Arch Pharm Res Vol 25, No 6, 923-931, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Sopungsungi-won (SP) Prevents the Onset of Hyperglycemia and Hyperlipidemia in Zucker Diabetic Fatty Rats

Your Young Kim[1], Hyo Joo Kang[1], Sung Kwon Ko[2], and Sung Hyun Chung[1]

[1]Laboratory of Pharmacology, School of Pharmacy, Kyung Hee University, Seoul 130-701, Korea and [2]Korea Ginseng Institute, Chung-Ang University, Gyeonggi-do, Ansungshi 456-756, Korea

(Received September 17, 2002)

Sopungsungi-won (SP) is a known formula for senile constipation and diabetes mellitus, based on traditional Korean medicine. The preventive effect of SP on the development of overt diabetes in Zucker diabetic fatty (ZDF) rats was evaluated. When administered orally through a diet for 8 weeks, diabetic conditions such as hyperglycemia, polydipsia and hypertriglyceridemia were all ameliorated in SP-treated rats. In parallel with the onset and progression of hyperglycemia in the ZDF control rats; there was a marked decline in plasma insulin concentrations from 26.1 μU/ml, at age 7 weeks, to 14.8 μU/ml at age 15 weeks. In the SP-treated rats, however, the plasma insulin concentrations did not decline, and SP at a dose of 5 g/kg significantly increased the insulin levels to 31.9 μU/ml. Early normalization of plasma insulin and a retained ability to subsequently increase plasma insulin were indicative of a pancreatic β cell protective action by the SP formula. In addition, expressions of an insulin-responsive gene and corresponding protein, glucose transporter 4 (GLUT4), in skeletal muscle, were also determined in SP- and rosiglitazone-treated ZDF rats. mRNA and protein levels of GLUT4 in SP-treated rats were upregulated in a dose dependent manner. Furthermore, when ZDF rats were treated with 2 g/kg of the SP formula, the activity of glucose-6-phosphatase was decreased by 49%, whereas the activity of glucokinase was increased by 196%, compared to the ZDF control rats. Taken together, these data provide evidence that the SP formula markedly lowered the plasma glucose levels, probably through an effect not only on improvement of insulin action, but through a combined stimulation of glycolysis and an inhibition of gluconeogenesis in the liver, and also suggest the validity of SP's clinical use in the treatment of type 2 diabetes mellitus following further toxicological investigation.

Key words: Sopungsungi-won, ZDF rat, Type 2 diabetes mellitus, Pancreatic β cell, Glucose-6-phosphatase, Glucokinase, GLUT4

INTRODUCTION

Type 2 diabetes mellitus (T2DM), also known as non-insulin dependent diabetes mellitus (NIDDM), develops in middle or later life, and affects 2-6% of adults in most Western societies (Amos *et al.*, 1997). The combined effects of insulin resistance and impaired insulin secretion cause metabolic disturbances resulting in hyperglycemia (DeFronzo, 1997; Polonsky *et al.*, 1996; Groop *et al.*, 1989). Different classes of antidiabetic agents offer a range of actions for reducing hyperglycemia. In spite of the intensive use of current antidiabetic agents, many T2DM patients still exhibit poor glycemic control, and some develop serious complications (Nathan, 1993; Klein, 1995; UK Prospective Diabetes Study Group, 1995).

A commonly practiced pharmacological treatment of diabetes mellitus includes oral hypoglycemic agents and insulin injections. However, for many years people in Korea have used medicinal plants empirically to treat diabetes. There are some traditional medicines, which have been reported for the treatment of diabetes, but there has been insufficient pharmacological study relating to them until now. Taking these facts into account, an attempt was made to find natural drugs with hypoglycemic activity. Traditional medicines and herbs would probably open new therapeutic avenues for multifactorial diseases, such as diabetes mellitus, since their complex components often provide versatile bioactivity and varied mechanisms of action.

Sopungsungi-won (SP), a traditional Korean formula documented in Donguibogam, has been used as a medicine

Correspondence to: Sung Hyun Chung, School of Pharmacy, Kyung Hee University, Seoul 130-701, Korea
E-mail: suchung@khu.ac.kr

for senile constipation. Recently, SP has also been used for the treatment of diabetes mellitus by Korean oriental medical doctors (personal communication).

In order to elucidate the potential of SP as an antidiabetic medicine, we have previously studied its antidiabetic activity in C57BL/KsJ *db/db* mice. With the oral administration of SP over a 4 week period, fasting blood glucose and triglyceride were both reduced compared with db/db control mice. SP-treated mice showed higher levels of immunoreactive plasma insulin than the control mice, although no statistical difference was observed (Lee *et al.*, 1999). However, there was no further experimental evidence on how SP improved hyperglycemia in a type 2 diabetic animal model. The purpose of this study was to investigate the potential of SP in the prevention of the development of diabetes in the Zucker diabetic fatty (ZDF) rat. We also wanted to examine whether the SP formula exhibited insulin-sensitizing activity, in a similar way to rosiglitazone, in ZDF rats. Since liver glucose metabolism is altered in diabetic state, the effect of the SP formula on the activity of the regulatory proteins involved in glycolysis and gluconeogenesis were also determined.

MATERIALS AND METHODS

Plant materials

Each raw ingredient was purchased from the Kyungdong Herbal Market in Seoul City, and were 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 to 112. Each crude drug was dried in the shade at room temperature, then cut into pieces, ground to powders, and mixed in a mortar in the ratios stated in Table I.

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

Animals and treatment

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

At age 8 weeks, the ZDF rats were randomly divided into to three groups of 7; the controls, and two treatment groups, one treated with Sopungsungi-won [SP] the other with rosiglitazone [RSG]. The control rats continued to receive a regular chow *ad libitum*. The SP rats were fed a 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 a regular chow mixed with 10 μmol/kg of rosiglitazone maleate (GlaxoSmithKlein, Korea). The chow was obtained from the manufacturer in the powdered form and mixed with an appropriate amount of either the SP or RSG. To achieve accurate dosing, the food intake of the rats was determined for 3 days prior to the study, and 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.

Blood sampling and plasma assay

Blood (~500 μl) was withdrawn from the orbital venous plexus, once per week, using a heparinized capillary tube without anesthesia. The blood samples were placed on ice and centrifuged, and plasma stored at -20°C until assayed. The plasma glucose concentration was determined using a glucose oxidase method (Sigma Diagnostics, USA). The plasma insulin concentration was measured by a radioimmunoassay (DPC, USA), which uses a specific anti-rat insulin antibody. Plasma triglyceride and free fatty acid concentrations were determined using commercially available diagnostic kits (Sigma Diagnostics, USA; Eiken, Japan). The activities of the hepatic enzymes alanine transaminase (ALT) and aspartate transaminase (AST), in the blood, were also assayed to evaluate the liver toxicity using commercially available kits (Asan Pharmaceutical Co., Korea).

Glycated hemoglobin (HbA_{1c}) in whole blood

At the end of the study, whole blood was obtained from the orbital venous plexus using a heparinized capillary tube, spun down, and the supernatant levels of HbA_{1c} determined using a commercially available kit (Glycated Hemoglobin Kit, Sigma Diagnostic, USA). The HbA_{1c} was separated using a cation exchange resin, based on the charge, and its absorbance measured at 415 nm (Boil *et*

al., 1980).

Pancreas removal and tissue processing

After fasting overnight, the rats were deeply anesthetized with urethane (0.9 ml/100 g body weight of 20% solution), and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The pancreas was removed postfixed overnight and subsequently embedded in paraffin. Paraffin sections with a thickness of 7 μm were prepared using a microtome (American Optical Company, USA) and mounted on double-gelatin coated slides. The tissue sections were deparaffinized in 0.01 mol/L citrate buffer (pH 6.0) and boiled for 10 min in a microwave for the HIER (Heat Induced Epitope Retrieval). Pancreatic tissue sections were incubated with the monoclonal antibody to insulin (Neomarkers, USA). Sections were washed, and then incubated with biotinylated secondary antibody for 1.5 hr. After further washing with PBS, avidin-biotin-peroxidase complex was applied for 1.5 hr. After washing, the peroxidase was reacted with 0.02% DAB (3,3-diaminobenzidine tetrahydrochloride) and 0.01% H_2O_2 for 3 min. Slides were dehydrated in alcohol and xylene, and then mounted using the Neo-mount (Merck, Germany).

RNA extraction and RT-PCR

Total RNA from skeletal muscle was prepared using easy-BLUE (Intron Co., Korea), according to the manufacturer's instruction. 1 μg of total RNA was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase, with random hexamers (Promega, USA) as primers. The specific primers were directed against the rat sequences for GLUT4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GLUT4: sense primer 5'-ACA GAA GCT GAT TGA ACA GAC-3', antisense primer 5'-ACC CGT CCA AGA ATG AGT ATC-3', GAPDH: sense primer 5'-GGA AAG ACA ACG GAC AAA TC-3', antisense primer 5'-GTC ATC TTC TGG AGC ACC TT-3'). Primers were added to a final concentration of 0.5 μM in a 25 μl reaction mixture containing: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5mM $MgCl_2$, 0.5 mM of each dNTP, 0.5-1.5 μl cDNA and 2.5 units of *Taq* DNA polymerase (TaKaRa medicals, Japan).

The PCR conditions were; denaturation at 95°C for 40 seconds annealing at 57°C for 40 seconds, and extension at 72°C for 40 seconds. Initial heating at 95°C for 5 min, and a final extension at 72°C for 10 min, were performed. The RT-PCR product was size fractionated on a 2% agarose gel and stained with 0.5 μg/ml ethidium bromide. The PCR product of GLUT4 was 285 base pairs. GAPDH was amplified as a control gene. The densities of the PCR products were measured using a GS-700 imaging densitometer. The level of mRNA was expressed as the ratio of the signal intensity for the target gene relative to that of GAPDH.

Western blot analysis

Skeletal muscle from an individual rat was homogenized in ice-cold buffer (250 mM sucrose, 10 mM Tris-HCl, 2 mM EDTA and 1% protease inhibitor mixture, at pH 7.4). The homogenate was centrifuged at 10,000 g for 20 min, and the supernatant centrifuged at 150,000 g for 60 min. The pellet was resuspended in the same buffer, and resolved in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X, 0.1% SDS and 1% sodium deoxycholate, at pH 7.4), then centrifuged at 100,000 g for 60 min. The resulting supernatant was collected, and the protein concentration determined by the Lowry method (Lowry, 1951).

An aliquot of protein was treated with the sample buffer, heated at 95°C for 5 min and resolved by 10% SDS-polyacrylamide gel and electrotransferred onto Hybond nitrocellulose membranes (Amersham Pharmacia Biotech, England). After blocking with 5% non-fat milk in TPBS (phosphate buffered saline containing 0.1% tween-20) overnight at 4°C, the blot was incubated with a 1:3000 dilution of the monoclonal antibody to GLUT4 (Santa Cruz Biotechnology, USA). The blot was then washed three times with TPBS and incubated with horseradish peroxidaseconjugated goat anti-rabbit IgG (Zymed, USA) at a 1:5,000 dilution for 1 h at room temperature. The blot was again washed three times with TPBS, and the protein band signal visualized using enhanced chemiluminescence (ECL) as described by the manufacturer (Pierce, USA). The relative amount of positive immunoreactive protein was quantified by densitometric analysis using a GS-700 imaging densitometer.

Glucose-6-phosphatase assay

Liver obtained from ZDF rats was minced and homogenized with a polytron homogenizer in 9 volumes of 0.25 M sucrose. The crude homogenate was centrifuged at 11,000 g for 30 min, and the precipitate discarded. The supernatant was further centrifuged at 105,000 g for 60 min, and the supernatant discarded. The solid precipitate was resuspended in ice-cold sucrose-EDTA solution, and stored at -20°C until required. The activity of glucose-6-phosphatase was assayed according to the method of Baginski *et al.* (1974), but with a slight modification. The assay mixture contained 0.25 M sucrose, 1 mM EDTA, 0.1 M glucose-6-phosphate and 0.1 M cacodylate buffer (pH 6.5), in a total volume of 0.3 ml. The reaction was started by the addition of a suitably diluted enzyme solution to the assay mixture. After incubation at 37°C for 5 min, the reaction was terminated by the addition of 2 ml of 2% ascorbic acid-10% TCA solution. The precipitate formed was removed by centrifugation after the mixture had been left to stand in an ice-bath. A 0.5 ml aliquot was pipetted

off, and the liberated inorganic phosphate in the supernatant determined using a commercially available reagent (Phosphor B-Test Waco, Waco Pure Chemical Industries, Japan) based on the molybdenum blue method (Wajima , 1973).

Glucokinase assay

Liver was homogenized in 9 volumes of a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM mercaptoethanol and 1 mM EDTA. After centrifugation at 12,000 g for 1 h, the supernatant was used to measure the glucokinase activity.

Glucokinase activity was measured by following the production of glucose-6-phosphate in an assay coupled to the reduction of nicotinamide adenine dinucleotide (NAD) in the presence of excess glucose-6-phosphate dehydrogenase (Hara *et al.*, 1986). The assay mixture contained 200 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 1 mM NAD, 5 mM ATP, 50 mM glucose, 100 mg/ml bovine serum albumin, 1 unit/ml glucose-6-phosphate dehydrogenase and the enzyme, in a final volume of 1 ml. The reaction mixture was preincubated for 1 min at 37°C, and the reaction started by the addition of the enzyme solution. The reaction velocity was measured as the rate of increase in absorbance at 340 nm over 2-3 min following the initiation of the reaction. One unit is the amount of enzyme that catalyzes the phosphorylation of 1 mmol of glucose per min.

Statistical analysis

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

RESULTS

Food intake and body weight

Food and water intakes were examined in the last week of treatment (Table II). There were no significant differences between controls and the SP groups in the food intake, but for RSG group, the food intake was slightly increased compared to the controls. The body weight gains for the control, SP2, SP5 and RSG groups, during the 8-week period, were 135, 186, 185 and 332 g, respectively, as shown in Fig. 1. As far as feed efficiencies (value calculated from body weight gain divided by total food intake) were concerned, the SP2-, SP5- and RSG-treated rats showed 111, 110 and 138%, respectively, with the feed efficiency of control group regarded as 100%. The reason for the high feed efficiency in the SP- or RSG-treated groups might be due to prevention of significant urinary glucose and albumin excretions of the ZDF rats.

The ZDF control rats did not gain further body mass since 13 week after birth, whereas the SP-treated rats continue to grow normally during the experimental period. Meanwhile, the RSG treatment of the ZDF rats induced a rapid weight gain, which was maintained throughout the study. When compared to the ZDF control rats, RSG-treated rats gained a further 197 g. Although the SP-treated rats also gained more weight than ZDF control rats, there was no significant difference in the relative fat mass between the SP-treated and control rats. By contrast, the relative fat mass in the RSG-treated rats was significantly higher than that in ZDF control rats (values calculated from epididymal fat divided by body weight for the control and RSG-treated groups were 2.22 ± 0.12 and 2.86 ± 0.12, $p<0.01$).

The water intakes were also markedly reduced in the SP-treated rats in a dose dependent fashion compared to the control rats, and those in the SP5-treated group were comparable to RSG-treated group.

Table II. Food and water intake of ZDF rats treated with SP or RSG

Characteristic	Control	SP		RSG 10 μmol/kg
		2g/kg	5g/kg	
Food intake (g/rat/day)	33.0 ± 0.7	33.4 ± 0.7	33.5 ± 1.3	35.4 ± 0.8*
Water intake (ml/rat/day)	111.0 ± 3.5	70.3 ± 3.8***	62.7 ± 4.0***	63.2 ± 1.2***

Food and water intakes were monitored in the last week of treatment. Data are the mean ± SE of seven animals examined. *$P < 0.05$, ***$P < 0.001$ *vs.* control

Blood glucose and HbA_{1c}

The plasma glucose was determined once a week, and

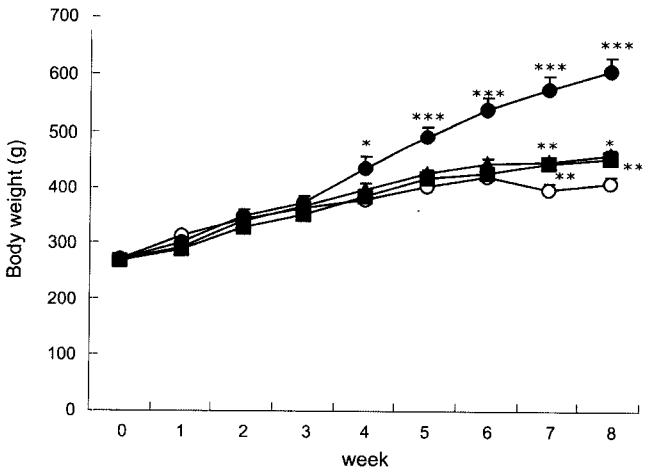

Fig. 1. Effect of SP or RSG on body weight. Each value shows the mean ± SE of seven animals examined. (○), ZDF control rats; (▲), 2 g/kg SP-treated rats; (■), 5 g/kg SP-treated rats; (●), 10 μmol/kg RSG-treated rats. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

compared between the groups (Fig. 2). At 3 weeks into the study, when the ZDF rats were age 10 weeks, the blood glucose concentration was already markedly elevated (288 mg/dl for ZDF control rats). This significant hyperglycemia eventually reached a peak of 387 mg/dl at age 12 weeks before declining slightly thereafter. By contrast, after an initial gradual increase in blood glucose, up to 3 weeks post treatment, the SP-treated rats became normoglycemic from age 11 weeks onwards.

Glycosylated hemoglobin (HbA_{1c}) in whole blood was also determined at the end of the experiment. Mean values of HbA_{1c} in the SP2- and SP5-treated rats were decreased from 6.85% to 6.26 and 5.64%, respectively. This data is in parallel with blood glucose levels, and suggests that SP persistently improved the hyperglycemic abnormality in a dose dependent fashion.

Plasma and pancreatic insulin

In parallel with the onset and progression of hyperglycemia in the ZDF control rats; there was a marked decline in the plasma insulin concentration, from 26.1 μU/ml at age 7 weeks, to 14.8 μU/ml at age 15 weeks. In the SP-treated rats, however, the plasma insulin concentrations did not declined, and in the SP5, to the contrary, significantly increased compared with the ZDF control rats (31.9 vs.14.3 μU/ml).

A morphological examination of the pancreas revealed a picture entirely consistent with progressive pancreatic β-cell failure in the ZDF control rats. Consistent with the plasma insulin data, the β-cell morphology was preserved in both the SP2- and SP5-treated rats. Thereafter, insulin contents in islet cells were determined by immunostaining with the antibody against insulin. As shown in Fig. 3, there was no evidence of depletion of the pancreatic insulin content in the SP-treated rats compared with the diabetic ZDF rats. This data is in parallel with the plasma insulin levels and the morphological examination, and suggests that SP prevented β-cell degeneration in a dose dependent fashion.

Triglyceride and free fatty acid

The effects of SP and RSG on plasma triglyceride and free fatty acid levels were examined once a week. While the ZDF control and SP2-treated rats showed triglyceride levels from 500 to 800 mg/dl, the triglyceride levels in SP5- and RSG-treated rats were continuously decreased from the first week of treatment (Fig. 4). At the end of the treatment, the SP5- and RSG-treated rats had lower plasma triglyceride levels, by 51 and 84%, respectively, compared to the ZDF control rats (control, 650 ± 70; SP5, 319 ± 34; RSG, 107 ± 7; $p<0.001$).

The plasma free fatty acid level in ZDF control rats was elevated, and fluctuated between 2.0 and 2.5 mEq/L throughout the study (Fig. 5). In contrast, the SP- and RSG-treated rats significantly decreased the free fatty acid levels from the second week of treatment compared to the ZDF control rats (control, 1.69 ± 0.05; SP5, 1.07 ±0.07; RSG, 0.39 ± 0.08; $p < 0.001$).

Hepatic glucose-6-phosphatase and glucokinase

The effects of SP or RSG on glucose-6-phosphatase and glucokinase were examined at the end of the treatment. Both the glucose-6-phosphatase and glucokinase activities were changed in the SP2-treated rats compared to the ZDF control rats. The glucose-6-phosphatase activity was decreased by 49%, whereas the glucokinase activity was

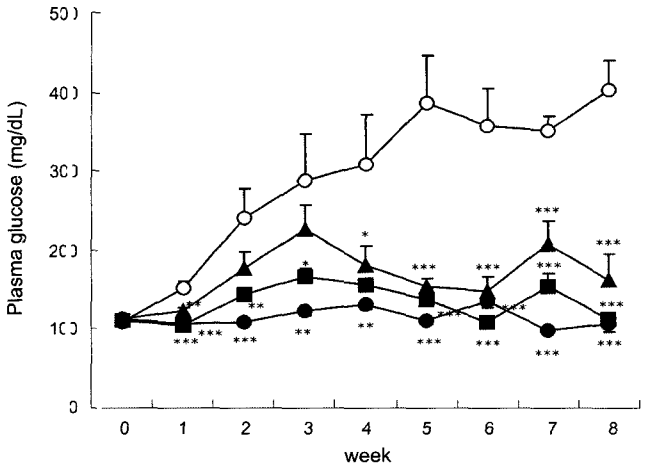

Fig. 2. Effect of SP or RSG on plasma glucose concentrations. Each value shows the mean ± SE of seven animals examined. (○), ZDF control rats; (▲), 2 g/kg SP-treated rats; (■), 5 g/kg SP-treated rats; (●), 10 μmol/kg RSG-treated rats. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control

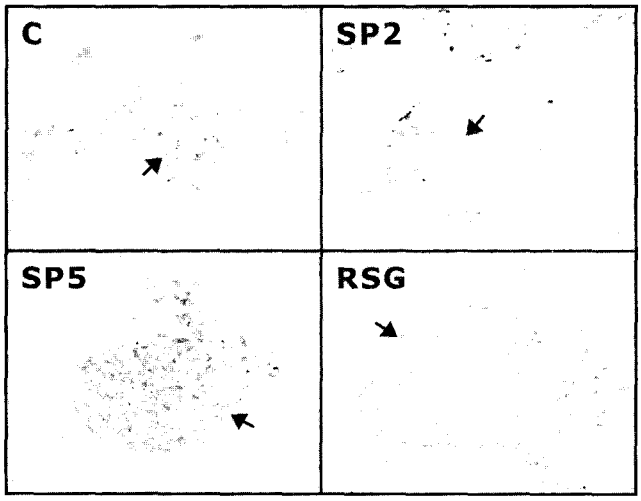

Fig. 3. Immunostaining of insulin in pancreatic islets of ZDF rats treated with SP or RSG, magnification ×100. (C) ZDF control rats, (SP2) 2 g/kg SP-treated rats, (SP5) 5 g/kg SP-treated rats and (RSG) 10 μmol/kg RSG-treated rats.

increased by 196% when the ZDF rats were treated with 2 g/kg of the SP formula. 5 g/kg of SP also modulated both enzyme activities, though the effects were not statistically significant. RSG, however, did not show any effect on either of the hepatic enzyme activities (Table III).

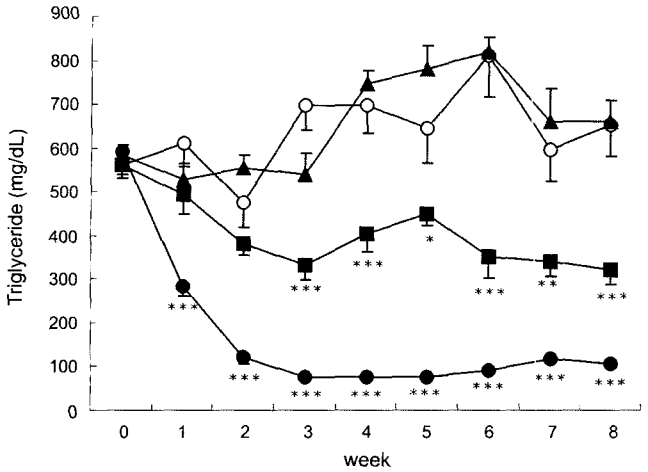

Fig. 4. Effect of SP or RSG on plasma triglyceride concentrations. Each value shows the mean ± SE of seven animals examined. (○), ZDF control rats; (▲), 2 g/kg SP-treated rats; (■), 5 g/kg SP-treated rats; (●), 10 μmol/kg RSG-treated rats. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

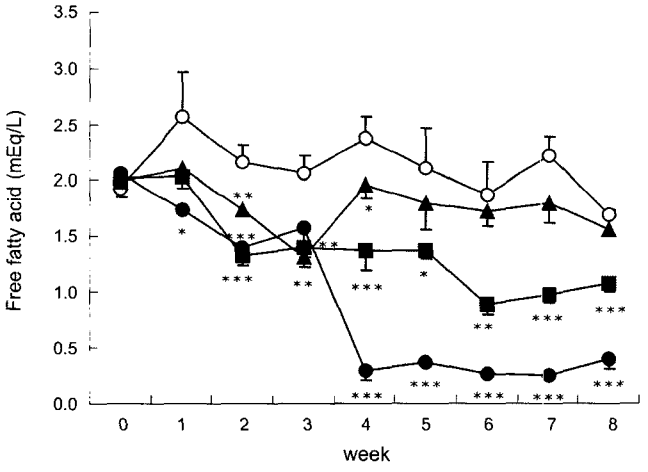

Fig. 5. Effect of SP or RSG on plasma free fatty acid concentrations. Each value shows the mean ± SE of seven animals examined. (○), ZDF control rats; (▲), 2 g/kg SP-treated rats; (■), 5 g/kg SP-treated rats; (●), 10 μmol/kg RSG-treated rats. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

GLUT4 mRNA and protein expressions

The mRNA and protein levels of GLUT4, from the skeletal muscle of each group, were determined. The SP2 and SP5 groups both had an increased GLUT4 mRNA level up to 2- and 5-fold compared to the ZDF control rats (control, 0.09 ± 0.02; SP2, 0.19 ± 0.08; SP5, 0.51 ± 0.01, Fig. 6). The protein levels of GLUT4 were also dose-dependently upregulated in the SP2-and SP5-treated rats, as shown in Fig. 7.

Hepatic enzymes

To ascertain whether SP and RSG induced hepatic damage, serum catalytic concentrations of ALT and AST were measured. 2- and 1.3-fold increases in ALT and AST activities were observed in the plasma from the RSG-

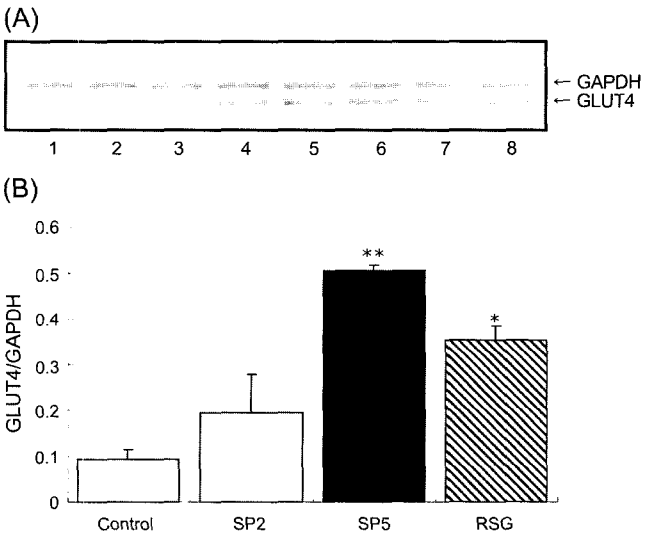

Fig. 6. Expression of GLUT4 mRNA in skeletal muscle of ZDF rats treated with SP or RSG. (A) Lanes 1~2, control; lanes 3~4, SP 2 g/kg; lanes 5~6, SP 5 g/kg; lanes 7~8, RSG. Bands representing 471 bp GAPDH PCR product and the 285 bp GLUT4 PCR product are indicated. (B) Quantification of mRNA was normalized 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 the means ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* Control.

Table III. Effects of SP and RSG on hepatic glucose-6-phosphatase and glucokinase activities

Enzymes	Control	SP		RSG 10ì mol/kg
		2 g/kg	5 g/kg	
Glucose-6-phosphatase (nmol/min/mg protein)	186.6 ± 11.8	95.2 ± 40.7*	152.0 ± 29.6	153.0 ± 17.5
Glucokinase (nmol/min/mg protein)	1.33 ± 0.30	2.61 ± 0.36*	2.52 ± 0.54	1.31 ± 0.40

At the end of the treatment, livers were removed, and enzymes assayed using commercially available kits. Data are the mean ± SE. $^{*}P < 0.05$ *vs.* control

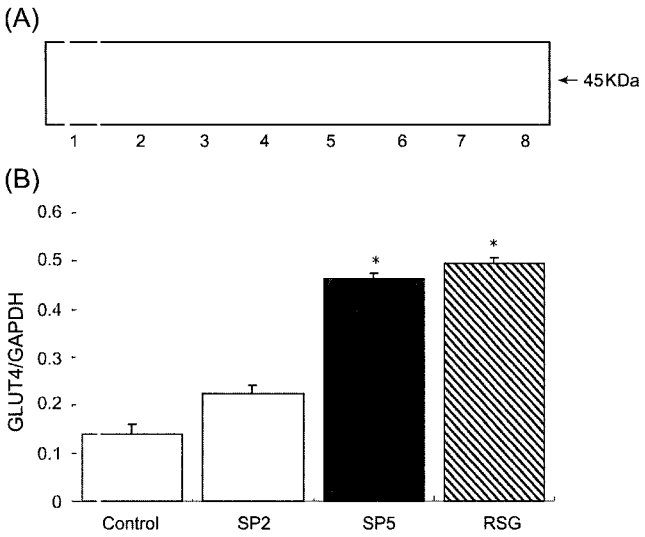

Fig. 7. Expression of GLUT4 protein in skeletal muscle of ZDF rats treated with SP or RSG. (A) Lanes 1~2, control; lanes 3~4, SP 2 g/kg; lanes 5~6, SP 5 g/kg; lanes 7~8, RSG. (B) Control, ZDF control rats; SP2, 2 g/kg SP-treated rats; SP5, 5 g/kg SP-treated rats; RSG, 10 μmol/kg RSG-treated rats. Data are the means ± SE. $^{*}P < 0.05$ *vs.* Control.

Table V. Parameters of liver toxicity in ZDF rats treated with SP or RSG

Characteristic	Control	SP		RSG 10 mol/kg
		2 g/kg	5 g/kg	
ALT (U/L)	35.8 ± 5.0	7.2 ± 1.6**	27.3 ± 2.9	69.5 ± 5.7**
AST (U/L)	37.0 ± 4.4	12.9 ± 2.3**	35.4 ± 2.9	49.3 ± 4.1*

At the end of the treatment, serum samples were collected, and enzymes assayed using commercially available kits. Data are the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control

treated rats compared with the ZDF control rats. In contrast, both enzyme activities in the SP-treated rats were significantly lower than those in the ZDF control rats (Table IV). This result suggests that SP does not exert any appreciable toxic effect on the liver.

DISCUSSION

Our data unequivocally show that administering SP formula, in prevention mode, blocked the transition to overt diabetes in male ZDF rats, an effect that was maintained throughout the course of the study. Unsurprisingly, attendant symptoms of diabetes in ZDF control rats, namely hyperglycemia, polydipsia, glycosuria and hypertriglyceridemia, were also absent in the SP-treated rats.

Early normalization of plasma insulin, and a subsequent ability to retained increased levels, in order to maintain normoglycemia, were indicative of the β cell protective

Table V. Effects of SP and RSG on plasma glucose, insulin and index of insulin resistance in ZDF rats

Characteristic	Control	SP		RSG 10 mol/kg
		2 g/kg	5 g/kg	
Glucose (mM)	22.4 ± 2.0	9.0 ± 1.8***	6.3 ± 0.4***	6.4 ± 0.4***
Insulin (μU/ml)	14.8 ± 1.9	23.9 ± 3.7*	31.9 ± 3.0**	14.0 ± 0.7
Index of insulin resistance	14.7 ± 1.7	8.6 ± 1.1**	8.8 ± 0.9**	3.7 ± 0.2***

Homeostasis Model Assessment was used to calculate an index of insulin resistance as insulin (μU/ml) × glucose (mM)/22.5. Data are the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control

action of the SP formula. Indeed, a protective or β cell sparing action is directly supported by the morphological finding and by the maintenance of the pancreatic insulin concentrations. Evidence of pancreatic islet degeneration, such as low insulin staining, a disorganized architecture (β-cells scattered throughout the islet), were all absent in the ZDF rats given the SP formula (Fig. 3).

Incorporation of the glucose and insulin data into the HOMA (Homeostasis model assessment; Matthews et al., 1985) model showed that the SP2 and SP5 treatments improved insulin sensitivity by 41% and 39%, respectively, and the insulin sensitivity was twice as effective in the rosiglitazone-treated rats, where there was a 75% improvement, as shown in Table V. While the improvement of insulin sensitivity in the RSG-treated rats is due to reduce plasma glucose levels without affecting insulin levels, the stimulation of insulin secretion may result in enhancement of the action of insulin in the SP-treated rats. The fact that the SP formula stimulated the insulin secretion was confirmed in an *in vitro* assay using the RINm5F pancreatic beta cell line (Control, 2.34 ± 0.39; SP 100 μg/ml, 3.32 ± 0.27; SP 200 μg/ml, 4.16 ± 0.28; SP 500 μg/ml, 4.12 ± 0.44).

Also of interest is that the proteinuria was reduced in the SP-treated rats (SP2 and SP5 reduced the albumin excretion by 29 and 44%, respectively). The fact that the blood glucose and urinary proteinuria increased in parallel in the ZDF control rats, suggests that the two processes may be linked, and that the glomerulus of the ZDF kidney may be sensitive to damage by elevated blood glucose. Moreover, a contribution to renal damage caused by coexistent hyperlipidemia cannot be ruled out, since such a connection has been reported in ZDF rats (Kasiske *et al.*, 1998). Our data have confirmed that both plasma triglycerides and free fatty acids are chronically reduced by the SP formula (Fig. 4 and 5). In light of these findings, we postulate that whilst the antihyperglycemic action may have been of pivotal importance, an antihyperlipidemic action may also have been contributory to the renal protective action of SP in this model.

It is also important to know that SP significantly reduced the supply of free fatty acids (FFAs) to the circulation in a dose dependent manner, since the fall in plasma FFAs reduces the substrate competition, between glucose and FFAs, for the uptake into skeletal muscle (Randle *et al.*, 1963), and it also reduces the intramuscular triglyceride and diacylglycerol contents (Oakes *et al.*, 1994). This effect is believed to underpin the improved muscle insulin sensitivity seen with SP treatment.

It is worth noting that whilst the RSG-treated rats revealed hepatotoxicity, as well as a marked weight gain, the SP treated-rats showed no appreciable toxic effect on the liver, and grow normally for the duration of the experiment period. Weight gain is a common accompaniment to other antidiabetic agents, particularly sulfonylureas and insulin (UKPDS group, 1998), and has also been observed with rosiglitazone, which is a member of thiazolidinediones (TZDs) (Shimizu *et al.*, 1998). As shown in Table II, food intake in the rosiglitazone-treated rats was significantly increased compared to that in the ZDF control rats, and this has been confirmed in several papers reporting an increased food consumption with TZDs in rodents (Wang *et al.*, 1997). The SP formula, however, did not increase the fat mass and food intake, although the body weight was marginally elevated compared to the ZDF control rats. It has to be addressed whether an elevated fat mass blunts the antidiabetic activity seen with long-term treatment of rosiglitazone.

Peripheral glucose utilization by skeletal muscle is impeded in most type 2 diabetics, and a decrease in GLUT4 translocation from the intracellular pool to the plasma membranes in skeletal muscle has been implicated as a possible cause of insulin resistance. GLUT4, which is a major enzyme present in skeletal muscle, is a responsible carrier protein for the uptake of plasma glucose in the presence of insulin. Thus, we examined whether SP has a positive effect on the expression of GLUT4 in skeletal muscle. As an enhancer of insulin action, SP upregulated the expressions of GLUT4 mRNA and protein in skeletal muscle.

The glucose metabolism in the liver is altered in untreated ZDF rats, and there is a decrease in the activity of the principal regulatory enzyme, glucokinase, and increases in the insulin-sensitive enzymes, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK). Reduced hepatic glucokinase activity in some obese humans with type 2 diabetes, when compared with nondiabetic normal weight or obese individuals, has been reported (Seoane *et al.*, 1999). Therefore, reduced glucokinase activity is a key feature in the low rate of glucose utilization, which is a characteristic of the diabetic phenotype. SP had the opposite effects on glycolytic and gluconeogenic enzyme activities (Table III). The combined effects of an increase in the glucokinase activity, and a decrease in the glucose-6-phosphatase activity, probably play an additional role in the mechanism by which the SP formula reduces hyperglycemia.

In summary, the SP formula showed potential as an antidiabetic agent in type 2 diabetic patients, and the antihyperglycemic action could be due, at least in part, to the enhancing of insulin action through stimulation of insulin secretion, and by improving the sensitivity of the liver and peripheral tissues to insulin, either directly or as the result of an improved insulin action. Our data, if applicable to humans, support the view that SP may have the potential to prevent the transition to T2DM in patients at risk of developing the disease, due to its capacity to restore glucose homeostasis and prevent on-going β-cell failure. Moreover, the SP formula shows a significant lack of side effects associated with the long-term administration in ZDF rats.

ACKNOWLEDGEMENT

This work was supported by the Oriental Medicine Research Fund of the Ministry of Health and Welfare, Republic of Korea (HMP-00-O-21400-0008).

REFERENCES

Amos, A. F., McCarty, D. J. and Zimmet, P., The rising global burden of diabetes and its complications: estimates and projections to the year 2010. *Diabetic Medicine*, 14, S1-S85 (1997).

Baginski, E. S., Foa, P. P. and Zak, B., "Methods of enzymatic analysis," Vol 2, ed. By H.U. Bergmeyer, Academic Press, New York and London, p. 876 (1974).

Boil, G., Compagnucci, P., Cartechini, M.G., Santeusanio, F., Cirotti, C., Scionti, L. and Brunetti, P., HbA_1 in subjects with abnormal glucose tolerance but normal fasting plasma glucose. *Diabetes*, 29, 272-277 (1980).

DeFronzo, R.A., Pathogenesis of type 2 diabetes: metabolic and molecular implications for identifying diabetes genes. *Diabetes Reviews*, 5, 177-269 (1997).

Groop, L. C., Bonadonna, R. C., DelPrato, S., Ratheiser, K., Zyck, K., Ferrannini, E. and DeFronzo, R. A., Glucose and free fatty acid metabolism in non-insulin dependent diabetes mellitus. Evidence for multiple sites of insulin resistance. *J. Clin. Invest.*, 84, 205-213 (1989).

Hara, H., Miwa, I. and Okuda, J., Inhibition of rat glucokinase by alloxan and ninhydrin. *Chem. Pharm. Bull.*, 34, 4731-4737 (1986).

Kasiske, B. L., ODonnell, M. P., Cleary, M. P. and Keane, W. F., Treatment of hyperlipidemia reduces glomerular injury in obese Zucker rats. *Kidney International*, 33, 667-672 (1998).

Klein, R., Hyperglycemia and microvascular and macrovascular

disease in diabetes. *Diabetes Care*, 18, 258-268 (1995).

Lee, S. H., An, S. Y., Du, H. K. and Chung, S. H., Blood glucose lowering activity and mechanism of Sopungsungi-won (SPSGW) in db/db mouse. *J. Appl. Pharmacol.*, 7, 335-341 (1999).

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., Protein Measurement with the Folin Phenol reagent. *J. Biol. Chem.*, 193, 265-275 (1951).

Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F. and Turner, R. C., Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentration in man. *Diabetologia*, 23, 412-419 (1985).

Nathan, D. M., Long-term complications of diabetes mellitus. *The New England Journal of Medicine* 328,1676-1685 (1993).

Oakes N. D., Kennedy, C. J., Jenkins, A. B., Laybutt, D. R., Chisholm, D. J. and Kraegen, E. W., A new antidiabetic agent, BRL 49653, reduces lipid availability and improves insulin actions and glucoregulation in the rat. *Diabetes*, 43, 1203-1210 (1994)

Polonsky, K. S., Sturis, J. and Bell, G. I., Non-insulin dependent diabetes mellitus. A genetically programmed failure of the B cell to compensate for insulin resistance. *The New England Journal of Medicine*, 334, 777-783 (1996).

Randle, P. J., Hales, C. N., Garland, P. B. and Newsholme, E. A., The glucose fatty-acid cycle: Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet*, 13, 7285-7290 (1963).

Seoane, J., Barbera, A., Telemaque-Potts, S., Newgard, C. B. and Guinovart, J. J., Glucokinase overexpression restores glucose utilization and storage in cultured hepatocytes from male Zucker diabetic fatty rats. *J. Biol. Chem.*, 272, 31833-31838 (1999).

Shimizu, H., Tsuchiya, T., Sato, N., Shimomura, Y., Kobayashi, I. and Mori, M., Troglitazone reduces plasma leptin concentration but increases hunger in NIDDM patients. *Diabetes Care*, 21, 1470-1474 (1998).

UK Prospective Diabetes Study Group, UK Prospective Diabetes Study 16. Overview of 6 tears therapy of type II diabetes: a progressive disease. *Diabetes*, 44, 1249-1258 (1995).

UK Prospective Diabetes Study Group, Intensive blood-glucose control with sulfonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet*, 352, 837-853 (1998).

Wajima, T., "Rinsyo Kagaku Bunseki," Vol 5, ed. By M. Kitamura, M. Satio, and M. Niwa, Tokyo Kagaku Dojin, Tokyo, p. 116 (1973).

Wang, Q., Dryden, S. and Frankish, H.M., Increased feeding in fatty Zucker rats by the thiazolidinedione BRL 49653 (rosiglitazone) and the possible involvement of leptin and hypothalamic neuropeptide Y. *Br. J. Pharmacol.*, 128, 1405-1410 (1997).