

Original Article

Effects of Jiangya Xiaoke prescription on TGF- β_1 in diabetic nephropathy rats with hypertension and its mechanisms

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Abstract: Objective: To observe the effects of Jiangya Xiaoke prescription on transforming growth factor- β_1 (TGF- β_1) in diabetic nephropathy (DN) with hypertension rats and to investigate its mechanisms. Methods: DN with hypertension models were made by 4 weeks high-salt diet with high sugar and fat for male Wistar rats, and intraperitoneal injection of streptozotocin (STZ). The model rats were randomly divided into three groups: untreated model group (n = 15); metformin group (n = 15), orally given metformin; Prescription group (n = 15), orally administrated JXR, for 8 weeks respectively. Blood pressure was measured before modeling and after treatment of 2, 4, 8 weeks. Fasting blood glucose (FBG), total triglyceride (TG) and total cholesterol (TC) and urine albumin excretion (UAE) of rats were observed and recorded. Renal histomorphology with PAS staining was observed by the light microscope. TGF- β_1 in kidney was detected by immunohistochemical assay and TGF- β_1 mRNA in renal cortex was detected by RT-PCR. Results: The base blood pressure of rats has no significant difference before modeling ($P > 0.05$). After 4 weeks of treatment, compared with model group, blood pressure in metformin group decreased ($P < 0.01$), blood pressure in Jiangya Xiaoke prescription group was slightly lower ($P < 0.05$). When 8 weeks, the rebound of blood pressure in metformin group is appropriate with the model, the blood pressure of Jiangya Xiaoke prescription reduced significantly ($P < 0.01$). Compared with model group, FBG, UAE and TG in Jiangya Xiaoke prescription group and metformin group significantly decreased ($P < 0.01$), TC levels also decreased ($P < 0.05$). The level of TGF- β_1 in Jiangya Xiaoke prescription group and the metformin group decreased ($P < 0.01$), and level of TGF- β_1 in Jiangya Xiaoke prescription group was lower significantly than that in metformin group ($P < 0.05$). The mRNA expression of TGF- β_1 in Jiangya Xiaoke prescription group and the metformin group was significantly lower than model group ($P < 0.01$). Pathological changes were ameliorated in Jiangya Xiaoke prescription and metformin group compared with model group. Conclusion: Jiangya Xiaoke prescription can regulate blood pressure and improve renal functional morphology through down-regulation of TGF- β_1 and its mRNA expression in DN rats with hypertension. We initially proved that the inhibition effect of TGF- β_1 in Jiangya Xiaoke prescription is better than metformin, and Jiangya Xiaoke prescription can lower blood pressure to normal levels with a better step-down smoothly and a long-term efficacy.

Keywords: Jiangya Xiaoke prescription, hypertension, diabetic nephropathy, transforming growth factor- β_1

Introduction

Diabetic nephropathy (DN) is a major microvascular complication of diabetes. DN of clinical stage shows hypertension, edema and proteinuria [1, 2]. Both DN patients and experimental DN rats could present hypertension with disease progression. Once DN is complicated by hypertension, the elevated blood pressure will increase the damage to the kidneys, hence hypertension become one of the important reasons leading to end-stage renal failure [3]. Therefore, throughout the course of DN associ-

ated with hypertension, hypertension and DN are causal.

Transforming growth factor- β_1 (TGF- β_1) is related to DN and hypertension and plays a key regulatory role in its occurrence and development. Thus, the way to inhibit mesangial cell proliferation and excessive secretion of extracellular matrix by regulating TGF- β_1 becomes a key to effectively delay the development of DN. And existing research results show that drugs such as angiotensin-converting enzyme inhibitor ACEI and Ang II receptor blocker can regulate

blood pressure by reducing the expression of TGF- β_1 [4, 5]. Currently, there has been no such Chinese patent medicine with exact effects. Therefore, it is significant to develop a new drug which can treat DN and is proven effective on hypertension.

We selected traditional Chinese medicine through long-term clinical experience and a lot of animal experiments and intended to develop a traditional Chinese herbal compound prescription which can reduce blood pressure and fat (hereinafter referred to as the Jiangya Xiaoke prescription formally), which is composed of ginkgo biloba, the root of red-rooted salvia, pberetima and other components [6-9]. Early pharmacological studies have shown that ginkgo biloba and the root of red-rooted salvia can significantly lower blood pressure. Some other clinical studies have shown that the Jiangya Xiaoke prescription has a certain effect on DN in addition to lowering blood glucose and lipid. The elements like eucommia and pberetima in the Jiangya Xiaoke prescription have an antihypertensive effect. The treatment using Chinese medicine is based on syndrome differentiation and treatment, and its recipe has multiple pharmacological effects [10, 11]. The Jiangya Xiaoke prescription address both the symptoms and the root causes of DN with hypertension starting from multi-targets, but the mechanism of the Jiangya Xiaoke prescription treating DN and lowering blood pressure by regulating TGF- β_1 is still unclear, which remains to be elucidated.

The study of effects of TGF- β_1 on DN with hypertension was initiated using DN-with-hypertension rat models, and effects of the Jiangya Xiaoke prescription on the mRNA expression and activity of TGF- β_1 were observed to make an attempt to clarify the pharmacological mechanism of the Jiangya Xiaoke prescription in lowering blood pressure and treating DN by regulating TGF- β_1 , thus preliminarily discussing the clinical application value of taking TGF- β_1 as a target in the treatment of DN associated with hypertension.

Methods

Preparation of prescription

The Jiangya Xiaoke prescription is composed of ginkgo biloba (Yunnan), the root of red-rooted salvia (Hubei) and other herbs. Ginkgo biloba in the Jiangya Xiaoke prescription was extracted

with water, the extract was concentrated to a thick paste, and the powder was obtained by pressure-relief and vacuum drying (the extract yield rate was about 12%). Salvia and other herbs were extracted with alcohol, the extract was concentrated to a thick paste, and the powder was obtained by pressure-relief and vacuum drying (19%). Before use, two kinds of powder were dissolved in distilled water, prepared in the concentration of 3 g crude drug/L and preserved at 4°C until use.

Reagents and drugs

Oligo (dT) primers, dNTP and MMLV reverse transcriptase were purchased from Sangon Bio-Technology Co. (Shanghai, China), Trizol from Waston Bio-Engineering Co. (Shanghai, China), and RNase inhibitor from Lingfei Company (Shanghai, China). Taq polymerase and DNA marker were obtained from Tiangen Biochemical Technology Co. (Shanghai, China). Primers were synthesized by Sangon Biotechnology Co. (Shanghai, China). Insulin radioimmunoassay kit was purchased from Guge Biotechnology Co. (Shanghai, China). Metformin hydrochloride was purchased from Sino-American Squibb Pharmaceuticals (Shanghai, China), prepared with distilled water as the suspension of 30 mg/mL before use.

Animals and diet

Fifty clean grade weaning Wistar male rats, with weight of 50 ± 5 g, were purchased from the Experimental Animal Center of Shanghai, China. The formula of standard rat chow contained the standard powder 35 g, wheat bran 15.5 g, soybean meal 20 g, corn flour 20 g, soybean oil 0.5 g, fish meal 5 g, bone meal 2.5 g, yeast extract 1 g, and salt 0.5 g in each 100 g, with total energy 14.03 KJ/g (protein 20.90%, fat 10.38%, and carbohydrate 68.72%). The formula of high-fat diet was as follows: each 100 g contains standard rat diet 60 g, lard 15 g, dried egg yolk powder 10 g, skim milk 8 g, casein 5 g, sugar 2 g, total energy 19.22 KJ/g (protein 19.45%, fat 49.85%, carbohydrate 30.70%) [12, 13]. The feed was mixed by adjustable high-speed electric homogenizer and stored at room temperature. A week worth of the feed was prepared at a time.

Grouping and induction of obesity

All rats were assigned randomly to one of two groups, the model group ($n = 50$) or the normal

control group ($n = 10$). After adaptation with free access to regular rat chow and tap water for one week, 50 rats in the model group were fed with high-fat diet, 25 g for each rat per day, and 8 rats in the normal group were fed with standard rat chow. All rats were maintained in the animal facility individually and housed in a controlled room, with a temperature of $20 \pm 5^\circ\text{C}$, humidity of $55 \pm 5\%$, and 12 h-dark and 12 h-light cycle. The criterion for judging successful induction of obesity was 20% or more increase in body weight as compared with the average weight of the control group. 45 rats in the model group that met the criterion were randomly divided into 3 groups: the model group ($n = 15$), the metformin group ($n = 15$), and the Prescription group ($n = 15$). The rest rats in the model group not meeting the criterion were not included in the rest of the study.

Drug delivery methods

The rats in the metformin group received metformin solution at 300 mg/kg/day, those in the Prescription group received Jiangya Xiaoke prescription at 4 g/kg/day, and those in the model group and the normal group received the same volume of distilled water. The treatment administration was performed by oral gavage at 8:30 a.m. every day for 8 weeks. The fur, feces, food intake, vitality and activities of rats were observed daily, and the rats were weighed once at 8:00 a.m. every other day by using an electronic analytical balance with 0.1 g accuracy. The dose of administration was adjusted according to recorded body weight of rats once a week.

Measurement of blood biochemical parameters

Serum fasting blood glucose (FBG), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured using an automatic biochemical analyzer (The Science and Technology Industrial Corporation, Japan).

Light microscopy of kidney tissue

A small block of kidney tissue was dissected from the left kidney and merged in 4% paraformaldehyde for 24 h. The fixed kidney tissue was embedded in paraffin and sliced for PAS stain-

ing. The quantitative analysis on the size of kidney cells was conducted by an automatic medical color image analysis system (HM IAS-2000, Champion Co., China).

Immunohistochemical analysis of kidney tissue

The kidney sections were deparaffinized, rehydrated, and incubated with 3% H_2O_2 followed by incubation with pancreatic enzyme at 37°C for 15 min. Rabbit anti-rat fibronectin antibody or rabbit anti-rat TGF- β_1 antibody was applied as the primary antibodies and peroxidase-labeled goat anti-rabbit IgG as secondary antibody. The staining was visualized by reaction with diaminobenzidine color reagent and then counterstained with hematoxylin. Finally, the sections were rinsed with phosphate buffer saline (PBS) and dried by gradient alcohol. Controls consisted of PBS in place of primary antibody followed by the procedure above. All the sections were examined by the light microscope. Optical density (OD) was identified as expression intensity of fibronectin or TGF- β_1 positive staining in renal tissue, which was semiquantitatively analyzed with HMIAS-2000 color medical image analysis system.

Real-time reverse transcription polymerase chain reaction

The left renal tissue was kept at -80°C in a freezing tube immediately after removal and weight measurement for total RNA extraction. Total RNA was isolated from renal cortex using Trizol reagent. Single-strand cDNA was synthesized by reverse transcription. The primers used were: TGF- β_1 , sense 5'-CCAACTATTGC-ATCAGCTCCA-3', antisense 5'-TTATGCTGGTTGT-ACAGGG-3'; β -actin, sense 5'-CCAAGGCCAACCGCAGAAGATGAC-3', antisense 5'-AGGGTACATGGTGGTGCCGCCAGAC-3'. The PCR products were visualized after electrophoresis using the GeneSnap gel image acquisition system and analyzed using GeneTools gel image analysis software. The intensity of each gene amplification product relative to beta-actin in the same reaction was used to compare expression levels.

Statistical analysis

All results were expressed as means \pm standard deviation for each experiment. The data

Table 1. Effects of Jiangya Xiaoke prescription on FEG, TG, TC and UAE at 8th week, ()

Groups	n	FBG (mmol/L)	TG (mmol/L)	TC (mmol/L)	UAE (μg/24 h)
Control	10	4.51 ± 0.72	0.85 ± 0.49	1.79 ± 0.61	69.72 ± 38.06
Model	15	16.29 ± 3.70**	1.86 ± 0.23**	4.98 ± 0.82**	596.74 ± 96.52**
Metformin	15	10.37 ± 2.12** [^]	1.27 ± 0.19 [^]	4.36 ± 0.69 [^]	255.95 ± 83.73 [^]
Prescription	15	9.46 ± 1.93 [^]	1.12 ± 0.17 [^]	4.23 ± 0.66 [^]	239.82 ± 91.96 [^]

NOTE: *P < 0.05, **P < 0.01 vs. Control groups; [^]P < 0.05, [^]^P < 0.01 vs. Model groups.

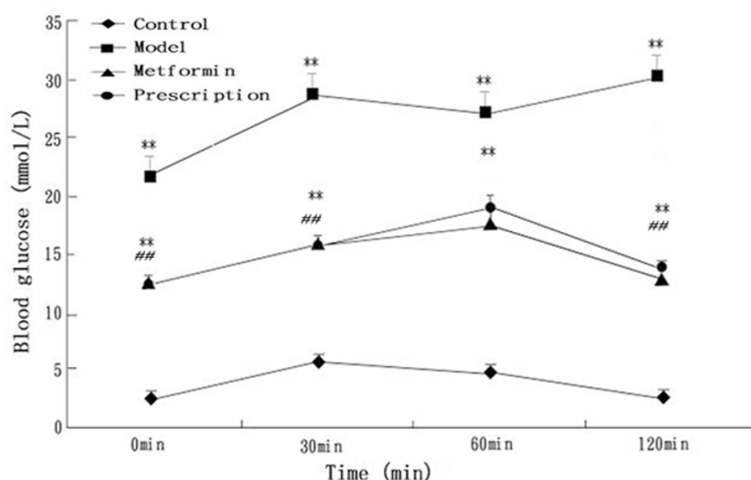


Figure 1. Curves of oral glucose tolerance test in different groups. Data are expressed as means S.E.M. NOTE: **P < 0.01 vs. Control groups, ##P < 0.01 vs. Model groups, and [^]P < 0.01 vs. Metformin rats 60 min.

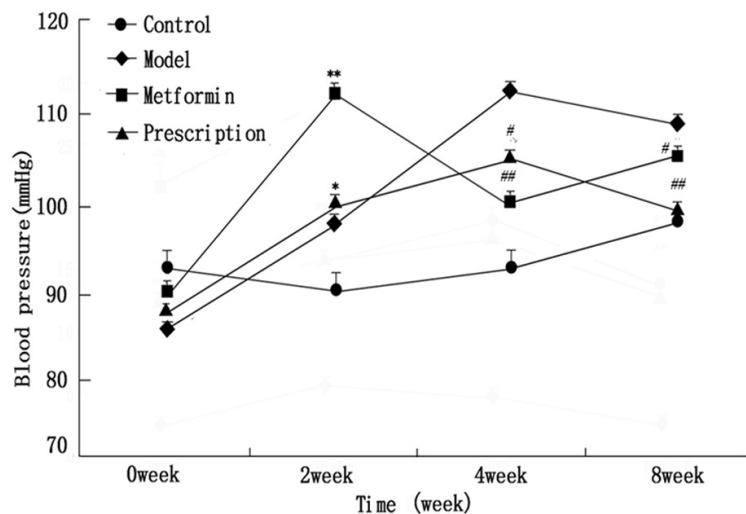


Figure 2. Curves of blood pressure test in different groups. Data are expressed as means S.E.M. NOTE: *P < 0.05, **P < 0.01 vs. Control groups; #P < 0.05, ##P < 0.01 vs. Model groups.

group comparison was done using analysis of variance (ANOVA). The analysis of Mann-Whitney U was used in the analysis of non-normal distribution data. The difference with P < 0.05 was considered to be significant statistically.

Results

Effects of ZQR on metabolic and renal parameters

After eight weeks of treatment, serum FBG, TG, TC and UAE of rats in all groups were compared. Compared with model group, FBG, UAE and TG in the Jiangya Xiaoke Prescription group and metformin group were significantly decreased (P < 0.01), and TC level was also decreased (P < 0.01) (see Table 1).

Effects of Jiangya Xiaoke prescription on oral glucose tolerance test

In OGTT conducted after 8-week treatments (Figure 1), the whole-body disposal of blood glucose was significantly delayed in all model groups, as compared to control groups. Blood glucose in untreated model rats reached the highest level at 60 min after oral glucose ingestion and failed to return to the level of 0 min (fasting level) at 120 min, indicating glucose intolerance. However, the blood glucose concentration reduced near to fasting levels at 120 min in groups treated

were analyzed by SPSS 13.0 statistical software as appropriate. Two-group comparison was analyzed using T-test, whereas multiple-

min, indicating glucose intolerance. However, the blood glucose concentration reduced near to fasting levels at 120 min in groups treated

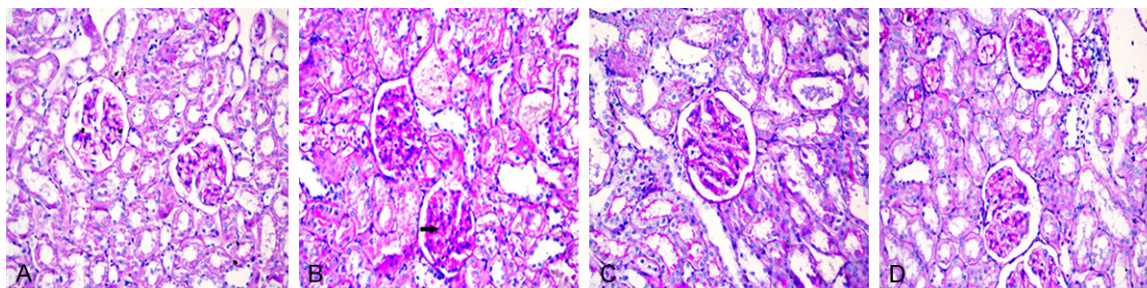


Figure 3. Light microscopy of renal tissues of rats (PAS staining \times 400 times). A: Normal; B: Model; C: Metformin; D: Prescription.

with Jiangya Xiaoke prescription and metformin, clearly demonstrating that treatment of Jiangya Xiaoke prescription improves glucose tolerance (see **Figure 1**).

Effects of Jiangya Xiaoke prescription on blood pressure test

Before modeling, blood pressure of rats in each group was measured in quiet awake states at the end of week 2, 4 and 8 after administration. The base blood pressure of rats had no significant difference in statistics before modeling ($P > 0.05$). After modeling, at week 2 after administration, the blood pressure of rats in other groups than normal group were increased in varying degrees and amplitude. At week 4 after administration, compared with model group, the blood pressure in metformin group was decreased ($P < 0.01$), and the blood pressure in the Jiangya Xiaoke prescription group was slightly lower ($P < 0.05$). At week 8, the rebound of blood pressure in metformin group was equal to that in the model group, and that in the Jiangya Xiaoke prescription group was reduced significantly ($P < 0.01$) (see **Figure 2**).

Light microscopy and analysis of renal PAS staining

In normal group, no stromal hyperplasia was observed in the glomerular mesangial area, and no basement membrane thickening was found in the glomerular capillary plexus. In model group, the glomerular mesangial area showed moderate hyperplasia; PAS staining showed significantly more lumps and the adhesion of some sphere bundles to glomerular wall; the glomerular capillary showed basement membrane thickening in a ring. In Jiangya Xiaoke Prescription group, matrix hyperplasia in the glomerular mesangium was significantly reduced, PAS staining showed rare lumpy sub-

stances, and no glomerular basement membrane thickening was found. In metformin group, mesangial matrix showed mild hyperplasia, PAS staining showed reduced lumpy substances, and no significant thickening of the glomerular basement membrane was found (see **Figure 3**).

Immunohistochemical observation and analysis of renal TGF- β_1

The observation under light microscopy showed only a weak positive reaction in renal tubular epithelial cells and negative reaction in glomeruli in the normal control group. In model group, TGF- β_1 expression was significantly enhanced, positive particles were yellow-brown, mainly presented in the glomerular capsule wall and glomeruli, and renal tubular epithelial cells were also expressed partly. Although there were tubular and glomerular expression in Jiangya Xiaoke prescription group and metformin group, the degree of positive staining was comparatively weaker. In model group, TGF- β_1 expression was significantly enhanced compared with the normal group, the mean optical density was increased ($P < 0.01$). Compared with model group, TGF- β_1 expression was decreased and the mean optical density was reduced in metformin group and Jiangya Xiaoke prescription group ($P < 0.01$), and TGF- β_1 expression in Jiangya Xiaoke prescription group was also significantly reduced compared with metformin group, indicating the Jiangya Xiaoke prescription was superior to metformin in inhibiting TGF- β_1 expression (see **Figures 4, 5; Table 2**).

Result of real-time reverse transcription polymerase chain reaction

The mRNA expression of TGF- β_1 in the renal cortex of animals in each group: Compared with

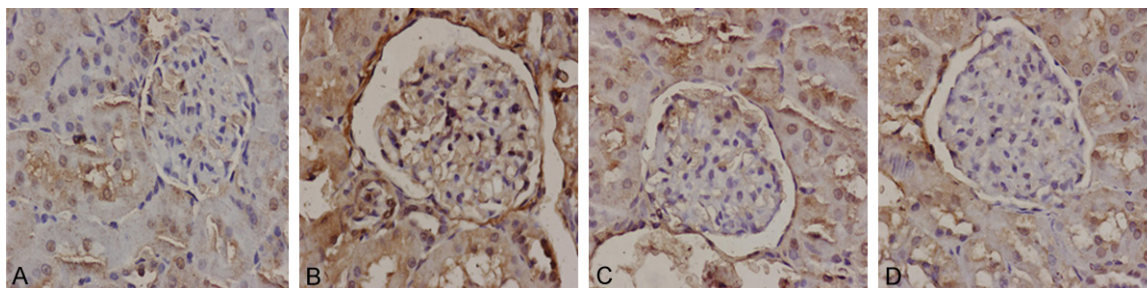


Figure 4. Immunohistochemical observation and analysis of renal TGF-β₁. A: Normal; B: Model; C: Metformin; D: Prescription.

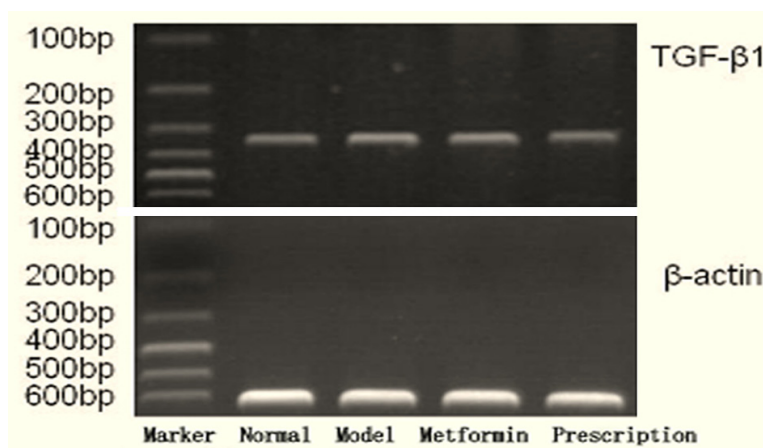


Figure 5. Optical density of TGF-β₁ in kidney. NOTE: ***P* < 0.01 vs. Control rats; #*P* < 0.05, ##*P* < 0.01 vs. Model; **P* < 0.05 vs. Metformin rats.

Table 2. Optical density of TGF-β₁ in kidney $\bar{x} \pm s$

Groups	n	TGF-β ₁ (OD)
Control	10	0.159 ± 0.0520
Model	15	0.274 ± 0.0615**
Metformin	15	0.225 ± 0.0561**^
Prescription	15	0.178 ± 0.0488**^v

NOTE: ***P* < 0.01 vs. Control rats; ^*P* < 0.05, ^^*P* < 0.01 vs. Model; v*P* < 0.05 vs. Metformin rats.

normal group, the mRNA expression of TGF-β₁ in the renal cortex of rats was increased in model group, metformin group and Jiangya Xiaoke prescription group; compared with model group, the mRNA expression of TGF-β₁ were reduced in both metformin group and Jiangya Xiaoke prescription group (**Figure 6; Table 3**).

Discussion

TGF-β₁ is a multifunctional cytokine. Clinical studies have shown that TGF-β₁ levels in hyper-

tensive patients are high than those of normal people, and the increased production of TGF-β₁ is associated with the damage of hypertensive target organs [14-16]. Basic research shows that TGF-β₁ and its mediated signal transduction are involved in the occurrence, development and prognosis of hypertension. TGF-β₁ can interact with renin-angiotensin system (RAS) and endothelial cells to affect blood pressure [17]. TGF-β₁ releases renin by stimulating juxtaglomerular cells, increasing the formation of angiotensin II (Ang II), thus resulting in high blood pressure. Ang II can induce in vitro TGF-β₁ gene expression of smooth muscle cells in the tunica media vasorum, up-regulate TGF-β₁ expression through the sequential activation of its type I receptors and stimulate TGF-β₁ to be transformed into the active state [18]. Endothelin-1 (ET-1) is the strongest vasoconstrictor factor in vivo. In-vitro studies show that TGF-β₁ can stimulate mRNA expression of ET-1 in vascular smooth muscle cells and endothelial cells [19]. These results suggest TGF-β₁ plays an important role in all types of hypertension. In this study, DN-with-hypertension rat models were prepared using high-sugar, high-fat and high-salt feed plus low-dose STZ to observe effects of the Jiangya Xiaoke prescription on the blood pressure of DN rats with hypertension. The study found that after eight weeks of administration, compared with model group, the blood pressure in Jiangya Xiaoke prescription group was decreased significantly. RT-PCR and immunohistochemistry show that TGF-β₁ mRNA expression and protein content are

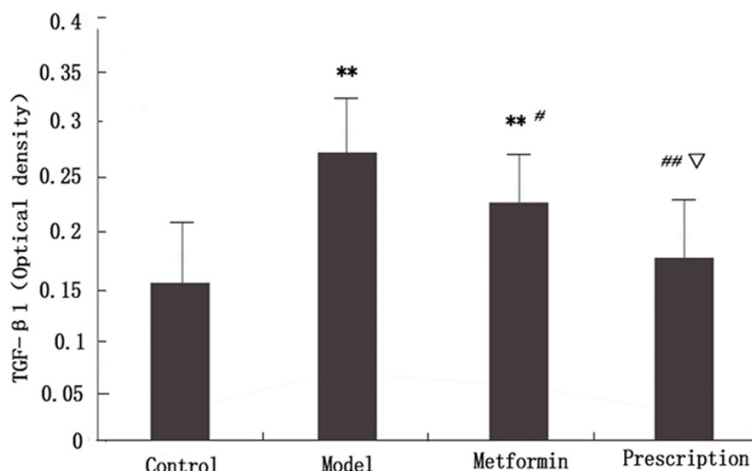


Figure 6. mRNA levels of TGF-β₁ in kidney.

Table 3. mRNA Levels of TGF-β₁ in kidney $\bar{x} \pm s$

Groups	n	TGF-β ₁
Control	10	0.350 ± 0.014
Model	15	0.624 ± 0.022*
Metformin	15	0.398 ± 0.031*^
Prescription	15	0.445 ± 0.020*^

NOTE: **P < 0.01 vs. Control rats; ^P < 0.05, vs. Model rats. The mRNA expression of TGF-β₁ in the renal cortex of animals in each group.

reduced significantly. We infer that the Jiangya Xiaoke prescription can directly reduce the blood pressure of DN rats with hypertension by inhibiting TGF.

Studies have shown that TGF-β₁ can be secreted by a variety of cells in vivo, and it can promote collagen gene expression in kidney cells and the synthesis of mesangial extracellular matrix (ECM) in the glomerular mesangium, as well as reduce its degradation, being a major regulatory factor in the metabolism of kidney ECM. The ECM accumulation in the kidneys causes tubulointerstitial fibrosis, glomerular basement membrane thickening and glomerular sclerosis, which are main characteristic pathological changes in DN [20]. The study found that after eight weeks of administration with the Jiangya Xiaoke prescription, tubular and glomerular TGF-β₁ expression in DN with hypertension rats indicated a weak level of staining of yellow-brown positive granules. The content of TG and TC was decreased, and UAE content was significantly decreased. PAS stain-

ing of renal pathology examination shows that in Jiangya Xiaoke prescription group, matrix hyperplasia in the glomerular mesangium was significantly reduced, PAS staining had rare lumpy substances, and no glomerular basement membrane thickening was found. We conclude that the Jiangya Xiaoke prescription can improve renal function and renal morphological lesions by down-regulating TGF-β₁ mRNA expression and protein levels, thus further regulating the blood pressure of DN rats with hypertension.

In this study, metformin was served as a control drug of the Jiangya Xiaoke prescription. Results show metformin also has a similar effect, and both drugs can lower blood pressure, improve kidney function and pathological damage and reduce urinary albumin excretion. However, at week 4 after administration, compared with model group, the blood pressure in metformin group was decreased, and the blood pressure in Jiangya Xiaoke prescription group was slightly lower. But at week 8, the rebound of blood pressure in metformin group was equal to that in the model group, and that in Jiangya Xiaoke prescription group was reduced significantly. Moreover, the immunohistochemical observation of renal TGF-β₁ under light microscopy shows that, compared with the model group, the mean optical density and TGF-β₁ expression were reduced in Jiangya Xiaoke prescription group and metformin group, and TGF-β₁ expression in Jiangya Xiaoke prescription group was also significantly reduced compared with metformin group, indicating the Jiangya Xiaoke prescription was superior to metformin in inhibiting TGF-β₁ expression. It can be seen from the study results above that the Jiangya Xiaoke prescription is more effective, long-lasting and stable than metformin in treating DN with hypertension.

In summary, it has better prospects and clinical value in studying the mechanism of effects of the Jiangya Xiaoke prescription on DN with hypertension by taking TGF-β₁ as a target, and is worthy of further study.

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Disclosure of conflict of interest

None.

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