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Long-Term Administration of Sopungsungi-won (SP) Prevents Diabetic Nephropathy in Zucker Diabetic Fatty Rats

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We investigated the long term effects of Sopungsungi-won (SP), a Korean traditional formula used for senile constipation and diabetes mellitus, on the development of diabetic nephropathy (DN) in Zucker diabetic fatty (ZDF) rats. ZDF rats were fed regular laboratory chow mixed with SP or rosiglitazone (RSG) for an 8-week period. Kidney hypertrophy was developed with increasing plasma glucose level, and glomerular hypertrophy was improved by 22% and 45% in SP- and RSG-treated rats, respectively. Urinary glucose and albumin excretions were also significantly lower in SP-treated rats than in ZDF control rats. Activation of the mitogen-activated protein kinase (MAPK)-transforming growth factor β1 (TGF β1)-fibronectin pathway in kidney, responsible for glomerular dysfunction, was markedly blunted by SP treatment in a dose dependent manner. Our findings, for the first time, provide strong evidence that long-term administration of SP formula prevents the development and progression of DN in ZDF rats. Human trials are needed to confirm these experimental results.

Key words: Fibronectin, Kidney hypertrophy, Mitogen-activated protein kinase, Sopungsungi-won, Transforming growth factor β1, Zucker diabetic fatty rat

INTRODUCTION

The results of the Diabetes Control and Complications Trial (The Diabetes Control and Complications Trial Research Group, 1993) have shown that strict glycemic control can prevent the onset and progression of diabetic complications. Several hypotheses such as hyperosmolarity, glycation end products formation, oxidant formation, abnormality of sorbitol and myoinositol metabolism, and diacylglycerol (DAG)-protein kinase C (PKC) activation have been proposed to explain the various pathologic changes induced by hyperglycemia (Greene *et al.*, 1987; Brownlee *et al.*, 1987; Koya and King, 1998; Craven and DeRubertis, 1989)

Diabetic nephropathy (DN) is the commonest cause of end-stage renal failure in the Western world. The early changes in diabetic kidney disease are characterized by an increase in kidney size, glomerular volume and kidney function, and later by the development of mesangial proliferation, accumulation of extracellular matrix (ECM) and increased urinary albumin excretion (UAE). Overt nephropathy is clinically characterized by proteinuria, hypertension and progressive renal insufficiency.

Sopungsungi-won (SP), a Korean traditional formula documented in Donguibogam (Korean traditional medical book), has been used for senile constipation and diabetes mellitus. We showed previously that administration of SP formula prevents the transition to overt diabetes in Zucker Diabetic Fatty (ZDF) rats and that its hypoglycemic activities are probably due to the stimulation of insulin secretion and improvement of insulin resistance through activation of fatty peroxisome-proliferator activated receptor γ.

The purpose of this study is to investigate the long-term effects of SP on the development of DN in ZDF rats.

MATERIALS AND METHODS

Plant material

All raw ingredients were purchased from the Kyungdong Herbal Market in Seoul City and botanically identified by Dr. Yook at the Department of Oriental Pharmaceutical Science, Kyung Hee University. A voucher specimen of each crude drug was deposited at the Medicinal Plants Herbarium of the School of Pharmacy, Kyung Hee University, with registration numbers 101-112. Each crude drug was left to dry in the shade at room temperature, cut

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Table I. Composition of crude drugs in Sopungsungi-won

Constituent	Weight Ratio
Rhei undulati Rhizoma	5
Plantaginis Semen	2
Pruni japonica Semen	2
Arecae Semen	2
Cannabis Semen	2
Cuscutae Semen	2
Achyranthes Radix	2
Dioscorea Radix	2
Corini Fructus	2
Ponciri Fructus	1
Ledebouriellae Radix	1
Angelicae pubescensi Radix	1

into pieces, ground to powder and mixed in mortar based upon the ratios stated in Table I.

Animals and treatment

Experiments were carried out on obese male ZDF rats (ZDF/Gmi, *fa/fa*) obtained from Genetic Models (Indianapolis, USA). Rats were obtained at age 7 weeks and were allowed to adapt to the local environment for 1 week before study. Rats were housed in separate cages, under a temperature (25± 2°C) and moisture (50%) controlled environment, and given free access to water. During the acclimatization period, each animal was fed regular laboratory chow (Samyangsa, Korea) *ad libitum*.

At age 8 weeks, ZDF rats were randomly divided into control and treatment (Sopungsungi-won [SP] or rosiglitazone [RSG]) groups (n = 7 for each group). The control rats continued to receive regular chow *ad libitum*. The SP treated rats were fed regular chow mixed with either 2 g/kg or 5 g/kg of SP (SP2 and SP5) for an 8 week-period. The RSG rats were fed regular chow mixed with 10 μmol/kg of rosiglitazone maleate (Glaxo SmithKlein, Korea). The chow was obtained from the manufacturer in the powdered form and mixed with an appropriate amount of either SP or RSG. To achieve accurate dosing, food intake was determined for 3 days prior to the study and then for 3 days every 2 weeks to re-set the dietary drug concentrations. New formulations of food mixed with either SP or RSG were prepared two to three times per week. Throughout the experiment, principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and Home Office regulations (Animal Scientific Procedures Act, 1986) were followed.

Urine sampling

At monthly interval, rats were transferred to metabolic cages for 24 h and urine was collected for determinations of albumin and glucose levels using commercial kits (Yeongdong Pharmaceutical Co., Korea; Sigma Diagnostic, USA).

RT-PCR for transforming growth factor-β1 (TGF-β1)

Total RNA from kidney tissue was prepared using easy-BLUE (Intron Co., Korea), according to the manufacturer's instruction. One micro-gram of total RNA was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, USA) and random hexamers as primers. The specific primers were directed against the rat sequence for TGF-β1 (by using a Primer3 program) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (TGF-β1: sense primer 5-TCA CTG GAG TTG TAC GGC AG 3, antisense primer 5-TCT CTG TGG AGC TGA AGC AA 3; GAPDH: sense primer 5'-GGA AAG ACA ACG GAC AAA TC-3', antisense primer 5'-GTC ATC TTC TGG AGC ACC TT-3'). PCR reactions were carried out at 95 for 40 s, at 57°C for 40 s, and at 72°C for 40 s, for 30°C cycles in a thermal cycler followed by an extension step at 72°C for 10 min. PCR product of TGF-β1 was 289 base pairs; GAPDH was amplified as a control gene. The amplified products were fractionated on a 2% agarose gel and stained with 0.5 μg/ml ethidium bromide. The densities of the PCR products were measured using Molecular Analyst software.

Immunohistochemistry for TGF-β1 and fibronectin

Anesthetized animals were perfused with 0.05 M phosphate-buffered saline (PBS, pH 7.4) and subsequently with 4% paraformaldehyde in PBS. The kidney was removed, postfixed in 4% buffered paraformaldehyde and embedded in paraffin. Paraffin sections (7 μm thick) of embedded kidney were cut on microtomes (American Optical, USA), and mounted on double-gelatin coated slides. Slides in 0.01 M citrate buffer (pH 6.0) were boiled for HIER (Heat Induced Epitope Retrieval), then incubated overnight with either rabbit polyclonal TGF-β1 antibody (Santa Cruz Biotechnology, USA) or mouse monoclonal fibronectin antibody (Neomarkers, USA) at a 1:200 dilution in a humidity box at 25°C. Slides were washed and then incubated with anti-rabbit or anti-mouse biotinylated secondary antibody (Vector Laboratories, USA) at a 1:200 dilution for 1.5 h. After further washes with PBS, Avidin Biotin Complex (Vector Laboratories, USA) was applied for 1.5 h after washing, then sections were developed in 0.01% DAB (3,3-diaminobenzidine tetrahydrochloride) for 5 min and rinsed with PBS. Slides were dehydrated in alcohol and xylene and mounted using the Neo-mount (Merck, Germany).

Western blot analysis for mitogen activated protein kinase (MAPK)

For determination of phospho-Erk1/2 or phospho-p38

protein, the kidney was homogenized in ice-cold lysis buffer (250 mM sucrose, 10 mM Tris-HCl, 2 mM EDTA, 1 mM sodium orthovanadate, 100 mM sodium fluoride, 1% protease inhibitor mixture, pH 7.4). The homogenate was centrifuged at 20,000× g for 20 min at 4°C. The resulting supernatant was removed and the protein concentration was determined by Lowry method (Lowry, 1951). Aliquots of 50 μg protein were treated with sample buffer, heated at 100°C for 5 min, and resolved by 10% SDS-polyacrylamide gels and electrotransferred to Hybond nitrocellulose membranes (Amersham Pharmacia Biotech, England). After blocking overnight with 5% non-fat milk in TPBS containing 0.1% Tween-20 at 4°C, the blots were incubated with a 1:2,000 dilution of polyclonal antibody to phospho-p38 or phospho-ERK1/2 (Cell signaling, USA). The blots were washed three times with TPBS and then incubated with horseradish peroxidaseconjugated goat anti-rabbit IgG (Zymed, USA) at 1:5,000 dilution for 1 h at room temperature. Blots were again washed three times with TPBS and the protein band signals were visualized using enhanced chemiluminescence as described by the manufacturer (Pierce, USA). The relative amount of positive immunoreactive proteins was quantified with densitometric analysis using a GS-700 imaging densitometer.

Statistical analysis

All data were expressed as mean ± S.E.M. Student's t-test was used to determine significant differences between groups. Results were considered significantly different at the $P < 0.05$ level.

RESULTS

Kidney hypertrophy

The index of kidney hypertrophy, as measured by the ratio of kidney weight to body weight, was measured at the end of the experiment (Table II). There was a trend for ZDF control rats to gain less weight than SP-treated rats. The average body weights of SP2- and SP5-treated rats were 12 and 11% heavier than controls, respectively. In contrast, the control rat kidneys were significantly heavier than those of SP2- and SP5-treated rats. The index of hypertrophy was 22% lower in SP-treated rats than in the ZDF control rats. Correlation between plasma glucose levels and index of kidney hypertrophy was observed (Fig 1, R = 0.831). The improvement of kidney hypertrophy shown in SP-treated rats is probably due to the plasma glucose-lowering activity of SP.

Urinary glucose and albumin

Urinary glucose and albumin excretions were measured monthly (Table III). Urinary glucose levels in SP2- and SP5- treated rats were decreased in a dose dependent manner; a result consistent with the plasma glucose lowering activity of SP.

UAE, which is responsible for glomerular dysfunction in diabetes, was gradually increased in ZDF control rats, but was significantly decreased by 29% and 43% in SP2- and SP5-treated rats, respectively.

TGF-β1 and fibronectin expressions

The most important histological character of DN is the expansion of glomerular mesangium composed of various ECM proteins. Thus, we examined the effect of SP on the mRNA and protein expressions of TGF-β1, which has been proposed to play an important role in the overproduction of ECM proteins in glomeruli of diabetic rats.

Renal expressions of TGF-β1 and fibronectin were determined by RT-PCR and immunohistochemistry. As

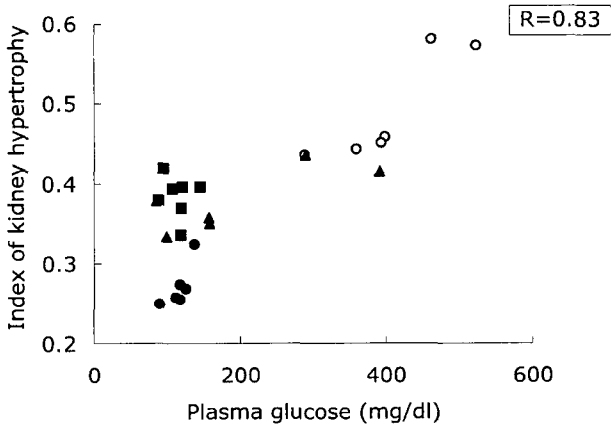

Fig. 1. Correlation between plasma glucose concentration and kidney hypertrophy. (○), ZDF control rats; (▲), 2 g/kg SP-treated rats; (■), 5 g/kg SP-treated rats; (●), 10 μmol/kg RSG-treated rats.

Table I. Effects of SP and RSG on the ratio of kidney to body weight in ZDF rats

Characteristic	Control	SP		RSG 10 mol/kg
		2 g/kg	5 g/kg	
Body weight (g)	407.4 ± 11.1	457.7 ± 10.1**	452.9 ± 7.7**	604.2 ± 24.2***
Kidney weight (g)	2.01 ± 0.06	1.73 ± 0.05**	1.75 ± 0.03**	1.63 ± 0.02***
Index of kidney hypertrophy (%)	0.49 ± 0.03	0.38 ± 0.02**	0.38 ± 0.01**	0.27 ± 0.01***

Data are mean ± SE. **P* < 0.01, ***P* < 0.001 vs. control

Table III. Effects of SP and RSG on urinary glucose and albumin levels in ZDF rats

Characteristic	Control	SP		RSG
		2 g/kg	5 g/kg	10 mol/kg
Urinary glucose (mg/day)	3,323 ± 305	660 ± 187***	279 ± 121***	3.0 ± 0.3***
Urinary albumin (mg/day)	64.2 ± 6.0	45.7 ± 8.4	36.4 ± 5.4**	40.4 ± 3.1**

Data are mean ± SE. **$P < 0.01$, ***$P < 0.001$ vs. control

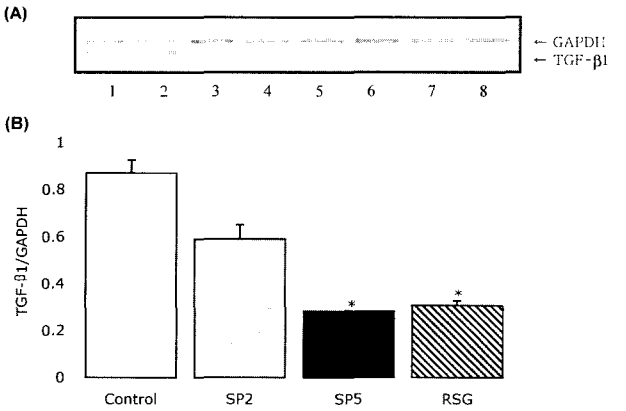

Fig. 2. Expression of TGF-β1 mRNA in kidney of ZDF rats treated with SP or RSG. (A) Lanes 1~2, control; lanes 3~4, SP 2 g/kg; lanes 5~6, SP 5 g/kg; lanes 7~8, RSG 10 μmol/kg. Bands representing the 471 bp GAPDH and the 289 bp TGF-β1 PCR products are indicated. (B) Quantification of mRNA was normalized with GAPDH. Control, ZDF control rats; SP2, 2 g/kg SP-treated rats; SP5, 5 g/kg SP-treated rats; RSG, 10 μmol/kg RSG-treated rats. Data are means ± SE. *$P < 0.05$ vs. Control.

shown in Fig. 2, the mRNA levels of TGF-β1 were reduced to as little as one third in SP5- and RSG-treated rats (ZDF control, 0.87 ± 0.06; SP5-treated rats, 0.28 ± 0.01; RSG-treated rats, 0.31 ± 0.02). Effects of SP and RSG on protein expressions of renal TGF-β1 and fibronectin were also remarkable. TGF-β1 protein expression in glomerular cells of SP5- and RSG- treated rats was much smaller that that in the ZDF control rats (Fig. 3). Fibronectin protein expressions in glomerular cells were also markedly attenuated in SP5- and RSG-treated rats (Fig. 4).

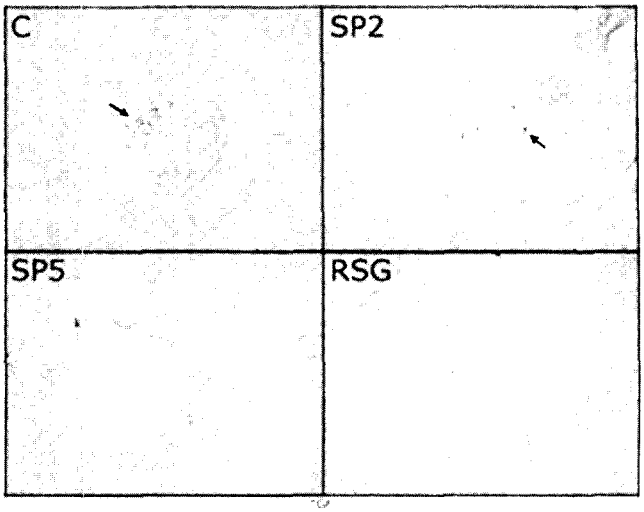

Fig. 3. Immunohistochemical staining for TGF-β1 in kidney from ZDF control (C), ZDF treated with 2 g/kg of SP (SP2), ZDF treated with 5 g/kg of SP (SP5), ZDF treated with 10 μmol/kg of RSG (RSG). Positive staining is shown in brown (indicated by arrow). Original magnification, × 200.

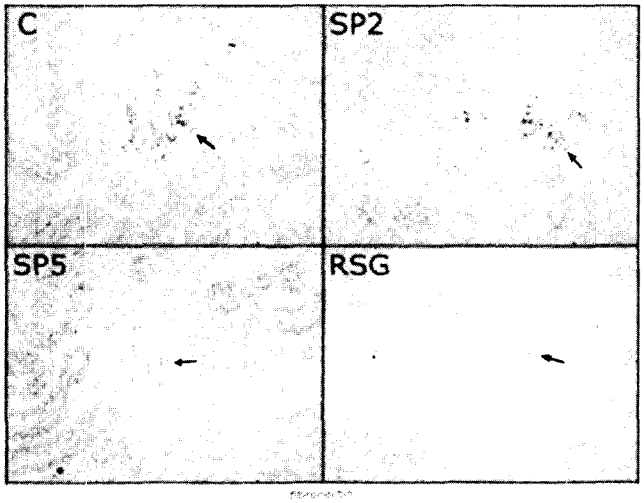

Fig. 4. Immunohistochemical staining for fibronectin in kidney from ZDF control (C), ZDF treated with 2 g/kg of SP (SP2), ZDF treated with 5 g/kg of SP (SP5), ZDF treated with 10 μmol/kg of RSG (RSG). Positive staining is shown in brown (indicated by arrow). Original magnification, × 200.

MAP kinase (ERK and p38) expressions

Because the activation of the DAG-PKC-MAPK pathway in diabetes has been suggested to be responsible for glomerular dysfunction in diabetic rats (Cooper, 2001), we examined whether SP prevents the activation of the ERK and/or p38 in glomeruli from ZDF diabetic rats. The immunoblot analysis using the anti-phospho ERK antibody, which recognizes phosphorylated threonine 202 and tyrosine 204 of p44/p42 ERK, showed that ERK activity was significantly decreased in SP2- and SP5-treated rats, in a dose dependent fashion, as compared with that in the ZDF control rats (Fig. 5A). Activity of p38, which is represented by the phosphorylated form, was also remarkably reduced in SP- treated rats as compared with that in the control rats (Fig. 5B).

DISCUSSION

In human and experimental diabetes, early renal involvement is characterized by hypertrophy of both glomerular

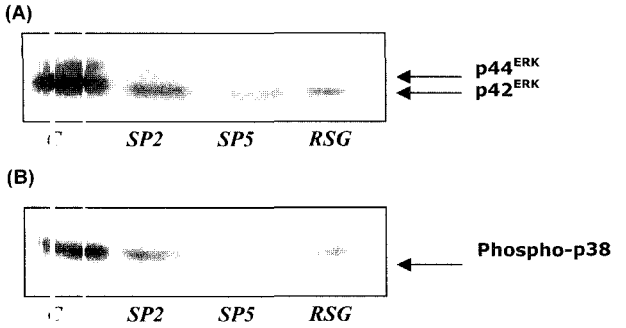

Fig. 5. Expressions of phospho-ERK (A) and phospho-p38 (B) protein in kidney from ZDF rats treated with SP or RSG. C, ZDF control rats; SP2, 2 g/kg SP-treated rats; SP5, 5 g/kg SP-treated rats; RSG, 10 μmol/kg RSG-treated rats.

and tubuloepithelial elements, thickening of the glomerular and tubular basement membranes, and accumulation of ECM components in the glomerular mesangium. As the disease progresses, increased mesangial expansion leads to obliteration of the glomerular capillary lumen, proteinuria, and loss of glomerular filtration. In this study, we examined the long-term effects of an SP formula on glomerular histology as well as on functional abnormalities in ZDF rats, a model for type 2 diabetes.

In the present study, we clearly demonstrated that SP was able to prevent glomerular dysfunction, such as albuminuria, and the enhancement of mRNA and protein expressions of TGF- β1 and fibronectin in ZDF rats. This effect of SP seems to be dependent on its plasma glucose-lowering activity (ZDF control rats, 403.8 ± 36.4; SP2-treated rats, 162.2 ± 32.6; SP5-treated rats, 113.1 ± 7.2 mg/dl). These findings provide the first evidence, to our knowledge, that SP exhibits potent hypoglycemic activity and might be useful in the prevention or treatment of DN.

Kidney hypertrophy was evidently developed as plasma glucose was increasing (Fig. 1), and the plasma glucose-lowering activity of SP caused an improvement in kidney hypertrophy by 22% and 45% in SP- and RSG-treated rats, respectively (Table II). Urinary glucose and albumin excretions were also significantly reduced in SP-treated rats as compared with those in ZDF control rats (Table III). These results clearly demonstrated that the amelioration in kidney hypertrophy and albuminuria is due to the hypoglycemic activity of SP.

DN is characterized histologically by an expansion of glomerular mesangium, which is composed of ECM proteins produced by mesangial cells. Because a contribution of TGF- β1 to the overproduction of ECM proteins in diabetic glomeruli has been proposed, we examined whether SP ameliorates these subsequent glomerular abnormalities. It has been previously reported that high glucose stimulates TGF-β1 production in mouse mesangial cells through a transcriptional mechanism that involves a specific glucose-responsive, DNA-binding element (Hoffman *et al.*, 1998). It has also recently been reported that high glucose stimulates the TGF- β type II receptor in mouse mesangial cells (Isono *et al.*, 2000). In this study, we showed that treatment of ZDF rats with SP significantly inhibits the high-glucose induced TGF-β1 mRNA level and protein production in a dose dependent manner (Fig. 2 and 3).

In the kidney, the critical role of TGF-β1 has been well recognized in several renal diseases characterized by progressive glomerular ECM accumulation leading to the development of glomerulosclerosis. TGF-β1 is a potent inducer of ECM protein synthesis and accumulation, and has been implicated as the key mediator of fibrogenesis in a variety of tissues. We also examined whether SP affects the protein expression of fibronectin, which is a major structural component of the ECM synthesized by glomerular mesangial cells. Immunostaining for fibronectin clearly showed that glomerular protein expression of fibronectin in SP5-treated rats was markedly decreased as compared with that in ZDF control rats (Fig. 4).

Recently, three mitogen-activated protein kinase (MAPK) families have been identified and characterized (Segar and Krebs, 1995); these are extracellular signal-regulated kinase-1/2 (ERK1/2 or p42/44 MAPK), stress-activated c-Jun N-amino terminal kinase (JNK/stress-activated protein kinase or SAPK), and p38 MAPK. These kinases can be activated by various extracellular stimuli, including growth factors and environmental stresses, and they play an essential role in the signal transduction cascades that lead to alterations in cell growth and other key functions (Segar and Krebs, 1995; Borkemeyer et al., 1996). With regard to the diabetic state, it has been reported that ERK is activated in glomeruli of diabetic rats as well as in mesangial cells cultured under high-glucose conditions (Haneda *et al.*, 1997; Awazu *et al.*, 1999). A recent report also demonstrated that p38 MAPK was activated by relatively high concentrations of extracellular glucose in various types of cells, including mesangial cells (Igarashi *et al.*, 1999). In contrast to these two kinases, JNK is not activated in the glomeruli of diabetic rats or stimulated in mesangial cells upon exposure to high glucose (Kang *et al.*, 1999).

In accord with these findings, we examined whether the ERK and/or p38 cascade is involved in the overexpression of TGF-β1 and fibronectin induced by hyperglycemia in ZDF rats. Immunoblot analyses using either anti-phospho-ERK antibody or anti-phospho-p38 antibody demonstrated attenuated phosphorylations of ERK and p38 in SP-treated rats as compared with those in the ZDF control rats (Fig. 5). Our data, taken together, provide evidence that the activations of both ERK and p38 MAPK by high glucose are required for the overproduction of ECM proteins.

Our findings provide the first evidence, to our knowledge, that long-term administration of SP formula can ameliorate the development of glomerular dysfunction in ZDF diabetic rats. Thus, SP might be useful to prevent the development and progression of DN in subjects with type 2 diabetes, by improving metabolic control, and in subjects with both types 1 and 2 diabetes, by preventing the activation of the MAPK-TGF-β1-fibronectin pathway. Human trials are necessary to confirm this hypothesis.

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