sham group. #P < 0.05, ###p < 0.001, vs. model group

DISCUSSION

Diabetic nephropathy (DN) arises from a complex interplay of various factors. This condition is influenced by abnormalities in glucose and lipid metabolism, enhanced oxidative stress, changes in kidney blood flow, buildup of advanced glycation end products (AGEs), genetic factors, and the involvement of diverse cytokines functioning through endocrine, autocrine, and paracrine pathways [7]. It stands as a primary cause of end-stage renal disease, significantly affecting the prognosis and life quality of patients [8]. Consequently, prompt intervention, precise diagnosis, and effective management are imperative in slowing the progression of DN. Traditional Chinese Medicine (TCM), with its rich historical background in treating DN, is increasingly acknowledged by scientific research, offering a range of prospective treatments. In this study, the utilized Tangzhiqing granules were a blend of Astragalus membranaceus, Codonopsis pilosula, Polygonatum sibiricum, Lycium barbarum, Bombyx batryticatus, Sinomenium acutum, Lysimachia christinae, and Salvia miltiorrhiza. Prior research has indicated the efficacy of this combination in addressing diabetes and its related complications via multiple mechanisms [9]. This study's objective was to explore how Tangzhiqing granules alleviate DN and decrease proteinuria.

In the treatment cohort, model mice group exhibited a reduction in body mass and a marked escalation in urine output over a 24-h period, alongside heightened consumption of both feed and water post a 12-week therapy period, relative to the sham control. Conversely, the mice receiving a high dose of Traditional Chinese Medicine demonstrated an upturn in body mass coupled with a decline in both 24-h urine production and their intake of feed and water when measured against model group. In addition, the levels of FBG, HbA1c, CHOl, LDL, CREA, BUN, mALB and 24 h UTP were decreased significantly, indicating that the modified sugar- and fat-clearing granules mitigated disorders in glucose and lipid metabolism, protected kidney function, and reduced proteinuria [10]. Results from histology suggest that at high-dose, the granules significantly reduced DN-induced pathological changes, and alleviated DN-mediated cell dysfunction and structural damage such as glomerular basement membrane thickening and renal tissue fibrosis [11]. The study found that abnormal infiltration and activation of T lymphocytes in renal tissue are potential immune system-related mechanisms involved in DN [12]. Activated T lymphocytes not only directly produce cytotoxic effects but also indirectly recruit or activate macrophages, leading to kidney damage. The Th17 and Treg cells are two important types of T lymphocytes. A balance in Th17: Treg ratio regulates immunity and inflammation, thereby playing an important role in the occurrence and development of diseases [13]. The Th17 cells represent a vigorously active subset of T lymphocytes, becoming pathogen-reactive through stimulation by cytokines like IL-6, IL-1β, IL-23, and TNF-β. Notably, Th17 cells produce IL-17A, a cytokine that amplifies chemokine expression within T cells and exacerbates conditions such as proteinuria as well as renal interstitial fibrosis. In addition, Toll-like receptor 4 secreted by Th17 activates Th17, thereby further aggravating kidney damage. On the other hand, Treg cells are critical to the maintenance of immune tolerance and protection against immune damage in DN. Treg cells protect the kidney by limiting the pro-inflammatory environment and by induction of the differentiation of monocytes to M2 macrophages which reduce inflammation and repair damaged tissue.

Research has demonstrated a rise in Th17 cell concentrations coupled with a reduction in Treg cell numbers in Diabetic Nephropathy (DN)

patients. This results in a significantly elevated Th17:Treg cell ratio. Such a ratio has been found to correlate directly with the severity of renal impairment in DN. Furthermore, another study highlighted a direct positive association between the Th17/Treg ratio and urinary microalbumin/creatinine ratio in individuals with DN, while showing an inverse relationship with Treg cell count. This further strengthens the link between Th17/Treg imbalance and DN pathology. Examination results indicated a proliferation of Th17 cells along with a decline in Treg cells in the experimental group, leading to an increased Th17:Treg ratio. Traditional Chinese Medicine (TCM) treatments, administered in different dosages, showed therapeutic effects. These were marked by a reduction in Th17 cell proportions, an increase in Treg cell quantities, and a lower Th17:Treg ratio. Notably, a higher dose of modified sugar- and fat-clearing granules demonstrated superior efficacy compared to irbesartan.

The JAK/STAT signaling pathway is known for its pivotal role in regulating cellular activities such as proliferation, differentiation, inflammation, and apoptosis. The importance of JAK2 and STAT3 in the progression of DN has been emphasized in various studies [13]. High glucose levels, ROS, and advanced glycation end-products are factors that activate JAK2 and STAT3, leading to increased phosphorylation and nuclear translocation, thereby influencing downstream genes like Bax and Bcl-2, and accelerating DN development. Diabetic rat models have shown significant upregulation in renal expressions of p-JAK2 and p-STAT3, as well as in the p-JAK2:JAK2 and p-STAT3:STAT3 ratios. Concurrently, there was an increase in Bax expression and a decrease in Bcl-2 levels [14]. TCM applications have been observed to reduce apoptosis in DN-affected renal tissues by inhibiting JAK2/STAT3 pathway. It is known that high glucose levels activate the JAK/STAT pathway via ROS, which promotes the growth of glomerular mesangial cells. Additionally, reports indicate that renal administration of SOCS2 adenovirus lessened renal impairment in a STZ-induced DN rat model, observable in the reduction of glomerular hypertrophy, inflammation, and fibrosis [15]. SOCS2 gene transfection notably decreased the renal protein expression levels of p-JAK2, p-STAT1, and p-STAT3, along with pro-inflammatory (MCP-1, IL-6, TNF-α) and pro-fibrotic (TGF-β1, type IV collagen, and FN) factors in DN rat kidneys [15]. Therefore, targeting JAK2/STAT3 signaling pathway to inhibit its activation could be a potential strategy to decelerate the progression of Diabetic Nephropathy (DN). Findings from the current study revealed increased protein expression of JAK2 and STAT3 in DN model group when compared to the control group. However, Jiawei Tongzhi Qingke granules significantly and dose-dependently reduced the protein levels of JAK2 and STAT3 compared to those in model group, suggesting their potential therapeutic impact on DN through modulating this crucial pathway.

Pharmacological studies have shown that the main bioactive components of *Jiawei Tangzhi Qing* granules ameliorate DN. *Astragalus* polysaccharides protect the kidneys from DN-induced renal injury by reducing renal tissue fibrosis, regulating disorders in lipid metabolism, and reducing oxidative stress response [16]. *Codonopsis* polysaccharides inhibit inflammatory reactions and suppress renal fibrosis. *Polygonatum sibiricum* polysaccharides significantly ameliorate clinical symptoms of diabetes and inhibit oxidative stress response [17]. *Lycium barbarum* not only regulates glucose and lipid metabolism but also exerts anti-oxidative stress and anti-inflammatory effects. *Bombyx mori* polysaccharides control blood sugar, lower lipid levels, and exert anti-coagulant effects [18]. *Gastrodia elata* enhances insulin secretion and reduces insulin resistance and blood rheology. Radix Lateralis Aconiti Praeparata lowers blood lipid levels, decreases blood viscosity and improves microcirculation. *Danshensu* exerts effects such as regulation of disorders in calcium and phosphorus metabolism, in addition to anti-fibrotic and anti-inflammatory effects, and clearing of oxygen free radicals [19].

Limitations of the study

Firstly, the study focused only on JAK2/STAT3 signal pathway. Other signal pathways may also be involved in the process of treating DN with this herbal formula. Secondly, the mechanism underlying the use of *Jiawei Tangzhi Qing* granules in DN treatment needs to be further verified in *humans*.

CONCLUSION

Jiawei Tangzhi Qing granules significantly alleviate disorders in glucose and lipid metabolism in DN mice, protect kidney function, and reduce proteinuria through inhibition of JAK2/STAT3 signal pathway and regulation of the balance between Th17 and Treg. There is need to find out if there is a correlation between Th17/Treg balance and JAK2/STAT3 signaling pathway.

DECLARATIONS

Acknowledgements

None provided.

Funding

Nantong Science and Technology Bureau Basic Science Research Program - Young Scientific and Technological Talent Innovation Special Project (No. JC12022084).

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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