

Tangshen formula improves inflammation in renal tissue of diabetic nephropathy through SIRT1/NF- κ B pathway

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Abstract. The present study investigated the mechanism underlying the anti-inflammatory effects of Tangshen formula (TS) in Sprague Dawley (SD) rats with diabetic nephropathy (DN). A rat model of DN was established by intraperitoneal injection of 1% (40 mg/kg) streptozotocin and administration of a high fat and glucose diet. Subsequently, SD rats were randomly divided into six groups (n=8): A DN group, a valsartan group, a high-dose TS group, a middle-dose TS group, a low-dose TS group and a control group with normal SD rats. Once rats received their allocated treatment for 12 weeks, body weight and kidney weight were recorded, and fasting blood glucose, ratio of urinary protein, β_2 -MG and creatinine clearance rate were determined. Furthermore, hemodynamic indices, including plasma viscosity and whole blood reduction viscosity were detected. Immunohistochemistry was used to detect the infiltration of macrophages in the kidneys of rats. Reverse transcription-quantitative polymerase chain reaction and western blotting were performed to investigate the activation; mRNA and protein expression levels of monocyte chemoattractant protein-1 (MCP-1), macrophage migration inhibitory factor (MIF), nuclear factor- κ B (NF- κ B) and sirtuin-1 (SIRT1) in each group. In comparison with the DN group, each biochemical indicator of rats in the high-dose TS group was significantly decreased (P<0.05). Blood viscosity in each treatment group was significantly decreased when compared with the DN group (P<0.01). Hematoxylin and eosin staining indicated that the infiltration of macrophages was significantly

decreased in the high-dose TS group when compared with the DN group (P<0.01). mRNA and protein expression levels of MCP-1 and MIF in the high-dose TS group were significantly decreased when compared with the DN group (P<0.05). In the treatment groups, SIRT1 mRNA expression levels were significantly increased, whereas the mRNA expression levels of NF- κ B were significantly decreased (P<0.01). Western blotting results indicated a significant decrease in the protein expression levels of acetylated NF- κ B in the treatment groups when compared with the DN group (P<0.01) and the propensity of protein expression of the other inflammatory factors were consistent with the mRNA findings. The results of the high-dose TS group were similar to those of the valsartan group. The present study indicates that TS was able to activate SIRT1, which lead to NF- κ B deacetylation, thus reducing the release of inflammatory factors and decreasing the severity of diabetic nephropathy.

Introduction

Diabetic nephropathy (DN) is the most common and serious complication of diabetes (1). China has around 400 million diabetic patients and 20-30% of these progress to DN, and 20-40% of patients with DN develop end-stage renal disease, which is severely harmful to human health (2). Western researchers have indicated that the pathogenesis of DN and glucose metabolism are predominantly caused by non-enzymatic glycation, polyol pathway activation, protein kinase C activation, dyslipidemia, hypertension, renal hemodynamic changes, oxidative stress, vasoactive substances, cytokines and several other genetic factors (3). However, researchers of traditional Chinese medicine believe that diabetic nephropathy belongs to the category of diabetes disease (4). In traditional Chinese medicine, it is believed that the pathogenesis of dryness and heat is due to a deficiency of Yin. Yin deficiency is the 'root' cause of the disease, while dryness and heat are the symptoms (5). A prolonged state of Yin deficiency may result in deficiency of both Qi and Yin, as well as a deficiency of Yin and Yang (4). This phenomenon may also cause incidental symptoms, including blood stasis, phlegmatic hygrois and turbidity toxin. Blood stasis or blood stagnation is an important underlying pathology of many diseases according to Traditional Chinese Medicine. It is described as a slowing or pooling of the blood due to disruption of the heart and is often

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Abbreviations: DN, diabetic nephropathy; DM, diabetic mellitus; PKC, protein kinase C; FBG, fasting blood glucose; UAER, urinary albumin excretion rate; Ccr, creatinine clearance rate

Key words: diabetic nephropathy, Tangshen formula, macrophages, nuclear factor- κ B, sirtuin

understood in biomedical terms as hematological disorders, including hemorrhage, congestion, thrombosis and local ischemia (microclots) or tissue changes (6). Phlegmatic dampness is created by impaired digestion and is primarily located in the lungs and large intestine (7). When the dampness is stored in the large intestine, it may lead to mucus-lined, loose or sticky stools that are difficult to clean up after or diarrhea with undigested bits of food (8). The Essential Pathogenesis of Diabetes Mellitus in traditional Chinese medicine refers to turbidity toxin as excess nutrition and the absorption of incompletely digested food, including protein and fat (9). It accumulates gradually to form a sticky sputum and ultimately the aggregated particles form a large block. This may lead to inflammation and suppuration, and may affect the transport and metabolism of nutrients (10). This mechanism is somewhat similar to the formation of atherosclerosis in Western medicine (4). Western medical practices have adopted various methods for controlling high blood glucose, blood pressure, low-protein diet, correcting lipid metabolism disorders and alternative symptomatic treatment for treating DN; however, none of these approaches are completely effective in preventing the progression of DN (11,12). The current study of traditional Chinese medicine for the treatment and prevention of DN is primarily based on compound prescriptions and treatment is performed using Tangshen capsules and Tangshenkang (13), which, in traditional Chinese medicine are believed to be beneficial in supplementing Qi, nourishing Yin, promoting circulation and removing stasis. Tong Luo Yi Shen Zi Yin prescription is used as an alternative treatment that has a key purpose in promoting blood circulation to remove obstructions in the blood vessels and to improve the blood supply to the kidneys (14). Chinese medicinal treatment applies multi-channel and multi-target activities to achieve positive and effective therapeutic effects, thus the application of traditional Chinese medicine has great potential in the prevention of DN.

According to the traditional Chinese medicine theory of Professor Kefu Chai, it is believed that the basic pathogenesis of early- and medium-stage DN is the deficiency of both Qi and Yin, which results in blood stasis (15). Previous results have indicated that a Chinese herbal compound prescription, Tangshen (TS) formula, has demonstrated good clinical efficacy in managing early-stage DN, as it is believed in traditional Chinese medicine that it supplements Qi, nourishes Yin, improves blood flow and removes obstructions in the blood vessels (16). In the present study, the molecular mechanisms of TS formula in a DN rat model were analyzed, providing a deeper insight into the effects of TS formula in the treatment of DN.

Materials and methods

Animals. A total of 50 specific pathogen-free male Sprague Dawley rats (age, 6–8 weeks; body weight, 160–200 g) were purchased from Shanghai Sippr-BK Laboratory Animal Co., Ltd. [Shanghai, China; (certificate of approval, SCXK (Shanghai); 2008-0016)]. Rats were bred in the Research Center of Zhejiang Chinese Medical University (Zhejiang, China), with the experimental license number of SYXK 2013-0184 (clean grade). Rats were maintained in the following experimental conditions: A 12-h light-dark cycle; a maintained

temperature of 18–22°C with a relative humidity of 40–70%; and access to food and water *ad libitum*. All animal experiments were approved by Zhejiang Chinese Medical University Animal Ethics Committee (Zhejiang, China).

Pharmacological agents. TS formula for one human body daily dosage is constituted of six different Chinese herbs, including *Radix puerariae* (15 g), *astragalus* (18 g), *Ligustrum lucidum* (15 g), *Lucid ganoderma* (15 g), *Salvia miltiorrhiza* (9 g) and *Rheum rhabarbarum* (6 g), which were all purchased from the Pharmacy Department of Zhejiang Provincial Hospital of Traditional Chinese Medicine (Zhejiang, China). Once herbs were combined, water (v/w) at eight times of the herbs weight was added. Subsequently, the medicinal herbs were decocted on a slow fire for 1 h and the remaining fluid was collected. The remaining pharmacological agents were continuously processed by adding water (v/w) six times for a further 1 h on the slow fire. The two remaining fluids were merged and the compounds were condensed at a final concentration of 2 g/ml and stored at 4°C. The positive agent, valsartan, was purchased from Beijing Pharma Ltd. (Beijing, China). Valsartan was dissolved in sodium carboxymethyl cellulose.

Reagents and instruments. Streptozotocin (STZ) was purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany) and the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) iTaq™ Universal SYBR® Green One-Step kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The primary antibody of monocyte chemoattractant protein-1 (MCP-1; sc-1785; 1:800), macrophage migration inhibitory factor (MIF; sc-20,121; 1:800), nuclear factor-κB (NF-κB; sc-372; 1:500), sirtuin-1 (SIRT-1; sc-15,404; 1:300) and β-actin were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The IgG-horse radish peroxidase (1:3,000; cat no. M209180) was obtained from Pierce (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Hemorheology was performed using a 7020-automatic biochemical analyzer (Hitachi, Ltd., Tokyo, Japan). Western blotting and PCR were performed using a T Personal thermocycler (Biometra GmbH, Göttingen, Germany) and Mini-PROTEAN electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Establishment of DN model and treatment. Prior to experiments, a total of 50 SD rats were fed for 1 week for adaptation and urine protein and blood glucose levels were detected. A total of 3 rats exhibited abnormal results and were subsequently sacrificed. The remaining 47 SD rats were intraperitoneally injected with 1% STZ at 40 mg/kg and fed high-fat glucose fodder, containing 10% egg yolk powder, 10% lard, 10% sucrose, 0.5% cholesterol and basic feed. This fodder was processed and disinfected by the Zhejiang Academy of Medical Sciences (Zhejiang, China). Another 8 rats were injected with an equal volume of vehicle (0.1 M citrate buffer; pH 4.2) to represent the normal control. A total of 72 h after the injection, tail vein blood was collected and serum glucose was detected in all rats. The diabetic rat model was successfully established with serum glucose ≥ 16.7 mmol/l. Diabetic rats were randomly divided into groups: Diabetic nephropathy group (DN group; n=8), valsartan group (valsartan group; n=8), and low-, medium- and high-dosage of TS formula

groups (all n=8). Rats injected with vehicle were considered as the normal control group (n=8). Rats of the valsartan group were treated with valsartan solution (25 mg/kg/day) and the rats in the TS formula groups were treated with TS formula, 6.5 g/kg/day (low dose), 13 g/kg/day (medium dose) and 26 g/kg/day (high dose), respectively. The rats in these groups were observed for 12 weeks; 12 weeks following treatment, all rats were sacrificed and the renal cortex was collected for pathological observation and further investigation.

Specimen collection. Following treatment, 24-h urine collection was performed. Under empty stomach conditions, 3% pentobarbital sodium (Sigma-Aldrich; Merck Millipore; cat no. P376; 45 mg/kg) anesthesia was administered to acquire serum, plasma and whole blood samples. Rat kidneys were weighed and either stored in 10% formalin solution for pathological detection or stored at -80°C for protein and mRNA detection.

Blood and urine biochemical index determination. The 24-h urine collected was centrifuged at 1,200 g/min for 10 min at 4°C and the supernatant was obtained. The automatic biochemical analyzer tested the blood for glucose, blood urine creatinine and serum creatinine levels and the endogenous creatinine clearance rate was calculated. The micro albuminuria and β 2-microglobulin (β 2-MG) levels were detected using the Rat β 2-microglobulin, BMG/ β 2-MG (Sangon Biotech Co., Ltd., Shanghai, China; cat no. D730498) and micro albuminuria ELISA kits (Wuhan Elabscience Biotechnology Co., Ltd., Wuhan, China; cat no. E-EL-R0025). Hemorheology indices, including blood viscosity and whole reduced blood viscosity, were determined using an automatic blood rheometer.

Hematoxylin and eosin (H&E) and periodic Schiff reaction (PAS) to observe pathological changes. Kidney tissues were fixed in 10% neutral formalin at 4°C overnight for dehydration. Tissues were heated at 120°C for 10 min in an autoclave for antigen retrieval and then dehydrated in an ascending grade of ethanol, washed with xylene and embedded in paraffin. The paraffin-embedded samples were cut into sections 3 μ m thick for histopathological examination. Tissue sections were de-paraffinized in xylene and stained with H&E for 20 min at room temperature. For PAS staining, paraffin slides were de-paraffinized, rehydrated and washed in water. Slides were placed in 2% Periodic Acid for 15 min and stained with Schiff's reagent for 25 min at room temperature. Counter-staining with filtered hematoxylin was then conducted. Following washing three times, slides were rapidly dipped in acid alcohol twice and slowly dipped in ammonia four times. Slides were then dehydrated and cover slipped. The glomerular, renal tubular structure and matrix hyperplasia were studied under a light microscope using H&E and PAS staining.

Infiltrating macrophages in renal tissues. EnVision two-step method was used for immunohistochemical staining. Following antigen retrieval, the sections were incubated with cluster of differentiation (CD) 68 primary antibody (1:50) at 4°C overnight. Samples were washed with PBS and incubated with EnVision working solution for 30 min. DAB and hematin

Table I. Changes in weight, kidney weight and kidney hypertrophy index following treatment.

Groups	N	Weight (g)	Kidney weight (g)	Kidney hypertrophy index ($\times 10^{-3}$)
Control	8	541.25 \pm 23.81	3.19 \pm 0.15	5.94 \pm 0.71
DN	8	291.62 \pm 14.58 ^a	3.85 \pm 0.07 ^a	13.24 \pm 0.82 ^a
TS ^{Low}	8	335.28 \pm 18.22	3.66 \pm 0.11 ^b	10.93 \pm 0.67 ^b
TS ^{Med}	8	346.63 \pm 10.64	3.50 \pm 0.09 ^b	10.10 \pm 0.35 ^b
TS ^{Hi}	8	362.25 \pm 22.68 ^b	3.49 \pm 0.15 ^b	9.66 \pm 0.3 ^b
Valsartan	8	355.25 \pm 17.94 ^b	3.48 \pm 0.07 ^b	9.81 \pm 0.58 ^b

^aP<0.05 vs. control group; ^bP<0.05 vs. DN group. Data are presented as the mean \pm standard error of the mean. TS, Tangshen; TS^{Low}, low dose (6.5 g/kg/day) of Tangshen formula; TS^{Med}, medium dose (13 g/kg/day) of Tangshen formula; TS^{Hi}, high dose of Tangshen formula (26 g/kg/day) of Tangshen formula; Valsartan, 25 mg/kg/day of valsartan; DN, diabetic nephropathy.

Table II. Changes in biochemical criterion following treatment (n=8).

Group	FBG (mmol/l)	UAER (μ g/min)	β 2-MG (ng/ml)	CCR
Control	9.25 \pm 0.98	0.28 \pm 0.09	45.02 \pm 2.79	121.25 \pm 6.57
DN	30.08 \pm 2.89 ^a	2.83 \pm 0.70 ^b	65.8 \pm 3.39 ^a	69.79 \pm 3.03 ^b
TS ^{Low}	28.96 \pm 2.61	2.39 \pm 0.31	59.15 \pm 3.6	80.00 \pm 2.09
TS ^{Med}	29.52 \pm 2.93	2.19 \pm 0.25 ^c	57.57 \pm 3.64	84.80 \pm 3.48
TS ^{Hi}	24.63 \pm 2.78 ^d	1.79 \pm 0.33 ^c	55.19 \pm 3.28 ^c	88.52 \pm 3.55 ^c
Valsartan	25.56 \pm 2.18 ^d	1.99 \pm 0.33 ^c	56.15 \pm 5.03	90.76 \pm 5.42 ^c

^aP<0.01 and ^bP<0.05 vs. control group; ^cP<0.05 and ^dP<0.01 vs. DN group. Data are presented as the mean \pm standard error of the mean. TS, Tangshen; TS^{Low}, low dose (6.5 g/kg/day) of Tangshen formula; TS^{Med}, medium dose (13 g/kg/day) of Tangshen formula; TS^{Hi}, high dose of Tangshen formula (26 g/kg/day) of Tangshen formula; Valsartan, 25 mg/kg/day of valsartan; DN, diabetic nephropathy; FBG, fasting blood glucose; UAER, urinary albumin excretion rate; CCR, creatinine clearance rate.

counterstaining were performed. Images were captured from five random fields under a light microscope (magnification, \times 400).

RT-qPCR for detecting mRNA expression levels. Total RNA was extracted from rat kidney tissue using TRIzol total RNA isolation reagent (Gibco; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed using the RT-PCR kit followed by qPCR detection. Primer sequences were as follows: SIRT1 (306 bp), forward 5'-CCCATTTCGGTTTCCATTGTA-3' and reverse 5'-TCATCA GCCAACAGGAGGTT-3'; NF- κ B (302 bp), forward 5'-TGC CTCCAGTGAGAAGAACA-3' and reverse 5'-GCACCAGAA GTCCAGGGTTA-3'; MCP-1 (302 bp), forward 5'-CAGCAG

Table III. Effect of TS formula on hemodynamic indices (n=8).

Group	Blood viscosity	Whole blood high shear reductive viscosity	Whole blood medium shears reductive viscosity	Whole blood low shear reductive viscosity
Control	0.9±0.6	3.33±1.06	3.87±1.19	6.95±1.93
DN	1.55±0.18 ^a	9.12±1.38 ^a	10.23±1.53 ^a	16.32±2.32 ^a
TS ^{Low}	1.17±0.22 ^b	5.7±2.21 ^b	6.99±2.82	12.93±3.55 ^b
TS ^{Med}	1.05±0.08 ^b	6.12±0.59 ^b	8.24±5.52	12.73±1.01 ^b
TS ^{Hi}	1.03±0.08 ^b	5.17±1.45 ^b	5.93±1.62 ^c	10.4±2.59 ^b
Valsartan	1.15±0.22 ^b	5.34±1.32 ^b	6.42±1.52	13.85±2.98 ^b

^aP<0.01 vs. control group; ^bP<0.01 and ^cP<0.05 vs. DN group. Data are presented as the mean ± standard error of the mean. TS, Tangshen; TS^{Low}, low dose (6.5 g/kg/day) of Tangshen formula; TS^{Med}, medium dose (13 g/kg/day) of Tangshen formula; TS^{Hi}, high dose of Tangshen formula (26 g/kg/day) of Tangshen formula; Valsartan, 25 mg/kg/day of valsartan; DN, diabetic nephropathy.

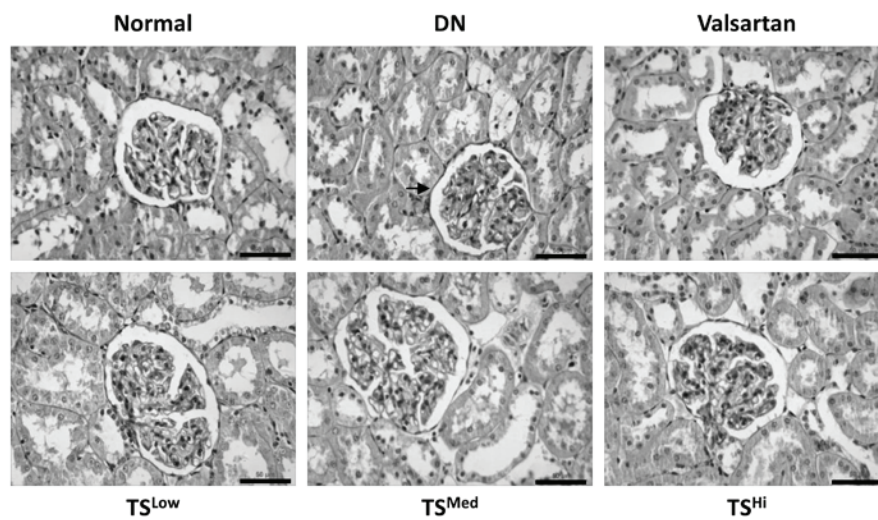


Figure 1. Periodic Schiff reaction staining of the pathological changes in the kidneys of rats in the control group, DN group, TS^{Low} group, TS^{Med} group, TS^{Hi} group and valsartan group. The arrow indicates the thickened basement membrane in the DN group. Scale bar, 50 μ m. TS, Tangshen; TS^{Low}, low dose (6.5 g/kg/day) of Tangshen formula; TS^{Med}, medium dose (13 g/kg/day) of Tangshen formula; TS^{Hi}, high dose of Tangshen formula (26 g/kg/day) of Tangshen formula; Valsartan, 25 mg/kg/day of valsartan; DN, diabetic nephropathy.

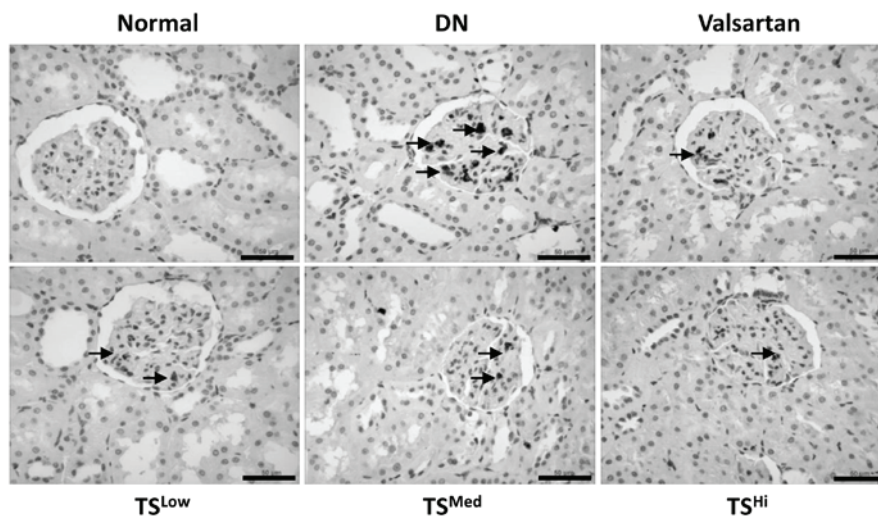


Figure 2. Envision method immunohistochemical staining of the expression of macrophages in the control group, DN group, TS^{Low} group, TS^{Med} group, TS^{Hi} group and valsartan group. Arrows indicate the infiltrated macrophages in different group. Scale bar, 50 μ m. TS, Tangshen; TS^{Low}, low dose (6.5 g/kg/day) of Tangshen formula; TS^{Med}, medium dose (13 g/kg/day) of Tangshen formula; TS^{Hi}, high dose of Tangshen formula (26 g/kg/day) of Tangshen formula; Valsartan, 25 mg/kg/day of valsartan; DN, diabetic nephropathy.

Table IV. Microphage infiltration in different groups.

Group	N	Microphage infiltration (count per field, x400)
Control	8	0.93±0.28
DN	8	8.80±1.16 ^a
TS ^{Low}	8	5.47±0.79 ^b
TS ^{Med}	8	5.33±0.69 ^b
TS ^{Hi}	8	4.1±0.52 ^b
Valsartan	8	6.47±0.61 ^c

^aP<0.01 vs. control group; ^bP<0.01 and ^cP<0.05 vs. DN group. Data are presented as the mean ± standard error of the mean. TS, Tangshen formula; TS^{Low}, low dose (6.5 g/kg/day) of Tangshen formula; TS^{Med}, medium dose (13 g/kg/day) of Tangshen formula; TS^{Hi}, high dose of Tangshen formula (26 g/kg/day) of Tangshen formula; Valsartan, 25 mg/kg/day of valsartan; DN, diabetic nephropathy.

GTGTCCCAAAGAAG-3' and reverse 5'-AGGCATCACATT CCAAATCAC-3'; MIF-1 (180 bp), forward 5'-CAGAACCGC AACTACAGCAA-3' and reverse 5'-CAGCAAGACTCGAAG AACAGC-3'; and GADPH (91 bp), forward 5'- GTGCTGAGT ATGTCGTGGAG-3' and reverse 5'-GTCTTCTGAGTGGCA GTGAT-3'. The RT-qPCR system contained: Sense primer 0.5 µl (10 µM), antisense primer 0.5 µl (10 µM), cDNA (1 µl), double distilled H₂O (8 µl) and SYBR Supermix from the iTaq™ Universal Syber green One-Step kit (10 µl). Reactions were incubated at 95°C for 2 min followed by 95°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec, for 40 cycles. Expression levels of mRNA were quantified by the 2^{-ΔΔC_q} method (8).

Western blot analysis. Samples were rinsed twice with cold PBS and then lysed on ice in radio immunoprecipitation assay lysis buffer and subsequently homogenized and centrifuged (4°C; 9,600 x g for 5 min) to collect the total protein. Protein content was determined using the BCA method. Supernatants were then transferred, mixed and boiled in sample buffer. Total protein (40 µg/lane) were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was incubated in blocking buffer consisting of 5% fat-free milk dissolved in 1X Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-Base, pH 7.5, 100 mM NaCl and 1% Tween 20) for 1 h at room temperature followed by incubation with monocyte chemoattractant protein-1 (MCP-1; sc-1785; 1:800), macrophage migration inhibitory factor (MIF; cat no. sc-20121; 1:800), nuclear factor-κB (NF-κB; cat no. sc-372; 1:500) and sirtuin-1 (SIRT-1; cat no. sc-15404; 1:300) primary antibodies at 4°C overnight and β-actin (cat no. sc-58673; 1:2,000) at room temperature for 1 h. The membrane was subsequently washed with TBST and incubated at 37°C with IgG-horse radish peroxidase for 1 h. The blot was visualized using an enhanced chemiluminescence reagent kit (GE Healthcare Life Sciences, Chalfont, UK) following four washes with TBS.

Statistical analysis. All data were presented as the mean ± or +standard error of the mean. Differences between multiple

groups were analyzed by one-way analysis of variance followed by Duncan's multiple range test. Differences between two groups were measured by Student's t-test, using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

General conditions. Following STZ injection for 72 h, the condition observed in DN rats gradually declined as the rats exhibited dull fur, weight loss, were apathetic, unresponsive, had an increased appetite, thirst and frequency of urination and urine protein level, compared with the control group. Local ulceration, including foot ulcers, and deep skin ulcers were observed. Furthermore, the DN group displayed the most severe psychotic symptoms such as manic behavior. To some extent, groups with treatment exhibited less complications.

Change in body weight and metabolic parameters following treatment. As indicated in Table I, following modeling with STZ and high-fat glucose fodder, the body weight of rats significantly decreased when compared with the normal control (P<0.05). Moreover, TS formula improved this condition in a dose-dependent manner, which was similar to the therapeutic effect exhibited by valsartan treatment (P<0.05). Conversely, kidney weight and kidney hypertrophy index were significantly increased in the DN group compared with the control; however, this was improved in a dose-dependent manner in the groups, which were treated with different doses of TS formula (P<0.05; Table I). Furthermore, these groups exhibited a similar weight and hypertrophy index as the valsartan treated group (Table I).

Fasting blood glucose (FBG), urinary albumin excretion rate (UAER), β2-MG and creatinine clearance rate (Ccr) were evaluated following TS formula or valsartan treatment. The present results indicated that a high dose of TS formula significantly decreased FBG (P<0.01), UAER (P<0.05) and β2-MG (P<0.05) levels. Furthermore, a high dose of TS formula significantly improved Ccr compared with the DN group and the rate was similar to the Ccr exhibited in the valsartan group (P<0.05; Table II). The whole blood low-, medium- and high-shear reductive viscosity and the blood viscosity were determined. The present results revealed that TS formula significantly reduced these hemorheology indices when compared with the DN group and a similar response was exhibited in the valsartan group (P<0.05; Table III).

Renal pathological changes and macrophage infiltration. PAS staining indicated clear glomerular and renal tubular structures in the control group, with opened glomerular capillaries. Conversely, the glomerular volume was significantly increased (P=0.025) and the basement membrane was thickened in the DN group, with mesangial cell hyperplasia, expansion and renal tubule vacuolization observed. In the TS formula and valsartan treatment groups, these pathological changes were markedly decreased (Fig. 1). In addition, immunohistochemical results from all TS treatment groups indicated that TS formula significantly ameliorated macrophage infiltration that was exhibited in the DN group and the valsartan group (TS^{Low} vs. DN group, P=0.006; TS^{Med}

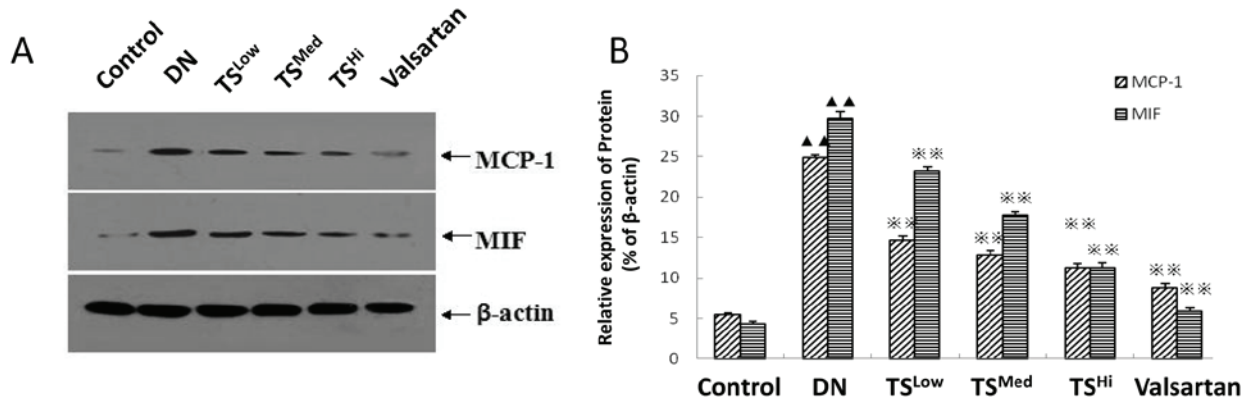


Figure 3. Protein expression levels of MCP-1 and MIF in the control group, DN group, TS^{Low} group, TS^{Med} group, TS^{Hi} group and valsartan group were detected by (A) western blotting. (B) Relative protein expression levels of MCP-1 and MIF in all groups. Data are presented as the mean + standard error of the mean. \blacktriangle $P < 0.01$ vs. control, ** $P < 0.01$ vs. DN group. TS, Tangshen; TS^{Low}, low dose (6.5 g/kg/day) of Tangshen formula; TS^{Med}, medium dose (13 g/kg/day) of Tangshen formula; TS^{Hi}, high dose of Tangshen formula (26 g/kg/day) of Tangshen formula; Valsartan, 25 mg/kg/day of valsartan; DN, diabetic nephropathy; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibitory factor.

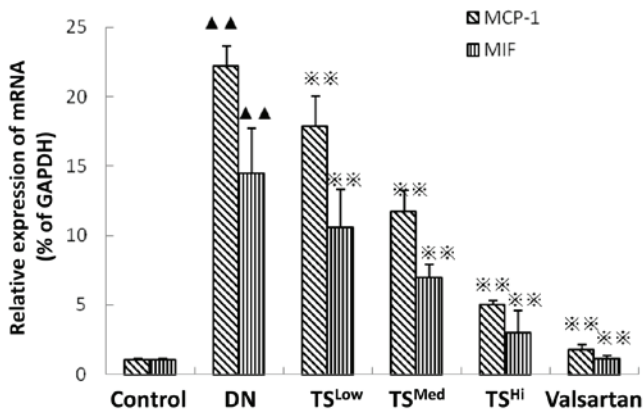


Figure 4. mRNA expression levels of MCP-1 and MIF in the control group, DN group, TS^{Low} group, TS^{Med} group, TS^{Hi} group and valsartan group. Data are presented as the mean + standard error of the mean. \blacktriangle $P < 0.01$ vs. control, ** $P < 0.01$ vs. DN group. TS, Tangshen; TS^{Low}, low dose (6.5 g/kg/day) of Tangshen formula; TS^{Med}, medium dose (13 g/kg/day) of Tangshen formula; TS^{Hi}, high dose of Tangshen formula (26 g/kg/day) of Tangshen formula; Valsartan, 25 mg/kg/day of valsartan; DN, diabetic nephropathy; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibitory factor.

vs. DN group, $P = 0.004$; TS^{Hi} vs. DN group, $P = 0.003$; Fig. 2, Table IV).

Effectiveness of TS formula on inflammatory proteins. RT-qPCR and western blotting results indicated that MIF and MCP-1 were significantly increased in the DN group compared with the normal control ($P < 0.01$; Figs. 3 and 4). TS formula groups exhibited significantly decreased levels of these inflammatory factors, both at the protein level (Fig. 3) and mRNA level (Fig. 4), in a dose-dependent manner when compared with the DN group ($P < 0.01$).

Furthermore, the SIRT1 and NF- κ B expression levels following different treatments were studied. Protein (Fig. 5) and mRNA (Fig. 6) expression levels of SIRT1 were significantly decreased and NF- κ B expression levels were significantly increased in the DN group when compared with the normal control ($P < 0.01$). Conversely, treatment with either

low- medium- or high-dose TS formula reversed this trend, with the high-dose TS group exhibiting similar expression levels exhibited in the valsartan group.

Discussion

DN is the most common microvascular complication of diabetes and is one of the leading causes of end-stage renal disease worldwide (17). The primary risk factors contributing to the development and/or progression of DN include hyperglycemia, hypertension and dyslipidemia (18). The occurrence of DN is predominantly mediated by high glucose levels and microvascular hemodynamic changes and is further mediated by a variety of inflammatory cytokines in diabetic kidneys, which activate the inflammatory pathways (19). Capillary basement membrane thickening and mesangial matrix expansion of glomerular sclerosis precedes the final stages of DN (20). It has been suggested previously that the blood rheology changes are primarily responsible for microcirculation disorder exhibited in patients with diabetes (21). Inflammation has a vital role in the development of DN. High levels of blood glucose and hemodynamic disorder may cause apoptosis and shedding of kidney cells, including podocytes and proximal tubular epithelial cells, leading to the activation of leukocyte, along with the increasing recruitment of inflammatory chemokine factors (22).

MCP-1 is the strongest chemotactic factor for monocytes and urinary MCP-1 secretion levels are upregulated in multiple renal diseases, including DN (23,24). MCP-1 has an important role in the pathogenesis of glomerular and progressive tubulointerstitial lesions via monocyte recruitment and activation (23). Increased urinary excretion of MCP-1 in patients with established DN (patients with micro- or macro albuminuria) is likely due to the enhanced production of MCP-1 in the renal tubules, which is presumably induced by excessive exposure to plasma protein filtered from the damaged glomeruli. However, MCP-1 may also upregulate the expression of CD11/18, which increases the adhesiveness of macrophages (25). Moreover, upregulated MCP-1 may lead to the activation of lysosomes and has a vital role in reactive oxygen species-induced kidney injury (20).

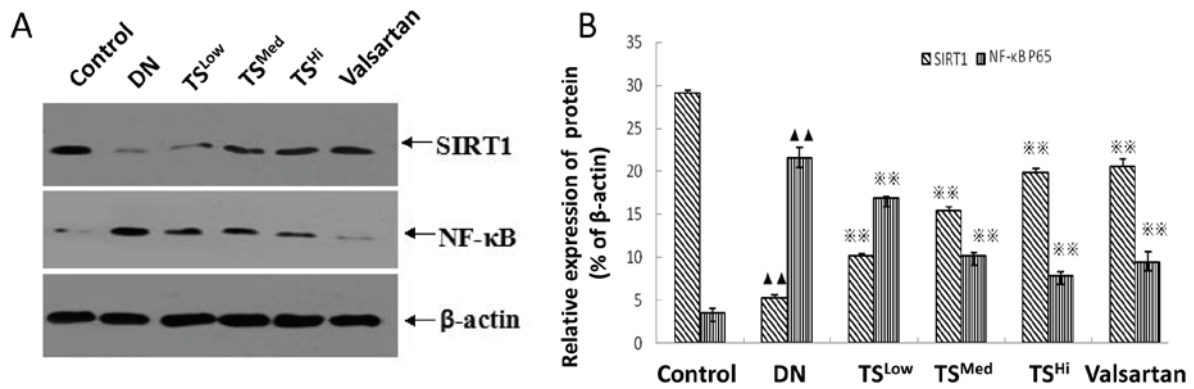


Figure 5. Protein expression levels of SIRT1 and NF-κB in the control group, DN group, TS^{Low} group, TS^{Med} group, TS^{Hi} group and valsartan group were detected by (A) western blotting. (B) Relative protein expression levels of SIRT1 and NF-κB in all groups. Data are presented as the mean + standard error of the mean. ^{▲▲}P<0.01 vs. control, ^{***}P<0.01 vs. DN group. TS, Tangshen; TS^{Low}, low dose (6.5 g/kg/day) of Tangshen formula; TS^{Med}, medium dose (13 g/kg/day) of Tangshen formula; TS^{Hi}, high dose of Tangshen formula (26 g/kg/day) of Tangshen formula; Valsartan, 25 mg/kg/day of valsartan; DN, diabetic nephropathy; NF-κB, nuclear factor-κB; SIRT1, sirtuin-1.

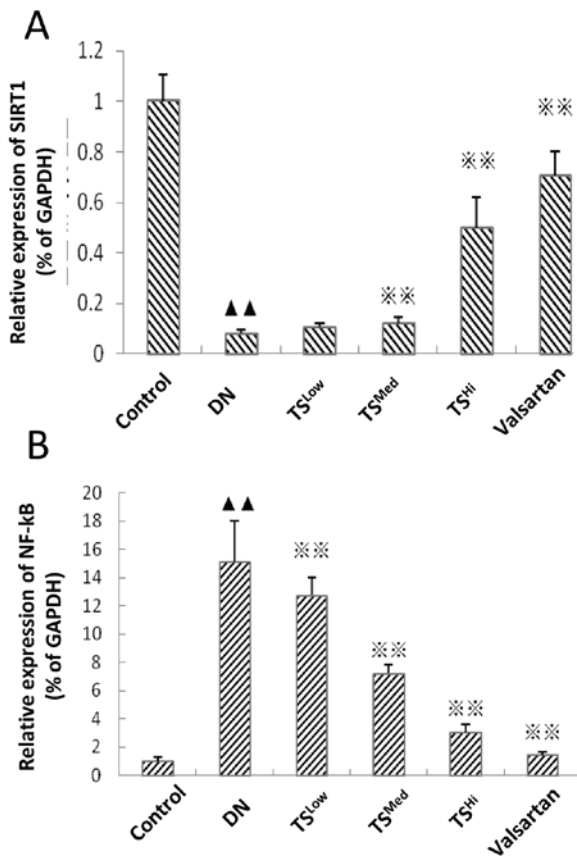


Figure 6. mRNA expression levels of (A) SIRT1 and (B) NF-κB in the control group, DN group, TS^{Low} group, TS^{Med} group, TS^{Hi} group and valsartan group. Data are presented as the mean + standard error of the mean. ^{▲▲}P<0.01 vs. control, ^{***}P<0.01 vs. DN group. TS, Tangshen; TS^{Low}, low dose (6.5 g/kg/day) of Tangshen formula; TS^{Med}, medium dose (13 g/kg/day) of Tangshen formula; TS^{Hi}, high dose of Tangshen formula (26 g/kg/day) of Tangshen formula; Valsartan, 25 mg/kg/day of valsartan; DN, diabetic nephropathy; NF-κB, nuclear factor-κB; SIRT1, sirtuin-1.

MIF has been described as a product of activated T cells that inhibits the migration of macrophages *in vitro* (26). Low levels of glucocorticoids upregulate MIF expression via a counter regulatory mechanism whereby MIF can override

the glucocorticoid effects, which suggests the presence of this component has a vital role in amplifying pro-inflammatory loop responses (27). Furthermore, Bruchfeld *et al* revealed that MIF are highly expressed in chronic nephrosis (28).

Previous results have indicated that SIRT1s may be identified and activated in the kidneys, liver, spleen, lung, heart, muscle, brain, testis, ovaries, thymus, pancreas and white or brown adipose tissue (29,30). Localization of SIRT proteins differs in cells and may develop various physiologic and pathologic metabolic effects under certain stress conditions (29). SIRT1 is the most studied member of the sirtuins, likely due to its generalized effects on the cell cycle, mitochondrial metabolism, energy homeostasis, inflammation, oxidative stress and apoptosis (30). SIRT1 may have a key role in the regulation of whole body metabolic homeostasis, and downregulation of SIRT1 in visceral adipose tissue may contribute to the metabolic abnormalities that are associated with visceral obesity in diabetic and obese women (31). SIRT1-deficient mice exhibit low levels of serum glucose and insulin (32). Molecular evidence has indicated that expression of SIRT1 is able to decrease the acetylation of NF-κB and reduce the toxic effect of cisplatin on kidney tubules. Additionally, a previous study indicated that abnormal activation of NF-κB may promote inflammatory responses and autoimmune responses, which results in the expansion of the extracellular matrix, inflammatory cell infiltration and renal tubule interstitial fibrosis (33).

Through long-term clinical practice, traditional Chinese medicine proposed a theory where kidney damage is caused by turbidity toxin called the 'damage of kidney by poison' theory. It is believed that phlegm stagnation implicates the renal microcirculation system, qi stagnation, stasis and pathogenic factor accumulation into turbidity toxins and that the toxins damage the renal microcirculation system (4). Damage to the renal microcirculation system is considered the most important pathogenesis factor of DN, which is a condition associated with the production of various inflammatory factors, including MCP-1 and NF-κB (34). The essence of stasis refers to thrombosis or embolism complications in the kidney, which is the typical pathology of DN in traditional Chinese medicine and its symptoms include a change in renal hemodynamics and involves mesangial cell proliferation

and extracellular matrix accumulation (34). This may lead to the onset of glomerulosclerosis in patients with DN (34). The accumulation of turbidity toxin is usually caused by blood stasis, phlegm and moisture (35). The mechanism of DN inflammatory pathogenesis proposed by modern western medicine provides theoretical circumstantial evidence for the pathological framework for the 'damage of kidney by poison' theory (35).

Western and traditional Chinese medicine researchers agree that the changes in hemodynamics and inflammatory factors are important pathological changes during the development of DN (35). TS formula is produced from six types of traditional Chinese medicine, namely *Astragalus*, *Radix puerariae*, *Lucid ganoderma*, *Ligustrum lucidum*, *Rheum officinale* and *Salvia miltiorrhiza*. *Astragalus* and *Radix puerariae* (15). *Astragalus* is beneficial for supplementing Qi and strengthening the immune system (36). *Radix puerariae* is used as an antipyretic and promotes the secretion of saliva (37). The combination of these two agents is beneficial in supplementing Qi and promoting the secretion of saliva (37). *Lucid ganoderma* could supplement Qi of the liver, heart, spleen, lungs and kidneys (37). *Ligustrum lucidum* fruit supplements kidney fluids, nourishes Yin of the liver and may reduce inflammation. In traditional Chinese medicine, Rhubarb cooked with rice wine is beneficial for promoting circulation, removing stasis, and when combined with *Salvia miltiorrhiza*, it may promote circulation and clear the body of phlegm senescence and blood stasis (37).

In the present study, following the successful establishment of the DN model, the effectiveness of TS formula was investigated. The present results revealed that TS formula significantly improved the physical condition of rats with DN and ameliorated kidney hypertrophy indices in a dose-dependent manner, which was similar to the effect exhibited with valsartan treatment. Furthermore, TS formula significantly decreased the pathological changes, including FBG, UAER, β 2-MG, hemorheology index, and mesangial cell hyperplasia and expansion. These results indicated that TS formula could effectively improve the pathological state of patients with DN and protect the kidneys.

In the present study, western blotting and RT-qPCR results demonstrated that the protein and mRNA expression levels of inflammatory factors, MIF and MCP-1, were significantly decreased following treatment with TS formula, which indicated that TS formula may have an effective role in preventing inflammatory responses. The anti-inflammatory effect may be closely associated with the upregulation of SIRT1 and the ensuing downregulation of NF- κ B that was exhibited in the present results, in order to block the key regulative protein in the inflammatory responses and protect the kidney.

In conclusion, TS formula elicited a prominent therapeutic effect by targeting two key pathological changes in symptoms: The changes in hemodynamics of DN; and the infiltration of inflammatory factors, which may be closely related to its pharmacological action on activating SIRT1 expression and NF- κ B downregulation, leading to a final inhibitory effect of pro-inflammatory factors. However, the necessary constituents of TS formula require further study and the functions of each constituent in the pathology of DN requires further elucidation.

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