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Original Article

Stilbene glucoside extract of *Polygonum multiflorum* ameliorates microalbuminuria and renal tubular injury in diabetic rats with nephropathy

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Abstract: Background: Oxidative stress caused by hyperglycaemia is known to be a major molecular mechanism of diabetic nephropathy. *2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside* (TSG), an active component extract from *Polygonum multiflorum* Thunb, is known to have antioxidant and anti-inflammatory properties. Aim of the study: We investigated the possible protective mechanisms of TSG in a diabetic nephropathy model in rats. Methods: Treatment with TSG significantly reduced 24-hour urine output, urinary albumin excretion rate (UAER), urinary α1-microglobulin (Uα1-MG), and the ratio of microalbuminuria and urine creatinine (MAU/Ucr) in rats with diabetic nephropathy. Results: The activities of superoxide dismutase (SOD) in renal homogenate were markedly increased, whereas malondialdehyde (MDA) and inducible nitric oxide synthase (iNOS) levels were decreased significantly after administration of high-dose TSG in diabetic rats. Furthermore, high-dose TSG dramatically inhibited diabetes-induced over-expression of nitrotyrosine (NT) and Sulfonylurea receptor 1 (SUR1). Conclusion: These findings indicate that the protective mechanism of TSG in diabetic nephropathy involves amelioration of microalbuminuria and alleviation of oxidative stress injury.

Keywords: 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside (TSG), diabetic nephropathy, oxidative stress, microalbuminuria, nitrotyrosine (NT), sulphonylurea receptor 1 (SUR1)

Introduction

Albuminuria is predictive both for progressive renal function loss to diabetic nephropathy, and for cardiovascular morbidity and mortality: the higher the albuminuria level, the more chance of end stage renal disease (ESRD) and cardiovascular complications [1]. Hyperglycemia is known to impair the cellular pathways that make up the critical microenvironment, which promotes apoptosis of cells, such as renal tubular epithelial cells, leading to renal functional and pathological changes that are associated with diabetic nephropathy [2]. High glucose levels are reported to induce oxidative insult and apoptosis of renal tubular cells, which is associated with a heightened inflammatory state in diabetic nephropathy [3]. Oxidative stress in the cell occurs from the enhanced generation of reactive species, such as reactive oxygen species and reactive nitrogen species, with increased lipid peroxidation and advanced glycation end (AGE) products in diabetic nephropathy [4]. High glucose levels generate reactive species that often play a significant role in glucose-induced cellular dysfunction in diabetes [2]. Auto-oxidation of glucose, glucose metabolism and formation of AGEs are possible sources of ROS. Therefore, antioxidants have a potential therapeutic role in the treatment of diabetic complications [5].

Polygonum multiforum, a traditional Chinese medicinal herb, has been widely used as a tonic and an anti-aging agent [6, 7]. 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside (TSG) (**Figure 1**) is one of the main bioactive constituents extracted from *P. multiflorum* Thunb and is known to have antioxidant, anti-inflammatory and anti-atherosclerotic effects, in addition to its unique action in improving memory and learning ability [8-10]. Studies have shown that

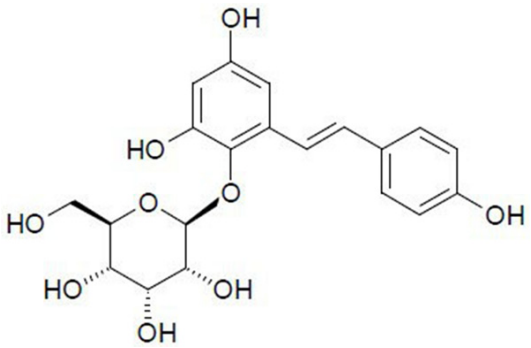

Figure 1. Chemical structure of *2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside.*

TSG may attenuate the oxidative stress-induced apoptosis of PC12 cells by inhibiting the generation of ROS and modulating the activation of Jun N-terminal kinase (JNK), PI3K/Akt and ROS-NO pathways [11, 12]. It has been demonstrated that inhibition of COX-2 ameliorates renal injury in diabetic and hypertensive rats [13]. TSG has also been reported to inhibit COX-2 enzyme activity and expression [9]. Moreover, TSG reportedly ameliorated diabetic nephropathy in rats through inhibition of SIRT1 and TGF-β1 pathways [14]. Besides, the polyphenolic structure of TSG is similar to that of resveratrol, which was reported ameliorating renal injury and enhancing mitochondrial biogenesis with Mn-SOD dysfunction in the kidney of *db/db* mice through improvement of oxidative stress via normalization of Mn-SOD function and glucose-lipid metabolism[5], prompting us to examine the role of TSG in diabetic nephropathy.

We propose that TSG may play a protective role in diabetic nephropathy by preventing oxidative stress. In the present study, we sought to investigate the protective effects of TSG on albuminuria and diabetic nephropathy. To verify the underlying mechanism of TSG against diabetic nephropathy, major oxidative stress and inflammatory markers, such as lipid peroxidation products and peroxynitrite ($ONOO^-$) were measured.

Materials and methods

Reagents

TSG (molecular weight 406, N98% purity, water soluble) was obtained from National Institutes for Food and Drug Control (Beijing, China). Streptozocin (STZ) was obtained from Sigma-Aldrich (Sigma Chemical Co. St. Louis, Mo., USA). Malondialdehyde (MDA) and superoxide dismutase (SOD) assay kits were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Anti-NT and anti-SUR1 antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-NOS antibodies, DAB horseradish peroxidase color development kit, normal goat serum blocking solution, phosphate buffered saline, and sodium citrate buffer were purchased from the Wuhan Boster Biological Technology (Wuhan, China). β-actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA), while the secondary antibodies were obtained from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd (Beijing, China). Acquada protein gel staining solution and ECL were obtained from Round-Biological Technology Co., Ltd. (HongKong, China). The protein sample buffer (4×) was obtained from the Beijing Soledad Symbol Technologies Ltd (Beijing, China).

Animals and treatment

Male Wistar rats, weighing 200±20 g, were obtained from the Weitonglihua Animal Technology Company (Beijing, China). The rats were housed in a temperature- and humidity-controlled environment, with free access to tap water and standard rodent chow. For the nephrectomy, the rat was anesthetized via intraperitoneal injection of urethane (1 mg/kg), and the left kidneys were removed after ligation of the renal artery, vein and ureter. A single dose (60 mg/kg) of STZ (Sigma Chemical Co. St. Louis, Mo., USA) in citrate buffer (pH 4.5) was injected intraperitoneally. Rats in the control rats were administered an injection of only the buffer. The levels of blood glucose were determined 72 h after injection of STZ, and the rats were confirmed to have developed diabetes, if fasting blood glucose levels were >16.7 mmol/L. Rats were randomly divided into five groups as follows: normal control group (NC), normal control rats treated with 20 mg/kg/d TSG group (NC+TSG), untreated diabetic nephropathy group (DN), low-dose TSG-treated group (10 mg/kg/d) (DN+TSG_1), and high-dose TSG-treated group (20 mg/kg/d) (DN+TSG_2). Body weight and blood glucose levels were monitored regularly. After treatment with TSG

Table 1. Effect of TSG on body weight among groups of rats

	N	2 weeks	N	4 weeks	N	6 weeks	N	8 weeks
NC	(12)	285.83±9.73	(12)	321.25±33.44	(12)	354.16±23.62	(12)	372.08±8.90
NC+TSG	(12)	275.83±10.18	(12)	286.66±10.94	(12)	289.58±10.96	(12)	292.91±17.11
DN	(20)	272.50±11.52#	(20)	292.25±12.51#	(20)	290.50±10.62#	(20)	277.89±11.21#
DN+TSG$_1$	(18)	271.38±14.93	(18)	293.88±22.98	(18)	282.77±7.11	(16)	281.56±7.23
DN+TSG$_2$	(20)	272.50±6.38	(20)	273.25±11.03♦	(20)	282.00±10.43	(17)	280.00±9.35

NC: Normal control; NC+TSG: normal control treated with TSG (20 mg/kg/d); DN: untreated diabetic nephropathy; DN+TSG$_1$: low-dose TSG-treated group (10 mg/kg/d); DN+TSG$_2$: high dose TSG-treated group (20 mg/kg/d); TSG: 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside. #P < 0.05 vs. NC; ♦P < 0.05 vs. DN.

Table 2. Fasting blood glucose levels at 2nd and 8th week by study group

Group	N	2 weeks	N	8 weeks
NC	(12)	5.79±1.01	(12)	6.49±0.55
DN	(18)	22.85±3.82#	(19)	25.46±3.95#
DN+TSG$_1$	(18)	20.50±2.99	(16)	23.87±2.39
DN+TSG$_2$	(20)	21.91±3.16#	(17)	24.78±3.00#

NC: Normal control; DN: untreated diabetic nephropathy; DN+TSG$_1$: low-dose TSG-treated group (10 mg/kg/d); DN+TSG$_2$: high-dose TSG-treated group (20 mg/kg/d). TSG: 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside. #P < 0.05 vs. NC.

for 8 weeks, 24 h urinary protein and blood samples were collected before the rats were sacrificed. Kidneys were removed and weighed. Renal cortex tissues were harvested and stored in liquid nitrogen until further processing.

MDA & SOD assay

Lipid peroxidation was assayed by the measurement of malondialdehyde (MDA) according to the lipid peroxidation MDA assay kit description. In brief, Kidney tissues were prepared by sonication in ice-cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 m MDTT). Then the homogenate were centrifuged at 1600× g for 10 min at 4°C to remove debris. The super natant was subjected to the measurement of MDA levels and the protein contents. We used a protein assay kit (Bio Rad Laboratories, Hercules, USA) to quantify protein concentration. MDA levels were then normalized to milligram protein. The samples were scanned with a spectrophotometer at absorbance from 530 to 540 nm.

The activity of total SOD was detected using Total Superoxide Dismutase Assay Kit with NBT. Kidney tissues homogenate were prepared according to the methods above. Samples were detected according to the manufacturer's protocol. The rate of change in absorbance was converted to units of enzyme activity, determined from a standard curve. Enzyme activity was then standardized to milligram protein. The samples were scanned with a spectrophotometer at absorbance 560 nm.

Histopathological examination

Kidneys were removed for histological analysis under a light microscope. Renal specimens from the used experimental groups were removed and immediately fixed in 10% phosphate buffered formalin (pH 7.4) for 24 hours, followed by dehydrating in ascending grades of ethyl alcohol, cleared in xylene and mounting in neutral gum. Four μm thick were cut and stained with hematoxylin/eosin.

Western blot analysis for SUR1 & NT

Western blot analysis was performed as described previously [15]. Briefly, 30 μg of protein extracted from each sample of renal cortical tissue was subjected to SDS-PAGE by electrophoresis under reducing conditions and transferred to PVDF membrane (Millipore Corporation, Bedford, MA, USA). The blotted membrane was then blocked with 5% nonfat dry milk in 1× TBS (0.1% Tween 20) for 1 h at room temperature and incubated overnight at 4°C with primary antibodies to SUR1 (Santa Cruz Biotechnology), NT (Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich). Following incubation with secondary antibodies (Promega) for 1 h at room temperature, the blotted membrane was detected by enhanced chemiluminescence method with SuperSignal® West Pico Chemiluminescent Substrate (Thermo SCIENTIFIC, USA) and captured on X-ray film. The densitometry assay was performed using Gel Doc XR System (Bio-Rad Laboratories, USA)

Table 3. Effect of TSG on urine output, UAER, Uα1-MG, MAU/Ucr among groups of rats at 2nd and 8th week

	N	Urine output		UAER		Uα1-MG		MAU/Ucr	
		2 weeks	8 weeks	2 weeks	8 weeks	2 weeks	8 weeks	2 weeks	8 weeks
NC	12	7.16±1.40	7.50±1.31	16.37±6.69	16.51±4.21	12.75±0.78	12.75±0.78	0.02±0.01	0.02±0.01
NC+TSG	12	7.75±2.00	8.00±1.47	17.35±5.89	16.53±3.61	12.98±0.86	12.98±0.86	0.02±0.01	0.02±0.00
DN	19	12.90±2.44#	13.52±2.31#	15.41±7.05	52.47±21.17#	12.55±1.00	50.82±9.21#	0.02±0.01	4.02±0.23#
DN+TSG$_1$	16	12.16±3.58	13.06±2.95	15.24±5.97	48.16±10.35	13.55±1.58	52.13±9.35	0.02±0.01	4.08±0.58
DN+TSG$_2$	17	11.65±2.18	11.64±2.14♦	15.41±5.16	33.37±5.99♦	12.74±2.65	33.04±5.89♦	0.02±0.01	0.45±0.29♦

TSG: 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside; UAER: Urinary albumin excretion rate; Uα1-MG: urinary α1-microglobulin, MAU/Ucr: microalbuminuria: urine creatinine ratio; NC: Normal control; NC+TSG: normal control treated with TSG (20 mg/kg); DN: untreated diabetic nephropathy; DN+TSG$_1$: low-dose TSG-treated group (10 mg/kg/d); DN+TSG$_2$: high-dose TSG-treated group (20 mg/kg/d). #P < 0.05 vs. NC; ♦P < 0.05 vs. DN.

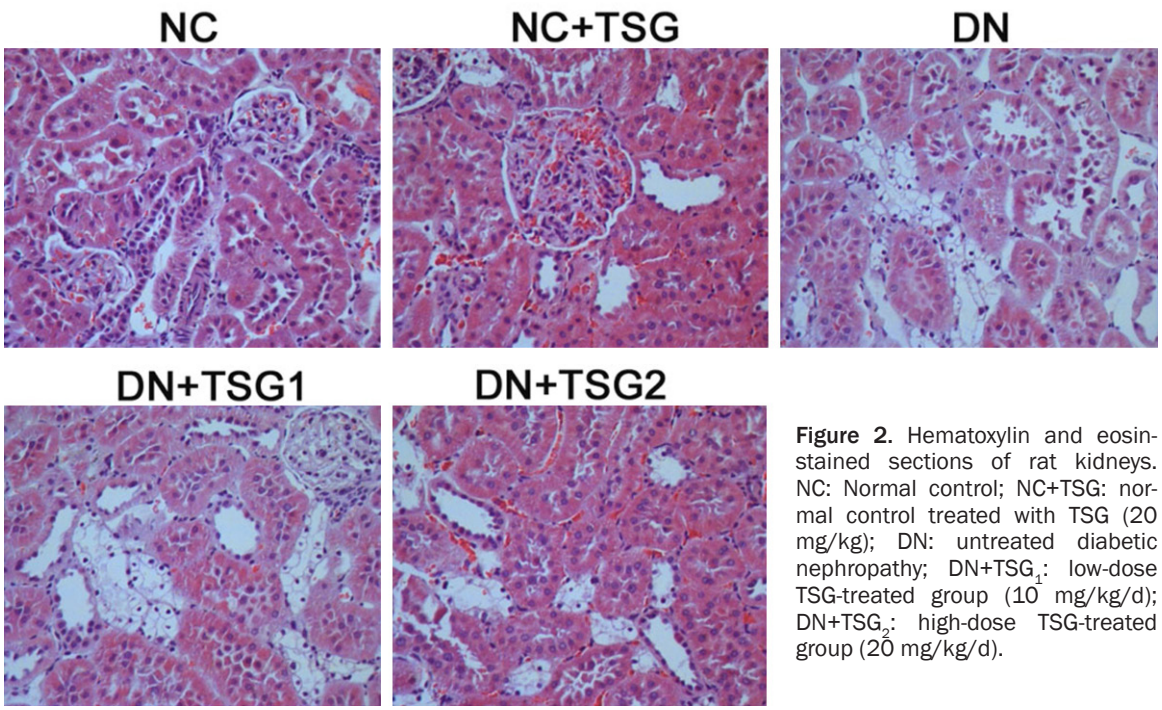

Figure 2. Hematoxylin and eosin-stained sections of rat kidneys. NC: Normal control; NC+TSG: normal control treated with TSG (20 mg/kg); DN: untreated diabetic nephropathy; DN+TSG$_1$: low-dose TSG-treated group (10 mg/kg/d); DN+TSG$_2$: high-dose TSG-treated group (20 mg/kg/d).

and analyzed by Quantity-one Protein Analysis Software (Bio-Rad Laboratories, USA).

Transmission electron microscopy

Kidneys were immersion-fixed in cold 2.4% glutaraldehyde in PBS buffer, post-fixed in 2% buffered osmium tetroxide, dehydrated in graded ethanols, and embedded in Spurr resin. Samples were sectioned at 70 nm, placed on copper for transmission electron microscopy, and stained with uranyl acetate and lead citrate. Percent foot process effacement was calculated as previously described [16]. Samples were screened on a Hitachi H-7100 Transmission Electron Microscope. Analytical measurements were performed using Axiovision software.

Statistical analysis

Values were expressed as mean ± S.E.M. Statistical differences between two groups were analyzed by the unpaired Student's *t* test and differences between multiple groups of data were analyzed by one-way ANOVA with Bonferroni correction (Graph Pad Prism 4.0, San Diego, CA, USA). A $P < 0.05$ was considered statistically significant.

Results

Effect of TSG on body weight among groups of rats

All animals completed the 8-week treatment and the body weight was recorded every two

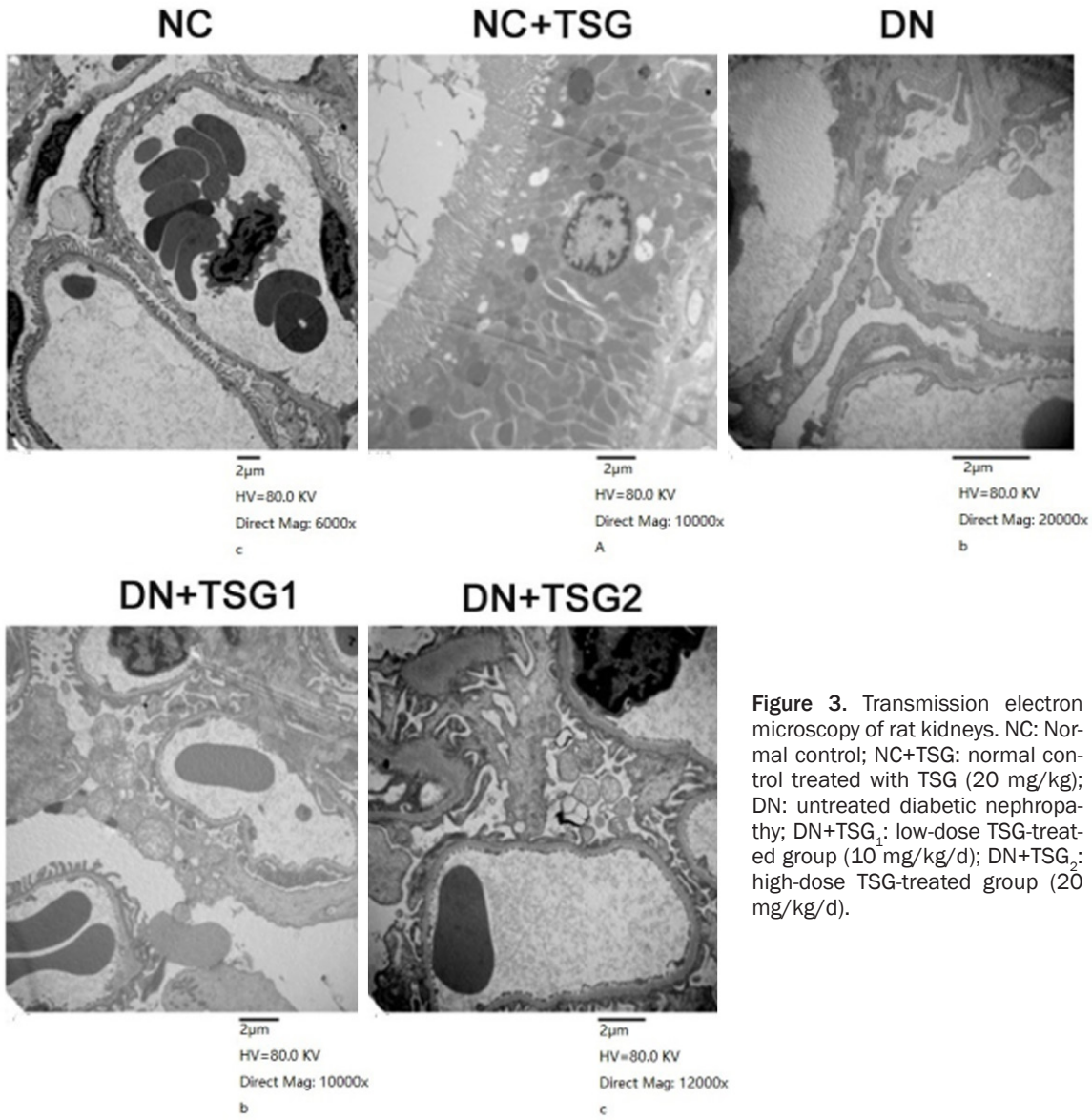

Figure 3. Transmission electron microscopy of rat kidneys. NC: Normal control; NC+TSG: normal control treated with TSG (20 mg/kg); DN: untreated diabetic nephropathy; DN+TSG_1: low-dose TSG-treated group (10 mg/kg/d); DN+TSG_2: high-dose TSG-treated group (20 mg/kg/d).

weeks. The diabetic nephropathy rats had a significantly lower body weight compared with controls. However, no significant differences were observed in the two TSG-treatment groups when compared with the DN group (**Table 1**).

Effect of TSG on fasting blood glucose in rats with diabetic nephropathy

Compared with the control group, the DN group showed significantly higher blood glucose levels. At 8 weeks, there were no differences in fasting blood glucose levels among the two TGS-treatment groups when compared with DN group (**Table 2**).

Effect of TSG on renal function of rats with diabetic nephropathy

Diabetic nephropathy rats developed polyuria, with their 24-hour urine output being substantially higher than that in the control group. Furthermore, diabetic nephropathy rats also had a substantially increased urinary albumin excretion rate (UAER), urinary α1-Microglobulin (Uα1-MG), and an increased microalbuminuria/urine creatinine (MAU/Ucr) ratio at 8 weeks; these parameters were significantly reduced in high-dose TSG-treated group when compared with that in the DN group (**Table 3**). These findings suggest that TGS may have improved the renal function in diabetic nephropathy rats.

Table 4. Comparison of MDA & SOD levels in kidney tissue among groups of rats

	N	MDA	SOD
NC	12	2.52±0.33	99.39±5.65
DN	18	4.99±1.18#	74.51±5.14#
DN+TSG$_2$	17	3.62±0.53♦	83.39±4.45♦

MDA, Malondialdehyde; SOD: Superoxide dismutase; NC: Control group; DN: untreated diabetic group; DN+TSG$_2$: high dose TSG group (20 mg/kg/d). #P < 0.05 vs. NC; ♦P < 0.05 vs. DN.

Table 5. Comparison of iNOS & SUR1 IOD by study group

	N	iNOS (IOD)	SUR1 (IOD)
NC	12	10698.92±3975.71	5800.54±1416.72
DN	18	21527.93±8319.30#	16126.10±9323.79#
DN+TSG$_2$	17	16771.24±5910.08♦	11107.19±4786.76♦

iNOS: inducible Nitric oxide synthase; SUR1: Sulfonylurea receptor 1; inducible Nitric oxide synthase integral optical density NC: Control group; DN: untreated diabetic group; DN+TSG$_2$: high dose TSG group (20 mg/kg). #P < 0.05 vs. NC; ♦P < 0.05 vs. DN.

Changes in kidney morphology

Figure 2 shows representative photomicrographs of renal tissue from rats, stained with hematoxylin and eosin. Injury was found to be more severe in the DN group and low dose TSG group (10 mg/kg/d), when compared with high dose TSG group (20 mg/kg/d). The DN group and low dose of TSG-treated group (10 mg/kg/d) showed a decrease in proximal tubular epithelial cells, vacuolar degeneration.

As shown in transmission electron microscopy (**Figure 3**), the untreated diabetic group and low dose TSG group (10 mg/kg/d) showed focal glomerular foot process fusion, mitochondrial degeneration, thickening of glomerular basement membrane, which was improved in the high dose TSG group (20 mg/kg/d).

Effect of GST on renal MDA and SOD levels

Diabetic nephropathy rats exhibited a significant increase in lipid peroxidation, as indicated by the marked increase in renal MDA levels and decrease in SOD levels when compared with the control rats. Treatment with high-dose TSG in diabetic nephropathy rats led to significant reduction in MDA levels accompanied with significant increase in SOD levels in kidney tissue, in comparison to that in the DN group (**Table 4**).

Immunohistochemical staining for iNOS and SUR1 protein

On immunohistochemical staining, the iNOS integral optical density (IOD) of the DN group was found to be markedly higher, but was reduced in the high-dose TSG group at 8 weeks. Similar to the iNOS, the SUR1 IOD values in DN group were higher than that in the control group, which was decreased in the high-dose TSG group (**Table 5** and **Figure 4**).

Expression of NT, SUR1 protein

In line with the immunohistochemical study, Western blot analyses revealed an increased expression of SUR1 in the DN group as compared to that in the control group, which was reversed after 8 weeks treatment with high-dose TSG. Consistent with the SUR1 expression, the NT expression in the untreated diabetic group was higher than that in the control group, and high dose TSG treatment was associated with a significant decrease (**Table 6** and **Figure 5**).

Discussion

In this study treatment with TSG, an active component extracted from *P. multiflorum* Thunb, was associated with attenuation of diabetic nephropathy lesions as reflected in the reduced 24 h urinary protein excretion, decrease in MDA expression, and increase in SOD levels. The histopathogical examination showed that TSG ameliorated diabetes-induced lesions. The protective mechanisms of TSG may involve inhibition of oxidative stress injury by decreasing iNOS, SUR1 and NT expression. These results indicate that TSG may prove to be a potential therapeutic agent for the treatment of diabetic nephropathy.

Streptozocin-injected rats are known to manifest typical characteristics of diabetes mellitus such as hyperglycaemia, polyuria, growth retardation and an increase in urinary albumin excretion [17]. In the present study, we have shown that unilateral renal ligation combined with STZ injection established diabetic nephropathy model in rats that was characterized by albuminuria, and increased levels of lipid peroxides in renal cortical tissue, suggesting increased oxidative stress in diabetic kid-

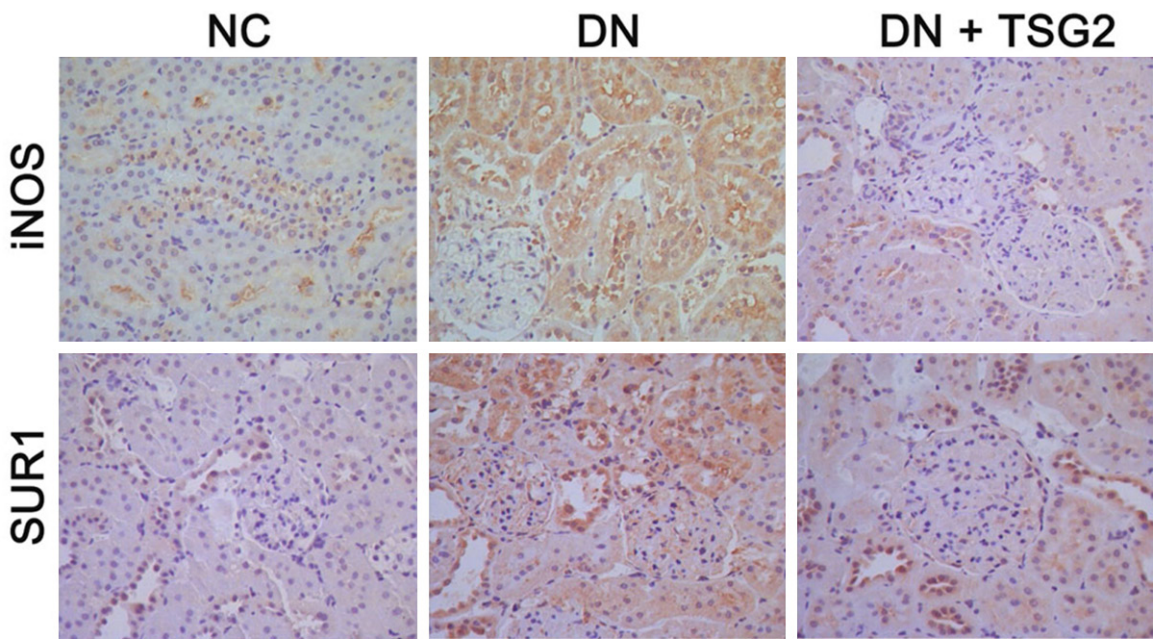

Figure 4. Immunohistochemical staining of inducible Nitric oxide synthase (iNOS), Sulfonylurea receptor 1 (SUR1) protein. iNOS: Inducible nitric oxide synthase; SUR1: Inducible nitric oxide synthase 1; NC: Normal control; DN: untreated diabetic nephropathy; DN+TSG_2: high-dose TSG-treaed group (20 mg/kg/d).

Table 6. Comparison of NT & SUR1 expression by study group

	SUR1/β-actin	NT/β-actin
NC	0.341±0.016	0.257±0.070
DN	0.921±0.078#	1.167±0.181#
DN+TSG_2	0.427±0.016♦	0.704±0.053♦

NT: Nitrotyrosine; SUR1: Sulfonylurea receptor 1; NC: Control group; DN: Untreated diabetic group; DN+TSG_2: high-dose TSG-treated group (20 mg/kg/d). #P < 0.05 vs. NC; ♦P < 0.05 vs. DN.

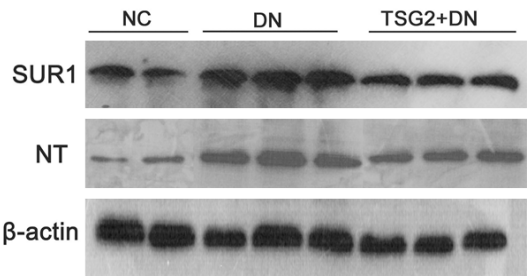

Figure 5. Western blot analyses of Sulfonylurea receptor 1 (SUR1) and NT protein. NT: Nitrotyrosine; SUR1: Inducible nitric oxide synthase 1; NC: Normal control; DN: untreated diabetic nephropathy; DN+TSG_2: high-dose TSG-treated group (20 mg/kg/d).

neys. Overproduction of oxygen species is believed to play a fundamental role in the pathogenesis of diabetic nephropathy because of their reactive chemical property to directly oxidize and damage DNA, protein, lipid or carbohydrate [14]. In our study, TSG treatment was associated with partial attenuation of the elevated MDA levels, which is a key marker of oxidative stress, and an increase in SOD levels, a key enzyme having antioxidative property. Further, high-dose TSG also attenuated the production of iNOS, a major subtype of NOS known to play a critical role in the early stage of DN. These results strongly suggest that the protective effect of TSG in diabetic nephropathy could be mediated by down-regulation of MAD and iNOS, and up-regulation of SOD.

Diabetic nephropathy is characterized by hypertrophy of both glomerular and tubular elements, leading to glomerulosclerosis and tubulointerstitial fibrosis due to increased synthesis and accumulation of extracellular matrix [18]. Overproduction of reactive oxygen species under hyperglycemic conditions [19] and is known to play a crucial role in the pathogenesis of chronic complications of diabetes including diabetic nephropathy. Therefore, enhancement of anti-oxidant defense is a potential therapeutic strategy in these patients [20]. TSG in this regard is a strong anti-oxidant and free radical-scavenging agent [21].

Three NOS isoforms have been found in the kidney of which only iNOS is known to be present in the mesangial cells [22]. Peroxynitrite (ONOO(-)), generated from iNOS, is a critical mediator in the causation of glomerular lesions in diabetic rats [23]. Sugimoto et al. reported increased AGE accumulation and iNOS expression in the glomerular mesangial tissue in STZ-induced diabetic mice, which was attenuated after administration of AGEs inhibitor, aminoguanidine [24]. In the present study, the expression of iNOS and NT, a specific marker of ONOO(-) was significantly increased in the kidneys of diabetic rats. However, TSG administration appeared to reverse the iNOS and NT upregulation in diabetic rats. In addition, the TSG treatment also appeared to attenuate the upregulation of SUR1.

Conclusion

To summarize, our study elucidates the molecular mechanism of TSG in the treatment of diabetic nephropathy through inhibition of oxidative stress. Our findings suggest that TSG may have potential therapeutic application in the treatment of diabetic nephropathy.

Disclosure of conflict of interest

None.

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