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Original Article

Effect of TongluoYishen formula on rearrangement of cytoskeleton of glomerular podocyte in diabetic nephropathy rats

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Abstract: To observe the effect of TongluoYishen formula on ultrastructure and cytoskeleton protein expression in glomerular podocyte in diabetic nephropathy (DN) rats. Rat DN model was prepared by removal of the right kidney plus intraperitoneal injection of streptozotocin. Model rats then received 7.2 mg/kg valsartan by gavage or TongluoYishen formula (13.6 g/kg by gavage) of Chinese medicine (N=10 each) daily for 6 consecutive weeks. 24-hour total urine protein, urine albumin, blood creatine and urea nitrogen were measured in all rats. Ultrastructure of glomerular podocyte was observed by transmission electron microscope. RT-PCR was used to quantify the mRNA expression of podocyte cytoskeleton proteins including α-actinin-4, synaptopodin, and F-actin. Compared with sham group, model rats had significantly elevated levels of 24-hour total urine protein, urine albumin, blood creatine and urea nitrogen ($P<0.01$). Both treatment methods remarkably decreased these indexes ($P<0.05$ or $P<0.01$). In addition, Chinese medicine suppressed urea nitrogen levels more significantly than valsartan ($P<0.01$) but not significantly in the levels of 24-hour total urine protein, urine albumin, or blood creatine ($P>0.05$). Both treatment improved podocyte injury, eliminated podocyte fusion, and alleviated irregular cytoskeleton fibers, as demonstrated by significantly elevated mRNA expression of α-actinin-4, synaptopodin, and F-actin ($P<0.05$) compared with sham group, but no difference was shown between the two treatment approaches. In conclusion, TongluoYishen formula can retard DN progression via up-regulating the expression of skeleton molecules in DN rat podocytes, thus protecting podocytes.

Keywords: TongluoYishen formula, diabetic nephropathy, podocyte, ultrastructure

Introduction

Diabetic nephropathy (DN) is the most severe microvascular complication of diabetic mellitus (DM). In the developed countries, DM has become a major reason for terminal nephropathy. About 43% of all patients with chronic renal failure requiring dialysis are DN cases, which has become a major death factor for DM. The incidence of DM in China is now rapidly increasing. DN occupies about 15% of all terminal nephropathy cases, and occurs in about 33% to 40% or 20% to 25% of type I or type II DM, respectively, becoming the major reason for renal failure [1-3]. DM affects glomerular podocytes, whose cytoskeleton rearrangement and oxidative stress are major research fields currently. Pathological features include thickening of glomerular basal membrane, widening of mesangium region, and the formation of K-W nodules, among which increased apoptosis is one important mechanism for absence of glomerular mesangial cells, and the major reason for renal function failure, whilst endoplasmic reticulum stress plays an important role in the cell apoptosis pathway [4, 5].

TongluoYishen formula derives from Didangtang and Shenqiwan, and consists of dried rehamnnia root, Chinese yam and ArillusCorni for supplementing the functions of liver and kidney, strengthening spleen and replenishing qi, strengthening kidney to stop emission, and leech, peach kernel and wine rhubarb for blood stasis, barrenwort for complementing kidney, achyranthes root for nourishing liver and kidney as well as activating blood flow and water. Based on the features of kidney insufficiency

and kidney-collateral blood-stasis, TongluoYishen formula is an effective treatment for DN [6-8]. The whole formula can have synergistic effects on nourishing yin and tonic yang, as well as activating blood dredging collaterals. Previous study showed that this formula could improve blood glucose, lipid and renal functions in both DN patients and DM rats [9]. However, the effect of TongluoYishen on glomerular podocyte of DN individuals remains unclear. Therefore, this study aimed to investigate the effect of TongluoYishen formula on ultrastructure and expression of cytoskeleton proteins including α-actinin-4, synaptopodin, and F-actin in glomerular podocyte in diabetic nephropathy (DN) rats, in an attempt to uncover the intervention effects and functional mechanism of TongluoYishen formula from sub-organelle ultrastructure and genetics perspectives.

Materials and methods

Experimental animals

A total of 40 healthy male adult SD rats (body weight 200±20 g) were provided by Laboratory Animal Center, Medical Faculty of Xi'an Jiaotong University (Certificate No. SCXK-2012-003). All procedures were approved by the Ethnic Committee of Xi'an Jiaotong University.

Chinese medicine

TongluoYishen formula consisting 30 g dried rehamnnia root, 15 g ArillusCorni, 15 g Chinese yam, 12 g peach kernel, 10 g wine rhubarb, 6 g leech, 10 g scorpion, 10 g Aconitum CarmichaeliDebx, 12 g RnizomaAlismatis and 12 g poriacocos were purchased from the Affiliated Hospital of Shaanxi University of Chinese Mdeicine. The dose on rats was calculated based on previous study [6]. All Chinese medicine was fried and condensed for gavage fluid containing 1.36 g/ml. Valsartan capsule (80 mg) was purchased from Novartis Beijing (China, lot number X1733) and was prepared into 0.72 mg/ml suspension using distilled water.

Reagents and equipment

Streptozotocin (STZ) was purchased from Sigma (US, lot number 201502). Trizol RNA extraction kit, primers were purchased from Invitrogen (US, lot number 110403 and 171-6471). 2 × SYBR green PCR mixture was purchased from QIAGEN (US, lot number 1510-40202). 6 × loading buffer was purchased from Weiao Bio (China, lot number 0508112). Gel imaging system GIS-1600 was purchased from Tianneng (China). ABI ViiA 7 Real Time PCR System was purchased from Applied Biosystem. Tecai G2 Spirit transmission electron microscope was purchased from FEI (US).

Animal model preparation and grouping

40 rats were acclimated for 7 d feeding, weighted and tested for urea glucose and urea protein. 10 randomly selected animals were included in the sham group, while the remaining rats were prepared for establishing DN model using removal of right kidney plus intraperitoneal injection of STZ (45 mg/kg in 0.1 mol/L citric acid buffer). In brief, rats were anesthetized with 10% hydrate chloral (3.5 ml/kg) via intraperitoneal injection. A surgical incision with 1-1.5 cm length was made on the back to dissect the right kidney. One week after surgery, STZ was injected into the peritoneal cavity while sham group received equal volume of citric acid buffer without kidney resection. 72 h after intraperitoneal injection of STZ, blood samples were collected from the tail vein to test blood glucose level. Successful DM model was generated with a blood glucose level ≥ 16.7 mol/L for three times. Three weeks later, 24 h urea samples were collected to quantify the urea protein level. Successful generation of DN model was achieved when the urea protein level being ≥ 30 mg/L. The time of experimental starting point was assigned as week 0.

DN rats were randomly assigned into three groups (N=10 each), named as DN model group, valsartan group, and TongluoYishen formula group, and treated with 10 mg/kg distilled water, 7.2 mg/kg valsartan, 13.6 g/kg TongluoYishen formula solution by gavage daily for 6 consecutive weeks, respectively. Rats in sham group were also treated with 10 mg/kg distilled water by gavage. In the following experiment, each group had one rat died except the sham group.

Sample collection

Rats were sacrificed at the end of 6th week. Before that 24 h urea samples were collected

Table 1. Primer sequence

Gene name	Primer sequence (5'-3')	Access number	Fragment length (bp)
α-actinin-4	F-CGACGAGAAGGCCATAATGA	NM_031675	119
	R-CATTTTCCTGGTTCACAGCC		
Synaptopodin	F-GACGCCCCTTAGGAAACTTT	NM_021695	172
	R-ATTCCTGTCTTGTTGGCAGG		
F-actin	F-ACAAAGGACATCGTCAACGG	NM_001005903	107
	R-TGCTGCTTTCTCTTCAAGGC		
GAPDH	F-GGCAAGTTCAACGGCACAGT	NM_017008	128
	R-ATGACATACTCAGCACCGGC		

from all rats in metabolic cage. Rats were anesthetized by intraperitoneal injection of 10% hydrate chloral (0.35 ml/100 g). 10 ml blood sample was collected from abdominal aorta to separate serum by centrifugation. Supernatant serum was kept at -20°C. Left renal cortex was immediately separated. Partial cortical tissues were kept in RNA storage buffer for RT-PCR assay. Other tissues were prepared into 1 mm^3 cubes, which were fixed in 3.7% glutaraldehyde buffer for electron microscopy analysis.

Biochemical indexes

24 h urea protein quantification was performed using biuret method; Urine albumin was tested by immune turbidimetry method. Serum urea and creatine were tested by a fully automatic biochemical analyzer.

Ultrastructure of glomerular podocyte was observed by transmission electron microscopy

TEM analysis of the ultrastructure of glomerular podocyte was performed as previously described [10]. Briefly, kidney tissue was removed and cut into small pieces followed by immersion in 2.5% glutaraldehyde for 3 hours and subsequent wash in cacodylate buffer. Then, the sample was post fixed with 1% OsO4 for 1 h followed by dehydration and embedding in Epon resin. Ultrathin sections (100 nm) were cut, collected and stained with uranyl acetate and lead citrate. The analysis of Ultrastructure was performed by a transmission Morgagni 268D electron microscopy (FEI Company, Eindhoven, The Netherlands).

RT-PCR for mRNA expression of podocyte cytoskeleton proteins

Total tissue RNA was extracted. A 20 μl reverse transcription reaction system containing 8 μl total RNA, 50 U/μl RNAse inhibitor, 2 μl random primer (50 pM/μl), 4 μl 5 × buffer (Invitrogen), 2 μl DTT and 1 μl AMV (200 U/μl) was prepared. The mixture was incubated at 42°C for 15 min, followed by 85°C heating for 5 s. Under 40°C, the mixture was incubated for 1 h, and then heated at 90°C for 5-10 min. 5000 g centrifugation for 5 s was performed following ice treatment for 5 min. Fluorescent quantitative PCR was performed using cDNA as the template under the following conditions: 95°C for 120 s, followed by 40 cycles consisting of 94°C 10 s, 59°C 10 s. Using GAPDH as the internal reference, $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression level. Primer sequences were shown in **Table 1**.

Statistical methods

SPSS17.0 software was used for statistical analysis. Data were presented as mean ± standard deviation (SD). Between-group comparison was performed by student t-test. A statistical significance was defined when $P<0.05$.

Results

Comparison of 24 h urea protein quantification, urine albumin, blood creatine and urea nitrogen levels in all groups of rats

Compared with sham group, model rats had significantly elevated levels of 24 h urea protein quantification, urine albumin, blood creatine and urea nitrogen ($P<0.01$). Compared with model group, both treatment groups had remarkably lowered indexes ($P<0.05$ or $P<0.01$). Furthermore, Chinese medicine group decreased urea nitrogen levels more significantly than valsartan ($P<0.01$) but had no statistically significant difference in 24 h urea protein quantification, urine albumin, blood creatine or urea nitrogen ($P>0.05$, **Table 2**).

Change of ultrastructure in rat glomerular podocyte

Under electron microscopy, rats in the sham group had complete podocyte with regular arrangement of foot process, vertical distribu-

Table 2. 24 h urea protein quantification, urine albumin, blood creatine and urea nitrogen levels

Group	N	Urea protein mg/24 h	Urine albumin mg/24 h	Creatine μmol/L	Urea nitrogen mmol/L
Sham	10	9.77±2.41**	0.15±0.03**	62.78±4.83**	7.85±1.69**
Model	9	127.23±42.10$^{\#\#}$	2.22±0.53$^{\#\#}$	133.51±36.24$^{\#\#}$	32.30±4.00$^{\#\#}$
Valsartan	9	44.10±11.60$^{\#\#,**}$	1.35 ±0.28$^{\#\#,**}$	81.45±5.26$^{\#\#,**}$	19.23±2.66$^{\#\#,**}$
Chinese medicine	9	57.53±13.57$^{\#\#,**}$	1.52±0.27$^{\#\#,*}$	78.32±9.78$^{\#\#,**}$	14.56±2.42$^{\#\#,**,\blacktriangle\blacktriangle}$

Note: $^{\#\#}$P<0.01 compared to sham group; *P<0.05, **P<0.01 compared to model group; $^{\blacktriangle\blacktriangle}$P<0.01 compared to valsartan group.

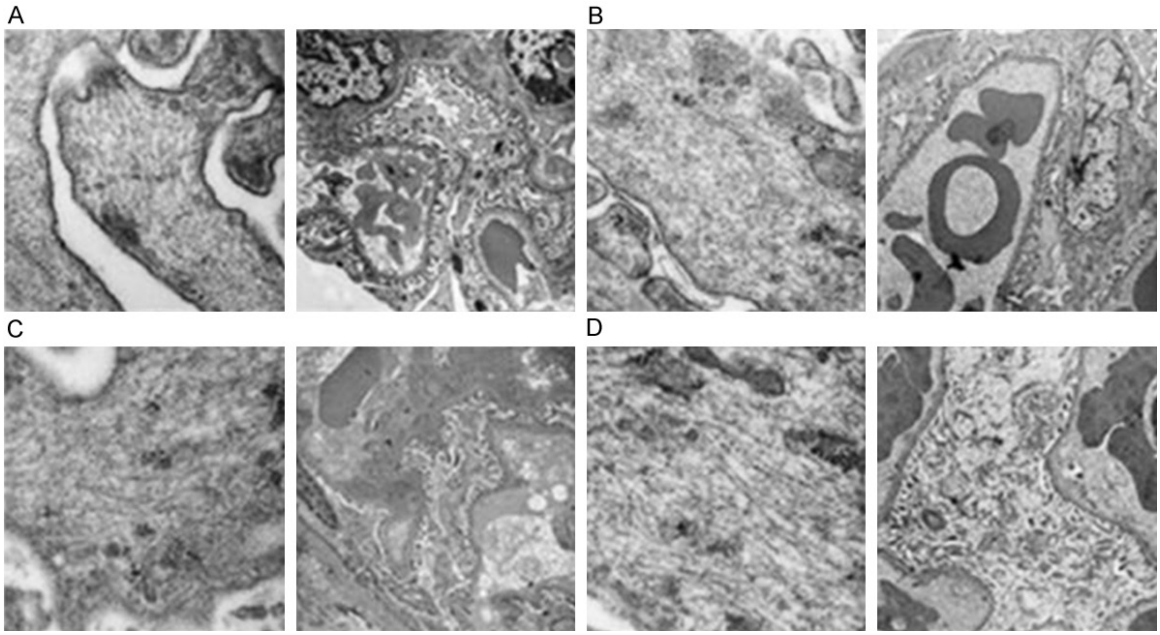

Figure 1. The ultrastructure of rat glomerular podocyte by electron microscopy. A. Sham group. The podocytes were intact and the foot processes and the fibers of the cytoskeleton were arranged regularly. B. Model group. The podocyte structure was incomplete, the density of which decreased, and the fibers of the cytoskeleton wer short and disarranged. C. Valsartan group. The changes of cells were obviously alleviated and the foot arrangement was relatively clear. D. Chinese medicine group. The fusion was alleviated and the arrangement of cytoskeleton fiber was relatively neat (x 6000).

tion between foot process and basal membrane without fusion, plus regular arrangement of cytoskeleton fiber in parallel fashion and orientation. However, model group had incomplete structure of podocyte, whose density was decreased, accompanied with lower cell numbers and more fusion. Cells showed belt-like distribution on the basal membrane or even disappearance. The foot process rupture pore membrane was thickened, with shortened cytoskeleton fiber and irregular arrangement. Both treatment groups had significantly ameliorated change of podocyte, displaying complete structure, clear vision of foot process arrangement and alleviated fusion. Cytoskeleton fibers had relatively regular arrangement. No significant difference was observed between these two treatment groups (**Figure 1**).

mRNA expression of podocyte cytoskeleton protein α-actinin-4, synaptopodin, and F-actin

Compared with sham group, model rats had significantly down-regulated mRNA levels of α-actinin-4, synaptopodin, and F-actin (P<0.01). Both treatment groups had elevated expression levels of those genes compared with the model group (P<0.01, P<0.05). However, Chinese medicine didn't significantly affect the expression of those cytoskeleton proteins (P>0.05, **Table 3**).

Table 3. mRNA expression of α-actinin-4, synaptopodin, and F-actin (N=3)

Group	α-actinin-4	Synaptopodin	F-actin
Sham	1.000±0.000**	1.000±0.000**	1.000±0.000**
Model	0.250±0.058$^{\#\#}$	0.286±0.067$^{\#\#}$	0.321±0.099$^{\#\#}$
Valsartan	0.725±0.091$^{\#\#,**}$	0.774±0.123$^{\#,**}$	0.822±0.120$^{\#,**}$
Chinese medicine	0.631±0.161$^{\#\#,*,\&}$	0.579±0.115$^{\#\#,*,\&}$	0.690±0.093$^{\#\#,**,\&}$

Note: #, P<0.05, ##, P<0.01 compared to sham group; *, P<0.05, **, P<0.01 compared to model group; &, P>0.05 compared to valsartan group.

Discussion

In traditional Chinese medicine, DN is classified as consumptive thirst nephropathy, and is frequently caused by genital weakness, inappropriate diet, excessive eparsalgia, and insufficient kidney. Basic pathogenesis mechanism refers to the deficiency of kidney yin and yang as well as obstruction of collaterals by blood stasis. Therefore activating blood and dissolving stasis, plus nourishing kidney and dredging collaterals are the major treatment approaches for DN [11]. TongluoYishen formula derives from Didangtang and Shenqi Capsule, and consists of dried rehamnnia root, ArillusCorni, Chinese yam, peach kernel, wine rhubarb, leech, scorpion, Aconitum CarmichaeliDebx, RnizomaAlismatis and poriacocos [12, 13]. Previous study showed satisfactory efficacy of this formula on the treatment of DN patients and model rats, as demonstrated by improved body signs and symptoms, improved renal function, lower levels of urea protein or urea β2 microglobulin [14], whose mechanism is associated with the inhibition of the proliferation of glomerular mesangial cells, down-regulation of TGF-β1 or TIMP-1, retarding the occurrence of endoplasmic reticulum, and inhibiting apoptosis of mesangial cells [15].

During DN process, dysregulation of high blood glucose, lipid metabolism disorder, change of hemodynamics, cytokines or oxidative stress may all lead to podocytes injury. Foot process fusion is the most common form of podocyte injury, with rearrangement of cytoskeleton of foot process as the intrinsic molecular mechanism [16, 17]. Cytoskeleton proteins of podocyte mainly include actin and associated regulatory proteins, which have interactions to maintain the structure and function of podocyte. Actin mainly consists of F-actin, with α-actinin-4 as the important actin cross-linking proteins to assemble actin fibers. Synaptopodinis the most important regulatory protein for podocyte. It can bind to actin, and interacts with α-actin-4 proteins to maintain the stability of cytoskeletons [18-21]. This protein is mainly expressed in matured podocyte processing and brain. Therefore, synaptopodin is a marker for mature podocyte. Electron microscopy observed significant change of podocyte morphology in DN model, with enlarged cell body, fusion of foot process, irregular cytoskeleton fiber arrangement. TongluoYishen formula and valsartan have certain effects on the improvement of the re-arrangement of podocyte cytoskeleton fibers. RT-PCR results also showed remarkably decreased mRNA expression of α-actinin-4, synaptopodin, and F-actin in DN rats. However, TongluoYishen formula significantly up-regulated the mRNA expression of α-actinin-4, synaptopodin, and F-actin in DN rats.

This study further demonstrated that TongluoYishen formula could decrease the level of urea proteins, and protect kidney functions, which might be through regulating the expressions of podocyte related cytoskeleton proteins, thus decreasing podocyte damage and maintaining stability of podocyte. This study, in combined with previous work, provided further evidences for the effect of this formula on the treatment of DN, and paved the basis for applying this formula in the treatment of deficiency of kidney and blood stasis in clinics.

TongluoYishen Formula could protect podocyte via up-regulating the expression of cytoskeleton molecules in DN rats, leading to the inhibition of DN progression.

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Disclosure of conflict of interest

None.

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