

Full Paper

Renoprotective Effect of Total Glucosides of Paeony (TGP) and Its Mechanism in Experimental DiabetesYonggui Wu^{1,*}, Kejun Ren¹, Chao Liang¹, Liang Yuan¹, Xiangming Qi¹, Jing Dong¹, Jijia Shen², and Shanyan Lin³¹Department of Nephrology, the First Affiliated Hospital, Anhui Medical University, Hefei, 230022, China²Department of Pathobiology, Anhui Medical University, Hefei, 230022, China³Department of Nephrology, Huashan Hospital, FuDan University, Shanghai, 240000, China

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Abstract. Total glucosides of paeony (TGP), extracted from the root of *Paeonia lactiflora* pall, has been shown to have ant-inflammatory and antioxidative actions. The aims of this study were to elucidate the renoprotective effect of TGP and its mechanism in experimental diabetes. Streptozotocin-induced diabetic rats were treated with TGP for 8 weeks. Treatment with TGP at 50, 100, and 200 mg/kg significantly lowered 24-h urinary albumin excretion rate in diabetic rats. TGP treatment in all doses markedly attenuated glomerular volume, and treatment with TGP at 100 and 200 mg/kg markedly reduced indices for tubulointerstitial injury in diabetic rats. Western blot analysis showed that the expressions of 1α (IV) collagen, intercellular adhesion molecule (ICAM)-1, interleukin (IL)-1, tumor necrosis factor (TNF)- α , NF- κ B p65, and 3-nitrotyrosine (3-NT) protein were increased in the kidneys of diabetic rats; the increases in these proteins were all dose-dependently and significantly inhibited by TGP treatment. The expression of nephrin protein was significantly reduced in the kidneys from diabetic rats and markedly increased by TGP treatment. The expression of transforming growth factor (TGF)- β 1 protein in the kidney was also significantly increased in diabetic rats, which was significantly inhibited by treatment with TGP at all doses. Our data suggest that TGP treatment ameliorates early renal injury via the inhibition of expression of ICAM-1, IL-1, TNF- α , and 3-NT in the kidneys of diabetic rats.

Keywords: diabetic nephropathy, total glucosides of paeony (TGP), intercellular adhesion molecule (ICAM)-1, interleukin (IL)-1, tumor necrosis factor (TNF)- α

Introduction

In recent years, a number of experimental and clinical studies have demonstrated the significant role of inflammation in the initiation and progression of diabetic nephropathy (1, 2). More important, several investigations in the setting of experimental diabetic nephropathy using different drugs, such as mycophenolate mofetil, methotrexate, and erythromycin, have shown that prevention of the development or amelioration of renal injury in diabetes is associated with ant-inflammatory

actions (3–6). More recently, much attention has also been focused on the role of oxidative stress, and it has been suggested that oxidative stress might constitute the key and common events in the pathogenesis of diabetic nephropathy (7, 8). Therefore, amelioration of diabetic oxidative stress may prevent or attenuate renal abnormalities associated with diabetes.

Paeonia lactiflora pall is a Chinese traditional herbal medicine. Total glucosides of paeony (TGP), extracted from the root of *Paeonia lactiflora* pall, has been used for gynecological problems and for cramp, pain, and giddiness for over 1,500 years in Chinese medicine. Effective parts and chemical constituents of TGP have been extracted and purified and their structures have been identified. TGP contains 96.2% of paconiflorin (PF)

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and other components such as hydroxyl-paeoniflorin, paeonin, albiflorin, benzoylpaeoniflorin, and so on (9). Anti-inflammatory, antioxidative, antihepatic injury, and immunoregulatory activities without evident toxic or side effects of TGP have been extensively proven in China (10–12). In 1998, TGP was approved by State Food and Drug Administration (SFDA) to enter the market as a disease-modifying drug for rheumatoid arthritis (RA).

Based on the therapeutic role of TGP which is considered to be attributed to the amelioration of inflammation and oxidative stress, the protective effect of TGP against diabetic renal damage and its molecular biological mechanism were investigated in this study.

Materials and Methods

Drugs and reagents

TGP was extracted and isolated from the root of *Paeonia lactiflora* pall by the methods of ethanol reflux, *n*-butanol extraction, and macroreticular absorption resin chromatography. It was determined to contain 96.2% PF by high performance liquid chromatography (HPLC, 600e pumps, 2996 PDA ultraviolet spectrophotometric detector; Waters Co., MA, USA) analysis. Streptozotocin (STZ) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The microalbumin assay kit was purchased from Exocell, Inc. (Philadelphia, PA, USA). Anti 3-nitrotyrosine (3-NT) was purchased from Upstate Technology (Lake Placid, NY, USA). Anti-intercellular adhesion molecule (ICAM)-1, anti-interleukin (IL)-1, anti-tumor necrosis factor (TNF)- α , anti-NF- κ B p65, anti-nephrin, and anti-transforming growth factor (TGF)- β 1 polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody was from Boster Biotechnology (Wuhan, China). The chemiluminescence kit was from Amersham Life Science (Little Chalfont, UK).

Animals

Male Munich-Wistar rats (weight: 180–200 g) were obtained from the Experimental Animal Center of Anhui Medical University. The research protocol was in accordance with the principles approved by the animal ethics committee of Anhui Medical University. Animals were housed in wire-bottomed cages under a 12-h light/dark cycle. Room temperature (about $24 \pm 1^\circ\text{C}$) and humidity (about 60%) were controlled automatically. They were allowed free access to standard laboratory chow and tap water.

Experimental design

After several days of adaptation, STZ diluted in citrate buffer (0.1 M, pH 4.0) was injected intraperitoneally at a dose of 65 mg/kg following overnight fasting. Two days later, the diabetic state was confirmed by measurement of tail blood glucose levels using a reflectance meter (one touch II; Lifescan Ltd., Jinan, China). Blood glucose levels were measured twice a week. Diabetic rats were divided into 4 groups ($n = 10$ per group), avoiding any inter-group differences in blood glucose levels. A normal group of rats was also included. The normal and control diabetic group was given 0.5% sodium carboxymethylcellulose (CMC-Na), while the other groups were given orally the TGP (suspended in 0.5% CMC-Na) at a dose of 50, 100, or 200 mg/kg daily using a stomach tube.

Blood sample and tissue collection

After 8 weeks, body weight was measured at the conclusion of the experiment. Rats were then anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and placed on a temperature-regulated table, the right jugular artery was catheterized, and this arterial catheter was used for blood sampling. The kidneys were perfused *in vivo* via the abdominal aorta with 100 ml of normal saline at 4°C , while the renal vein was punctured to permit the perfusate to drain; then the kidneys were removed immediately and sectioned midfrontally into two pieces. One fragment was fixed in 10% formalin and processed in paraffin for subsequent histologic assessment and immunohistochemical studies, while the other was stored at -70°C until they were assayed.

Assays of blood sample

Blood glucose, triglycerides, cholesterol, and creatinine (Cr) were determined using commercial reagents. The serum activity of aspartate aminotransferase and alanine aminotransferase were also estimated by commercially available kits.

Urinary albumin excretion and Cr clearance

Prior to sacrifice, rats were placed in metabolic cages for collection of urine over 24 h for measurement of albumin concentrations. Urine samples from each rat were combined and measured. After centrifugation, aliquots of the supernatant were frozen at -70°C for subsequent analysis of albumin and Cr concentrations. Urinary albumin concentrations were measured by enzyme-linked immunosorbent assay using an anti-rat albumin antibody and 24-h protein excretion was calculated by multiplying the urinary protein excretion by the 24-h urine volume. Urinary Cr concentrations were determined using commercial reagents. Cr clearance

(Ccr) was calculated on the basis of urinary Cr, serum Cr, urine volume, and body weight using the following equation: Ccr (ml/min/kg body weight) = [urinary Cr (mg/dl) × urine volume (ml) / serum Cr (mg/dl)] × [1000 / body weight (g)] × [1 / 1440 (min)].

Renal pathology

Formalin-fixed kidney sections (2 μm) were stained with periodic acid-schiff (PAS) reagent to identify kidney structure and hematoxylin to distinguish cell nuclei. Digital images of glomeruli and interstitial areas were obtained from microscopy (magnification: ×400). The glomerular cross-sectional area (A_G) was measured in 50 glomerular profiles per rat by using a computerized image analysis system (Beijing Aeronautic and Aerospace University, Beijing, China). The glomerular volume (V_G) was then calculated as: $V_G = \beta/K[A_G]^{3/2}$, where $\beta = 1.38$ is the size distribution coefficient and $K = 1.1$ is the shape coefficient for glomeruli idealized as a sphere. Tubulointerstitial area in the cortex was evaluated and graded as follows: 0, normal; 1, the area of interstitial inflammation and fibrosis, tubular atrophy, and dilation with cast formation involving <25% of the field; 2, lesion area between 25% and 50% of the field; and 3, lesions involving >50% of the field. The indices for tubulointerstitial injury (TII) were calculated by averaging the grades assigned to all tubule fields. All measurements and scoring were performed on blinded slides.

Western blot analysis

Kidney samples were homogenized and lysed in SDS-PAGE sample buffer, boiled, centrifuged, and the supernatant recovered. The protein content was estimated by the dye binding assay of Bradford, with bovine serum albumin used as a standard. Samples were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes, incubated with blocking buffer for 1 h, and incubated with primary antibody overnight at 4°C. After the blots were washed, they were incubated with a HRP-labeled goat anti-rabbit IgG. The bound secondary anti-

body was detected by enhanced chemiluminescence. Housekeeping protein β -actin was used as a loading control. Positive immunoreactive bands were quantified densitometrically (Leica Q500IW image analysis system; Leica Microsystems, Wetzlar, Germany) and expressed as ratio of collagen type IV, ICAM-1, IL-1, TNF- α , NF- κ B p65, 3-NT, and nephrin to β -actin in optical density units.

Immunohistochemistry

Immunoperoxidase staining for TGF- β 1 was performed on 3-μm sections for formalin-fixed tissue using antigen retrieval (microwave oven heating in 0.1 M sodium citrate pH 6.0 for 12 min) followed by a three-layer streptavidin-biotin-peroxidase complex staining method. Immunostaining of TGF- β 1 in glomeruli and tubulointerstitium was evaluated as described previously (13, 14).

Statistical analyses

Data were expressed as the mean ± S.E.M unless otherwise specified. One-way analysis of variance (ANOVA) with pairwise comparisons according to the Tukey method was used in this study. Since urinary albumin excretion rate followed a non-normal distribution, log transformation was performed prior to statistical analysis of this parameter. Differences were considered significant if the *P* value was less than 0.05.

Results

Clinical and metabolic parameter

Table 1 shows the effects of TGP on clinical and metabolic parameters in diabetic rats induced by STZ. Rats in the control diabetic group had reduced body weight and increased blood glucose level. No effects on body weight and blood glucose were observed with TGP treatment. The ratio of kidney weight to body weight in control diabetic rats was significantly higher than that in normal rats. TGP treatment for 8 weeks did not have any effect on the increase in the ratio of kidney weight to

Table 1. Clinical and metabolic parameters in five groups of rats

Group	Dose (mg/kg)	Blood glucose (mg/dl)	Body weight (g)	Kidney weight / body weight (g/100 g BW)	Creatinine clearance rate (ml/min/100 g BW)	Albumin excretion rate ^a (mg/24 h)
Normal	–	123.53 ± 29.19	458 ± 27.47	0.30 ± 0.04	0.91 ± 0.06	0.38×/÷1.3
Control diabetic	–	469.07 ± 74.58**	271.75 ± 16.86**	0.56 ± 0.05*	0.68 ± 0.05*	1.87×/÷1.1**
Diabetic + TGP	50	445.04 ± 77.43	281.75 ± 25.01	0.52 ± 0.02	0.73 ± 0.06	1.32×/÷1.1 [#]
	100	470.49 ± 75.47	266.4 ± 27.87	0.50 ± 0.06	0.78 ± 0.06	1.15×/÷1.1 [#]
	200	484.16 ± 75.65	318.0 ± 17.8	0.50 ± 0.04	0.78 ± 0.06	0.65×/÷1.1 ^{###}

P*<0.05, *P*<0.01, compared with the normal group; [#]*P*<0.05, ^{###}*P*<0.01, compared with the control diabetic group. Data are expressed as means ± S.E.M. ^aShown as geometric mean tolerance factor. Number of rats in each group was 10.

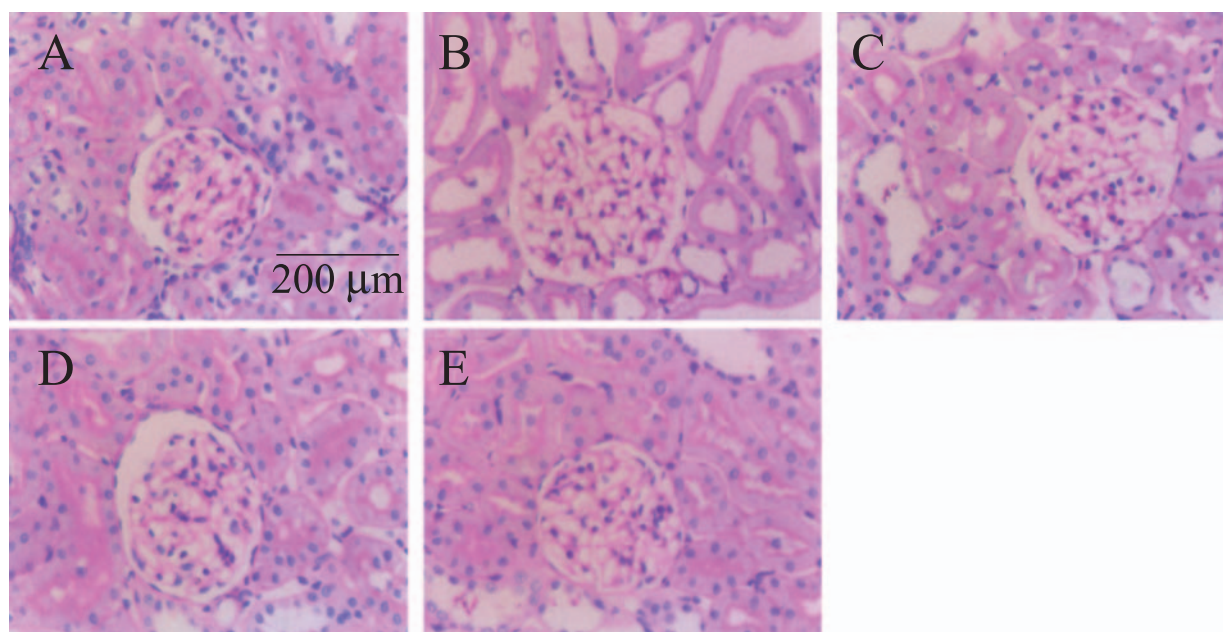


Fig. 1. Representative micrographs of kidney tissue stained with Periodic acid-Schiff from normal rats (A), control diabetic (B), diabetic + TGP 50 mg/kg (C), diabetic + TGP 100 mg/kg (D), and diabetic + TGP 200 mg/kg (E). Original magnification $\times 400$.

Table 2. Morphologic parameters in glomerular volume and tubulointerstitial injury index in five groups of rats

Group	Dose (mg/kg)	Glomerular volume ($10^6 \mu\text{m}^3$)	Tubulointerstitial injury index ^a (Score)
Normal	–	1.00 ± 0.22	0 (0–1)
Control diabetic	–	$1.50 \pm 0.17^{**}$	3 (2–3)**
Diabetic + TGP	50	1.36 ± 0.10	2 (1–3)
	100	$1.23 \pm 0.15^{\#}$	1.5 (0–3) [#]
	200	$1.06 \pm 0.14^{##}$	0.5 (0–1) ^{##}

** $P < 0.01$, compared with the normal group; [#] $P < 0.05$, ^{##} $P < 0.01$, compared with the control diabetic group. Data are expressed as means \pm S.E.M. ^aShown as the median. Number of rats in each group was 10.

body weight of diabetic control rats. In the control diabetic group, Ccr was significantly decreased when compared with normal rats, and the administration of TGP promoted no significant change in Ccr when compared with control diabetic rats. In the control diabetic group, albuminuria was significantly increased when compared to normal rats; treatment with TGP dose-dependently attenuated the increase in albuminuria from the diabetic rats, but this level was still higher than that observed in normal rats.

Plasma lipid and liver function parameters

Compared with normal rats, diabetic rats developed elevated plasma triglycerides and cholesterol levels, and administration of TGP could not change the levels of cholesterol and triglycerides. Treatment with TGP did not affect the serum activity of alanine aminotransferase and aspartate aminotransferase (data not shown).

Renal histology

Figure 1 and Table 2 show histological observations of kidney sections stained with PAS. Rats in the control diabetic group had an increase in V_G when compared with the values in normal rats. Elevated V_G was significantly attenuated by TGP at 50, 100, and 200 mg/kg. Rats in the control diabetic group also had an increase in TII when compared to normal rats; the increased TII was only ameliorated by TGP at 100 and 200 mg/kg.

Renal 1α (IV) collagen expression

Renal 1α (IV) collagen expression is shown in Fig. 2A. Protein band intensities of 1α (IV) collagen are corrected by β -actin and shown in Fig. 2B. Control diabetic rats had 2.7-fold higher expression of 1α (IV) collagen than normal rats; TGP at 50, 100, and 200 mg/kg reduced 1α (IV) collagen protein expression by approximately 47.9%, 60.4%, and 72.9%, respectively.

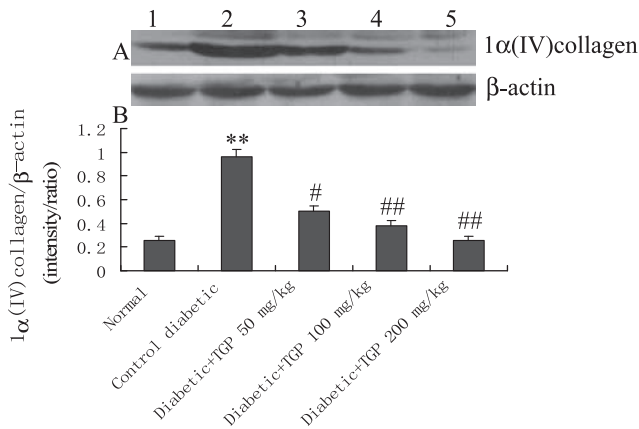


Fig. 2. Western blot analysis of 1α (IV) collagen protein (A) and densitometric analysis (B) in renal tissue from normal, control diabetic, diabetic + TGP 50 mg/kg, diabetic + TGP 100 mg/kg, and diabetic + TGP 200 mg/kg rats. Lane 1: normal, lane 2: control diabetic, lane 3: diabetic + TGP 50 mg/kg, lane 4: diabetic + TGP 100 mg/kg, and lane 5: diabetic + TGP 200 mg/kg. Values are the means ± S.E.M. ** $P < 0.01$ vs normal; # $P < 0.05$, ### $P < 0.01$ vs control diabetic.

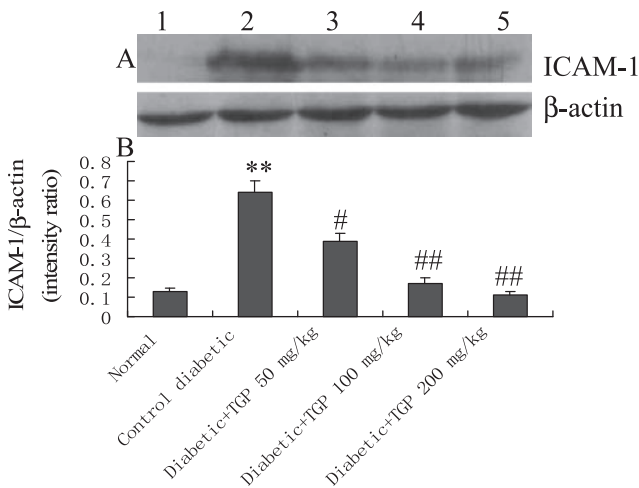


Fig. 3. Western blot analysis of ICAM-1 protein (A) and densitometric analysis (B) in renal tissue from normal, control diabetic, diabetic + TGP 50 mg/kg, diabetic + TGP 100 mg/kg, and diabetic + TGP 200 mg/kg rats. Lane 1: normal, lane 2: control diabetic, lane 3: diabetic + TGP 50 mg/kg, lane 4: diabetic + TGP 100 mg/kg, and lane 5: diabetic + TGP 200 mg/kg. Values are the means ± S.E.M. ** $P < 0.01$ vs normal; # $P < 0.05$, ### $P < 0.01$ vs control diabetic.

Renal ICAM-1 expression

Renal ICAM-1 expression is shown in Fig. 3. Western blot analysis showed that the amount of immunoreactive peptide was increased in the kidneys of control diabetic rats compared to that from normal rats. Densitometric analysis of the Western blot showed a 3.9-fold increase in the amount of ICAM-1 from control diabetic rats compared to that in normal rats; treatment with TGP at

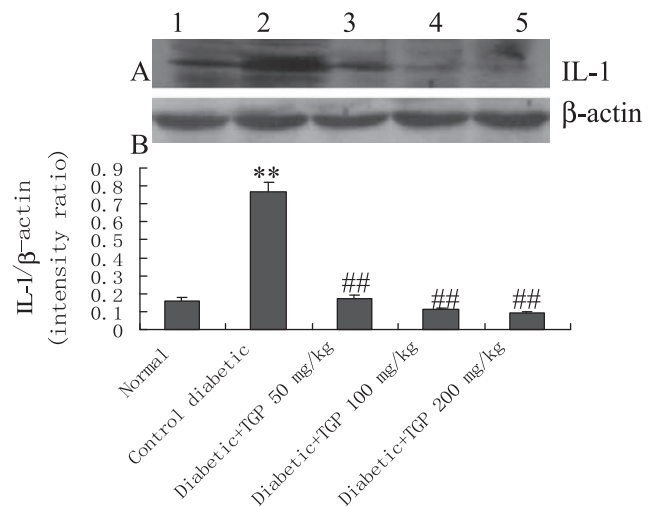


Fig. 4. Western blot analysis of IL-1 protein (A) and densitometric analysis (B) in renal tissue from normal, control diabetic, diabetic + TGP 50 mg/kg, diabetic + TGP 100 mg/kg, and diabetic + TGP 200 mg/kg rats. Lane 1: normal, lane 2: control diabetic, lane 3: diabetic + TGP 50 mg/kg, lane 4: diabetic + TGP 100 mg/kg, and lane 5: diabetic + TGP 200 mg/kg. Values are the means ± S.E.M. ** $P < 0.01$ vs normal, ## $P < 0.01$ vs control diabetic.

50, 100, and 200 mg/kg reduced ICAM-1 protein expression by 39.2%, 72.7%, and 83.8%, respectively.

Renal IL-1 expression

Figure 4 shows the effect of TGP on renal IL-1 expression in diabetic rats induced by STZ. Densitometry of the Western blots revealed a 3.8-fold increase of IL-1 protein expression in the control diabetic rats kidneys compared with normal rats. Treatment with TGP at 50, 100, and 200 mg/kg inhibited diabetic-induced increments in IL-1 protein expression by 77.7%, 86.4%, and 88.6%, respectively.

Renal TNF-α expression

Figure 5 shows the effect of TGP on renal TNF-α expression in diabetic rats induced by STZ. Western blot analysis indicated an increase in the amount of immunoreactive peptide in the kidneys of control diabetic rats compared to that from normal rats. Densitometric analysis of the Western blot showed an 8.2-fold increase in the amount of TNF-α from control diabetic rats as compared to the amount in normal rats; treatment with TGP at 50, 100, and 200 mg/kg reduced TNF-α protein expression by 46.5%, 86.0%, and 93.8%, respectively.

Renal NF-κB p65 expression

Figure 6 shows the effect of TGP on renal NF-κB p65 expression in diabetic rats induced by STZ. Densito-

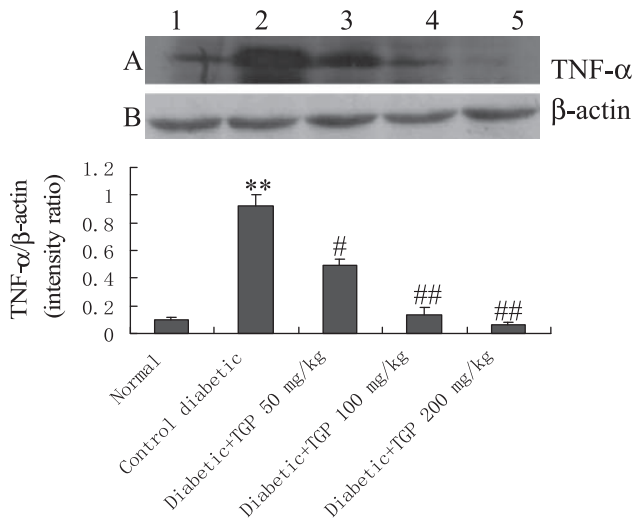


Fig. 5. Western blot analysis of TNF- α protein (A) and densitometric analysis (B) in renal tissue from normal, control diabetic, diabetic + TGP 50 mg/kg, diabetic + TGP 100 mg/kg, and diabetic + TGP 200 mg/kg rats. Lane 1: normal, lane 2: control diabetic, lane 3: diabetic + TGP 50 mg/kg, lane 4: diabetic + TGP 100 mg/kg, and lane 5: diabetic + TGP 200 mg/kg. Values are the means \pm S.E.M. ** P <0.01 vs normal; # P <0.05, ## P <0.01 vs control diabetic.

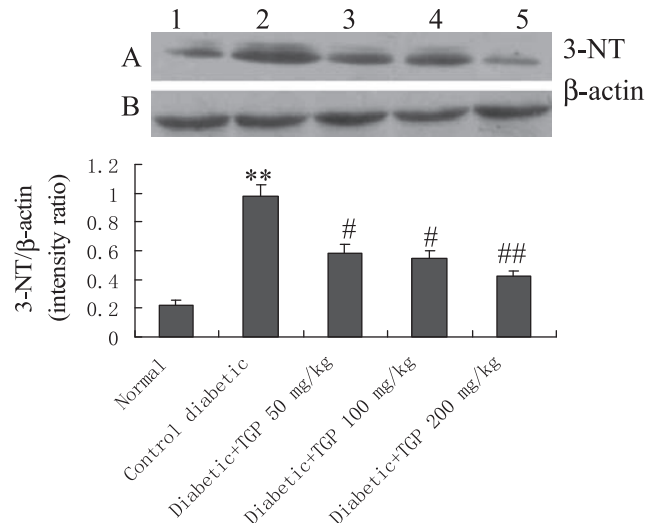


Fig. 7. Western blot analysis of 3-NT protein (A) and densitometric analysis (B) in renal tissue from normal, control diabetic, diabetic + TGP 50 mg/kg, diabetic + TGP 100 mg/kg, and diabetic + TGP 200 mg/kg rats. Lane 1: normal, lane 2: control diabetic, lane 3: diabetic + TGP 50 mg/kg, lane 4: diabetic + TGP 100 mg/kg, and lane 5: diabetic + TGP 200 mg/kg. Values are the means \pm S.E.M. ** P <0.01 vs normal; # P <0.05, ## P <0.01 vs control diabetic.

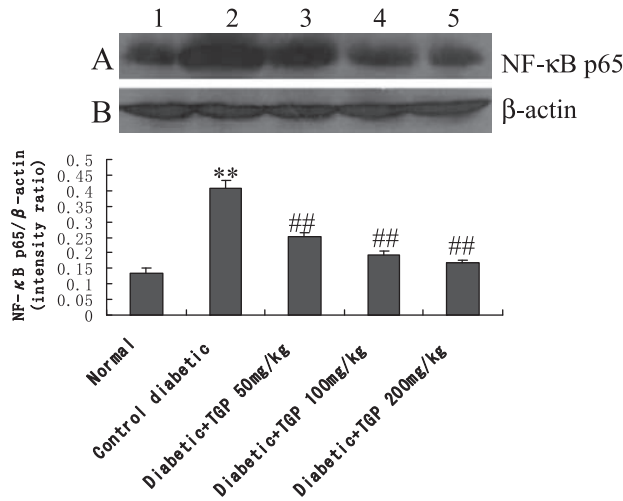


Fig. 6. Western blot analysis of NF- κ B p65 protein (A) and densitometric analysis (B) in renal tissue from normal, control diabetic, diabetic + TGP 50 mg/kg, diabetic + TGP 100 mg/kg, and diabetic + TGP 200 mg/kg rats. Lane 1: normal, lane 2: control diabetic, lane 3: diabetic + TGP 50 mg/kg, lane 4: diabetic + TGP 100 mg/kg, and lane 5: diabetic + TGP 200 mg/kg. Values are the means \pm S.E.M. ** P <0.01 vs normal, ## P <0.01 vs control diabetic.

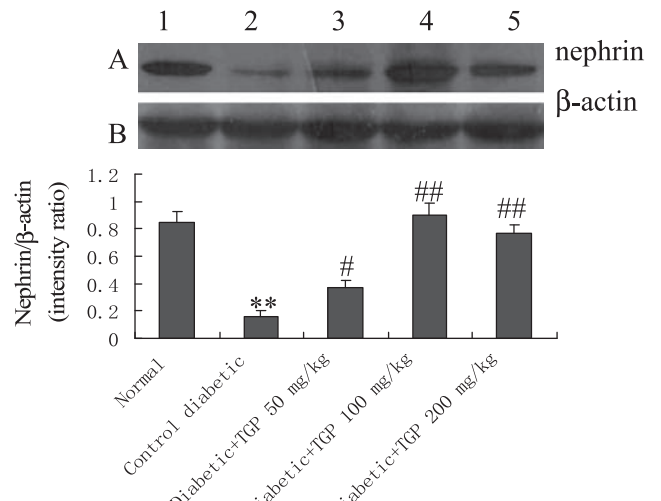


Fig. 8. Western blot analysis of nephrin protein (A) and densitometric analysis (B) in renal tissue from normal, control diabetic, diabetic + TGP 50 mg/kg, diabetic + TGP 100 mg/kg, and diabetic + TGP 200 mg/kg rats. Lane 1: normal, lane 2: control diabetic, lane 3: diabetic + TGP 50 mg/kg, lane 4: diabetic + TGP 100 mg/kg, and lane 5: diabetic + TGP 200 mg/kg. Values are the means \pm S.E.M. ** P <0.01 vs normal; # P <0.05, ## P <0.01 vs control diabetic.

metric measurement of Western blot analysis revealed a 2.0-fold increase in NF- κ B p65 protein expression in control diabetic rats kidneys compared with normal rats. TGP at 50, 100, and 200 mg/kg inhibited diabetic-induced increments in NF- κ B p65 protein expression by 38.4%, 53.0%, and 59.0%, respectively.

Renal 3-NT expression

Figure 7 shows the effect of TGP on renal 3-NT production in diabetic rats induced by STZ. Western blot analysis showed that there was an increase in the amount of immunoreactive peptide in the kidney of control diabetic rats compared to that from normal rats.

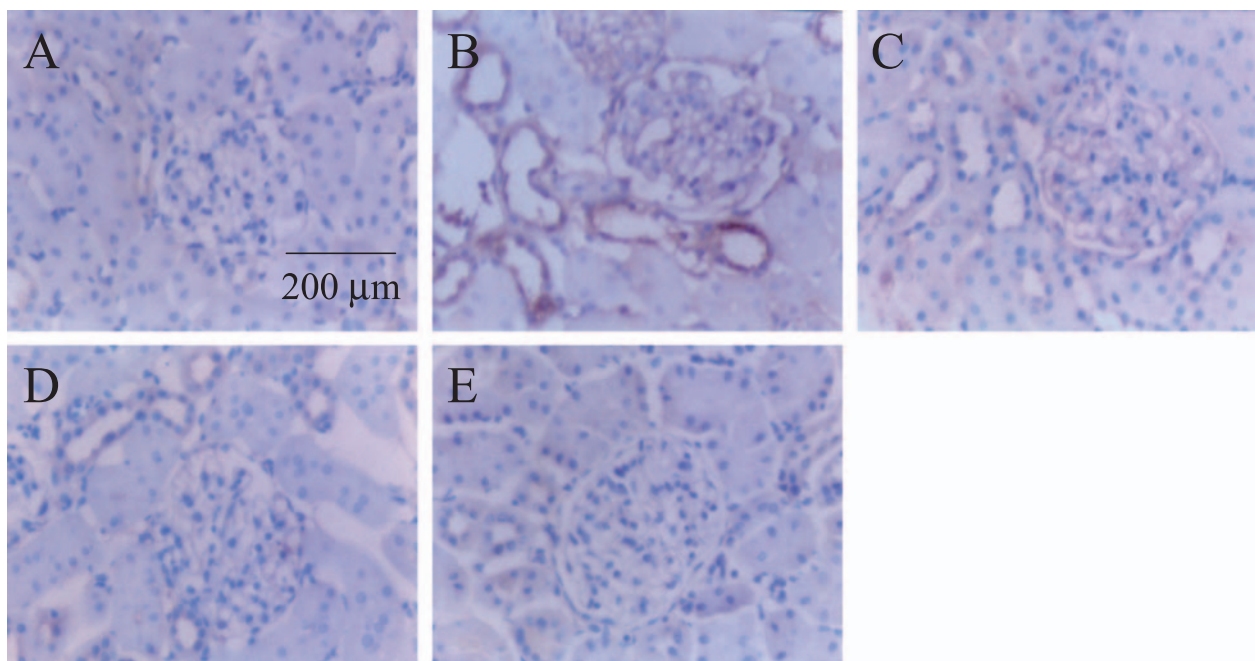


Fig. 9. Representative micrographs of immunostaining for TGF- β 1 in renal tissue from normal rats (A), control diabetic (B), diabetic + TGP 50 mg/kg (C), diabetic + TGP 100 mg/kg (D), and diabetic + TGP 200 mg/kg (E) rats. Original magnification \times 400.

Table 3. Histopathological evaluation of the kidney for TGF β -1 in five groups of rats

Group	Dose (mg/kg)	Glomeruli ^a (Score)	Tubuliinterstitium (%)
Normal	–	0.5 (0–1)	5.33 \pm 0.49
Control diabetic	–	2 (1–3)**	21.42 \pm 2.52**
Diabetic + TGP	50	1.7 (1–3)	15.82 \pm 1.24
	100	1 (0–2) [#]	12.79 \pm 1.69 [#]
	200	0.5 (0–1) ^{##}	7.02 \pm 0.95 ^{##}

** P <0.01, compared with normal group; [#] P <0.05, ^{##} P <0.01, compared with the control diabetic group. Data are expressed as means \pm S.E.M. ^ashown as median. Number of rats in each group was 10.

Densitometry of the Western blot showed a 3.4-fold higher amount of 3-NT in the control diabetic rats as compared to that in normal rats, but it was significantly and dose-dependently reduced by TGP administration.

Renal nephrin expression

To further reveal the mechanism responsible for the prevention of albuminuria in TGP-treated diabetic rats, we used Western blotting to study the expression of nephrin, a key protein of the glomerular slit membrane. The results are shown in Fig. 8. The expression of nephrin in the kidney of diabetic rats was significantly lower than that in normal rats. The decreased nephrin in control diabetic group was significantly increased by TGP treatment.

Renal TGF- β 1 expression

Renal TGF- β 1 expression is shown in Fig. 9 and Table 3. In the kidneys of normal rats, there was minimal staining for TGF- β 1. Diabetes was associated with a prominent increase in renal TGF- β 1 immunostaining, which was seen in glomeruli and tubulointerstitium; increased expressions of TGF- β 1 in glomeruli and tubulointerstitium were only ameliorated by TGP at 100 and 200 mg/kg.

Discussion

Features of early diabetic renal changes are glomerular hyperfiltration, glomerular and renal hypertrophy, increased urinary albumin excretion, increased basement membrane thickness, and mesangial expansion with the

accumulation of extracellular matrix proteins such as collagen, fibronectin, and laminin. Our present study shows that TGP treatment provides renoprotection in a model of experimental diabetes. This has been shown for a range of functional and structural parameters including albuminuria and glomerular and tubulointerstitial injury, without changing blood glucose and plasma triglycerides and cholesterol levels in STZ-induced diabetic rats. TGP markedly reduced the expression of 1α (IV) collagen in diabetic kidneys. Our findings suggest that TGP could prevent the development of diabetic nephropathy, independently of the metabolism factor. Our study was in agreement with the findings of Nakagawa and coworkers (15) who demonstrated that keishibukuryogan, which contains TGP, protects the kidney function of diabetic WBN/Kob rats. In this study, we further investigated the mechanism by which TGP protects against renal injury in diabetic rats.

ICAM-1 is one of the major adhesion molecules that promote leukocyte attachment to the endothelium and their transmigration by its binding to β -integrins on leukocyte cell surfaces. Previous studies showed that the expression of ICAM-1 was rapidly induced and maintained for a long time in renal tissues after induction of diabetes in experimental type 1 diabetic rats (16). Furthermore, macrophage infiltration was blocked by anti-ICAM-1 antibody, confirming that ICAM-1 mediated macrophage infiltration into the diabetic kidney (16). Recent studies have demonstrated that ICAM-1-deficient mice were protected from renal injury after induction of diabetes, suggesting that the inflammatory process was a critical factor for the development of diabetic nephropathy (17). In the present study, we for the first time reported that TGP could inhibit ICAM-1 expression in diabetic kidney. Increased levels of inflammatory parameters, including pro-inflammatory cytokines such as IL-1 and TNF- α , have been found in diabetic patients with renal injury (18). Regarding IL-1, its increased expression in diabetic glomeruli has been related to an overexpression of adhesion molecules and chemotactic factors. Furthermore, IL-1 could also act as a growth factor on mesangial cells to increase expression of collagen, TGF- β , and platelet-derived growth factor. An increase in TNF- α has been related to cytotoxicity to in glomerular, mesangial, and epithelial cells and might induce direct renal damage. In our study, we found that administration of TGP might lower increased expression of IL-1 and TNF- α in diabetic kidney. The expression of activated NF- κ B p65 was also markedly increased in diabetic kidney, which was inhibited by treatment with TGP. A number of studies have demonstrated a key role for NF- κ B in the stimulation of glucose-stimulated ICAM-1, IL-1, and TNF- α expression. Thus, the inhibited

effect of TGP on ICAM-1, IL-1, and TNF- α was likely to be mediated through inhibition of NF- κ B activation (2, 19). Recently, Zhu et al. (20) suggested that TGP (25, 50, and 100 mg/kg) inhibited the secondary inflammatory reaction, bone destruction, and ultrastructure ultrastructural changes of synoviocytes in adjuvant arthritis (AA) rats. Furthermore, the administration of TGP (50 and 100 mg/kg) in AA rats significantly decreased the production of IL-1 and TNF- α by macrophages-like synoviocytes (MLS) (21). In addition, the increased phosphorylation of mitogen-activated protein kinases (MAPKS), and cell proliferation in fibroblast-like synoviocytes (FLS) stimulated by supernatants of MLS in AA rats could also be inhibited by TGP (50 and 100 mg/kg). Their study suggested that TGP possess exerts anti-inflammatory effects by modulating the pro-inflammatory mediators production from MLS and phosphorylation of MAPKS from FLS (21). Further studies are required to determine to investigate whether the effect of TGP on IL-1 and TNF- α is also through the above-mentioned mechanism in diabetic kidney.

TGP has been proved to have efficacy of antioxidative actions though inhibiting TBA-reactive substance, an index of endogenous lipid peroxidation, and increasing antioxidant enzyme activities such as superoxide dismutase, and catalase as well as glutathione peroxidase following cerebral ischemia and immunological liver injury (22, 23). Furthermore, Liao et al. (24) reported that extract of paeony showed antioxidative activity and inhibited the production of nitric oxide in vitro. 3-NT, a by-product of the reaction between ONOO $^-$ and proteins, is a potential biomarker of reactive-nitrogen species and increases in diabetic renal tissue (25). The 3-NT level was also significantly ameliorated by TGP administrations, suggesting that TGP alleviated oxidative stress by inhibiting the generation of reactive-nitrogen species such as ONOO $^-$.

An early marker for diabetic nephropathy is the occurrence of microalbuminuria. Nephritin, a member of the immunoglobulin superfamily, has been recently proven to be an integral membrane protein of the slit diaphragm mutated in the Finnish type 1 congenital nephritic syndrome. Previous studies showed reductions in nephritin mRNA and protein expression in human and STZ diabetic rat models (26, 27). In the present study, administration of TGP restored the level of nephritin protein in diabetic animals. Recently, using conditionally immortalized reporter podocytes, Takano et al. (28) found that bystander macrophages as well as macrophage-derived cytokines IL-1 β and TNF- α markedly suppressed the activity of the nephritin gene promoter in podocytes. Our studies showed that TGP might

upregulate expression of nephrin in the kidney from diabetic rats through a mechanism that may be at least partly correlated with downregulated expressions of IL-1 and TNF- α protein. TGF- β 1 has been consistently implicated as playing a pivotal role in the pathogenesis of diabetic nephropathy (29, 30). In the present study, as glomerular and tubulointerstitial expansion increased and proteinuria progressed, the expression of TGF- β 1 was markedly up-regulated; this overexpression of TGF- β 1 observed in the kidneys of diabetic rats was attenuated by TGP. These data suggested that TGP inhibited glomerular and tubulointerstitial expansion by controlling renal overexpression of TGF- β 1 in diabetic rats.

Potential toxic effects of TGP should also be investigated in detail. The mean body weight of animals treated with TGP was slightly higher, although it is not statistically different than that of diabetic rats. Data (not shown) from the present study indicated that TGP did not affect survival, and serum alanine aminotransferase and aspartate aminotransferase activities in either diabetic or control rats.

In conclusion, the present study provides scientific evidence for the preventive and therapeutic potential of TGP against renal damage associated with anti-inflammatory and antioxidant effects in diabetic kidneys.

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