Protective Effect of Sun Ginseng against Diabetic Renal Damage

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Received February 3, 2006; accepted April 28, 2006

The effect of sun ginseng (SG, heat-processed Panax ginseng C. A. MEYER at 120 °C) on diabetic renal damage was investigated using streptozotocin-induced diabetic rats. The diabetic rats showed loss of body weight gain, and increases in food and water intake and urine volume, while the oral administration of SG at a dose of 50 or 100 mg/kg body weight/d for 15 d attenuated water intake and urine excretion induced by diabetes. In addition, the diabetic rats given SG at a dose of 100 mg/kg body weight showed significant decreases in serum glucose, serum glycosylated protein and urinary protein levels, suggesting that SG improves the abnormal conditions that lead to oxidative stress. Furthermore, SG significantly reduced advanced glycation endproduct (AGE) formation and thiobarbituric acid-reactive substance levels elevated in the kidneys of diabetic rats. This implies that SG would alleviate the oxidative stress under diabetes through the inhibition of lipid peroxidation. SG also decreased the levels of 3-nitrotyrosine, carboxymethyllysine and receptors for AGE which increase under diabetes. These findings indicate that oxidative stress is increased in the diabetic rat kidney and that SG can prevent renal damage associated with diabetes by attenuating the oxidative stress.

Key words Panax ginseng; sun ginseng; diabetes; oxidative stress; advanced glycation endproduct (AGE); nuclear factor-kappa B (NF-κB)

Diabetes mellitus is characterized by hyperglycemia. An abnormally elevated blood glucose level causes oxidative stress and the formation of advanced glycation endproducts (AGEs), which have been closely linked to diabetic complications such as neuropathy, retinopathy and nephropathy. In particular, diabetics are at increased risk for several types of kidney disease, and the predominant cause of end stage renal disease in this disorder is diabetic nephropathy. However, recent clinical trials suggest that there is no effective treatment for diabetic nephropathy. Therefore, prevention of the occurrence and progression of diabetic nephropathy has become a very important issue. Therefore, great effort has been focused on traditional and herbal medicine without toxic effects to find a novel therapeutic agent for diabetic nephropathy. Panax ginseng C. A. MEYER is one of the most widely used herbal medicines in the Orient. It has a wide range of pharmacological and physiological actions, such as antiaging, immunoenhancement, antistress and antitumor. In addition, several investigations strongly support the evidence that ginseng root possesses anti-diabetic properties, such as inhibition of intestinal glucose absorption, increase in energy expenditure, improving sensitivity to insulin and stimulation of sugar metabolism, etc. Moreover, ginseng root has been shown in clinical studies to have beneficial effects in diabetic patients.

Of the two kinds of ginseng, white ginseng (WG) is air-dried ginseng, and red ginseng (RG) is produced by steaming raw ginseng at 98—100 °C for 2—3 h. RG is reportedly more pharmacologically active than WG. These improved biological activities of ginsengs result from changes in the chemical constituents that occur during steaming treatment. Ginseng saponins, referred to as ginsenosides, are believed to play a pharmacologically important role. Several investigators have reported new ginsenosides from RG that are not usually found in WG. Recently, a method which can enhance the yield of these RG specific components by steaming ginseng at a temperature higher than RG has been developed. This heat-processed ginseng, termed sun ginseng (SG), has been reported to have more potent pharmacological activities, such as vasorelaxation, antioxidative and anti-tumor activities. Furthermore, SG showed stronger peroxynitrite (ONOO−) and hydroxyl radical scavenging activities than WG and RG in our previous in vitro study. However, the effects of SG on diabetic rats have not been reported yet, and the enhanced radical scavenging activities of SG are thought to be beneficial against diabetic oxidative damage caused by hyperglycemia. Therefore, the protective effect of SG against renal damage caused by oxidative stress or the formation of AGEs under diabetes and its molecular biological mechanism were investigated in this study.

MATERIALS AND METHODS

Reagents Phenylmethylsulfonyl fluoride (PMS) and β-actin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protease inhibitor mixture DMSO solution was purchased from Wako Pure Chemical Industries (Osaka, Japan). Nuclear factor-kappa Bp65 (NF-κBp65), inhibitor binding protein xB-α (xB-α), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), receptors for AGE (RAGE) and goat anti-rabbit and/or goat anti-mouse IgG horseradish peroxidase (HRP) conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, California, U.S.A.). 3-Nitrotyrosine (3-NT) was purchased from Upstate Technology (Lake Placid, NY, U.S.A.). The primary monoclonal antibody against carboxymethylly-
sine (CML) was kindly provided by Dr. Nagai of Kumamoto University. The other chemicals and reagents used were of high quality and obtained from commercial sources.

Preparation of SG Extract  WG (Panax ginseng C. A. Meyer, four-years-old) was purchased from a local ginseng market in Seoul. SG was made by autoclaving WG at 120°C for 3 h by the reported method. The product was pulverized and extracted three times with MeOH under reflux at 70°C for 2 h, and the solvent was evaporated in vacuo to give a MeOH extract with a yield of about 20%, by weight, of the original ginseng powder. The major pharmacologically active components of SG, so far reported, are ginsenoside-Rg3, -Rg5, -Rg1, -Rk 1 and maltol, and these compounds are absent or exist in comparably small quantities in conventional WG and RG.

Animals and Treatment  The Guidelines for Animal Experimentation, approved by the University of Toyama, were followed in these experiments. Male Wistar rats (120—130 g) from Japan SLC, Inc. (Hamamatsu, Japan) were used. They were kept in a plastic-bottomed cage under a conventional lighting regimen with a dark night. The room temperature (about 25°C) and humidity (about 60%) were controlled automatically. They were allowed free access to laboratory chow (CLEA Japan Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid and 60.5% carbohydrate) and water. After several days of adaptation, streptozotocin (STZ, Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in citrate buffer (10 mM, pH 4.5) was injected intraperitoneally at a dose of 50 mg/kg body weight following overnight fasting. Ten days after the injection, the glucose level of blood from the tail vein was determined and the rats were divided into 3 groups (each group consisted of 8 rats), avoiding any inter-group differences in blood glucose levels. The control group was given water (vehicle), while the other groups were given the SG extract orally at a dose of 50 or 100 mg/kg body weight daily using a stomach tube. After administration for 15 consecutive days, urine was collected from the metabolic cage and blood samples were collected from the abdominal aorta. The serum was separated immediately from the blood samples by centrifugation. Subsequently, the renal arteries of each rat were perfused with ice-cold physiological saline (0.9% NaCl, pH 7.4), and the kidneys were removed, quickly frozen and kept at −80°C until analysis.

Assays of Serum and Urine Samples  Serum glucose, albumin, urea nitrogen and creatinine (Cr) were determined using commercial reagents (Glucose CII-Test Wako and A/G B-Test Wako were obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan; BUN Kainos and CRE-EN Kainos were obtained from Kainos Laboratories Inc., Tokyo, Japan). The serum glycosylated protein level was measured using a modified thiobarbituric acid (TBA) assay of Fluckiger and Winterhalter. Urine component levels were determined as follows: protein by the sulfosalicylic acid method and quantified as the ratio to BSA as the standard.

Data Analysis  The results for each group are expressed as mean±S.E. values. The effect on each parameter was examined using one-way analysis of variance. Individual differences between groups were evaluated using Dunnett’s test and those at p<0.05 were considered significant.
shows the effects of SG on changes in physico-metabolic symptoms associated with diabetes over the 15-d experimental period. The body weight gain of STZ-induced diabetic rats was significantly lower than that of normal rats. However, there were no significant changes in body weight between diabetic control and SG-treated groups. In addition, the levels of food and water intake and urine excretion were markedly elevated in diabetic control rats. Food intake amounts showed no changes between diabetic control and SG-treated groups. However, water intake and urine excretion levels were significantly reduced by SG administrations.

**Biochemical Features of Serum and Urine** Table 2 shows the effects of SG on general biochemical parameters of serum and urine. Diabetic control rats showed a remarkably higher blood glucose level than normal rats, while the elevated glucose level was significantly reduced in diabetic rats given 100 mg/kg body weight/d of SG. The glycosylated protein level of the control rats was also significantly increased compared to normal rats, but it was significantly decreased by administration of 100 mg/kg body weight/d of SG. In addition, the serum urea nitrogen level increased from 15.0 mg/dl in normal rats to 26.0 mg/dl in diabetic control rats. It was slightly reduced by administration of 50 mg/kg body weight/d of SG. In the case of serum Cr and urinary protein, these levels were no significant changes in diabetic control rats compared to normal rats, but significantly decreased in SG administered groups. However, there were no significant changes in 

**Renal AGEs and TBA- Reactive Substance Levels** The effects of SG on renal AGEs and TBA-reactive substance levels are shown in Table 3. The renal AGEs level in diabetic control rats was significantly higher than in normal rats, but it was effectively lowered by SG administrations to an almost normal level. It declined from 0.96 to 0.81 and 0.80 arbitrary units (AU) by the administration of 50 or 100 mg/kg body weight/d of SG, respectively. Similarly, the renal TBA-reactive substance level was significantly elevated under the diabetic condition, but it was dose-dependently reduced from 1.45 to 0.90 or 0.76 nmol/mg protein by administration of 50 or 100 mg/kg body weight/d of SG, respectively.

**Electrophoretic Patterns of Proteinuria** Figure 1 shows the effects of SG on the proteinuria pattern in STZ-induced diabetic rats. The albumin band is strongly expressed at about 65 kDa in Fig. 1A, and its band intensities are shown in Fig. 1B. There were significant increases in the albumin band intensity of diabetic control rats compared to normal rats, but it was decreased significantly by SG administrations.

**Western Blotting** The protein expressions related to oxidative stress-induced damage in renal tissue are shown in Fig. 2. The albumin band intensity of diabetic control rats was significantly higher than in normal rats, but it was dose-dependently reduced from 1.45 to 0.90 or 0.76 nmol/mg protein by administration of 50 or 100 mg/kg body weight/d of SG, respectively. Similarly, the renal TBA-reactive substance level was significantly elevated under the diabetic condition, but it was dose-dependently reduced from 1.45 to 0.90 or 0.76 nmol/mg protein by administration of 50 or 100 mg/kg body weight/d of SG, respectively.

**Table 1. Physico-Metabolic Symptoms**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg BW/d)</th>
<th>Body weight (Initial, g)</th>
<th>(Final, g)</th>
<th>(Gain, g)</th>
<th>Food intake (g/d)</th>
<th>Water intake (ml/d)</th>
<th>Urine volume (ml/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>239.2±3.6</td>
<td>313.3±11.0</td>
<td>72.8±6.6</td>
<td>18.8±0.8</td>
<td>35.9±3.1</td>
<td>15.2±1.3</td>
</tr>
<tr>
<td>Diabetic</td>
<td>—</td>
<td>196.9±4.6</td>
<td>222.0±7.2</td>
<td>25.1±4.9</td>
<td>28.6±0.9</td>
<td>147.1±6.0</td>
<td>120.2±5.2</td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>196.7±7.6</td>
<td>222.8±11.5</td>
<td>26.1±5.1</td>
<td>28.1±0.8</td>
<td>134.8±4.2</td>
<td>107.9±3.1</td>
</tr>
<tr>
<td>SG</td>
<td>100</td>
<td>196.5±5.6</td>
<td>221.5±10.1</td>
<td>24.4±3.9</td>
<td>28.1±0.8</td>
<td>124.9±3.0</td>
<td>98.8±2.8</td>
</tr>
</tbody>
</table>

a) p<0.001 compared with normal rats; b) p<0.01, c) p<0.001 compared with diabetic control rats.

**Table 2. Biochemical Features of Serum and Urine**

<table>
<thead>
<tr>
<th>Item</th>
<th>Normal (mg/dl)</th>
<th>SG (50 mg/kg BW/d)</th>
<th>SG (100 mg/kg BW/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum glucose</td>
<td>112±4</td>
<td>558±22</td>
<td>526±26</td>
</tr>
<tr>
<td>Serum glycosylated protein</td>
<td>15.5±0.5</td>
<td>20.9±0.9</td>
<td>21.6±0.4</td>
</tr>
<tr>
<td>Serum urea nitrogen</td>
<td>15.0±0.7</td>
<td>26.0±0.6</td>
<td>24.5±1.0</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>0.31±0.01</td>
<td>0.32±0.01</td>
<td>0.28±0.01</td>
</tr>
<tr>
<td>Urinary protein</td>
<td>11.2±2.3</td>
<td>13.0±0.6</td>
<td>9.9±1.0</td>
</tr>
<tr>
<td>Ccr, ml/kg BW/min</td>
<td>7.7±0.06</td>
<td>7.29±0.38</td>
<td>7.89±0.36</td>
</tr>
</tbody>
</table>

a) p<0.05, b) p<0.01, c) p<0.001 compared with normal rats; d) p<0.05, e) p<0.01, f) p<0.001 compared with diabetic control rats.

**Table 3. Renal AGEs and TBA-Reactive Substance Levels**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg BW/d)</th>
<th>AGEs (AU)</th>
<th>TBA-reactive substance (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>0.77±0.02</td>
<td>0.83±0.01</td>
</tr>
<tr>
<td>Diabetic</td>
<td>—</td>
<td>0.96±0.03</td>
<td>1.45±0.11</td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>0.81±0.03</td>
<td>0.90±0.04</td>
</tr>
<tr>
<td>SG</td>
<td>100</td>
<td>0.80±0.01</td>
<td>0.76±0.05</td>
</tr>
</tbody>
</table>

a) p<0.001 compared with normal rats; b) p<0.001 compared with diabetic control rats.
cantly decreased by SG administrations. In the case of the iNOS level, it was mildly but significantly reduced in rats administered 100 mg of SG. On the other hand, there were no significant changes in the 1kB-α level between normal and diabetic control groups, and it was slightly increased in SG administered groups (Fig. 2B).

Figure 3 shows the 3-NT level of renal cortex tissue and the band intensities, corrected by β-actin. Nitrotyrosine Immunoblotting Control (NIC) in Fig. 3A indicates nitrated BSA (ca. 65 kDa). The 3-NT level of diabetic control rats was about 1.4 times higher than that of normal rats, as shown in Fig. 3B, but it was significantly reduced by SG administrations.

DISCUSSION

Diabetes mellitus is a disorder characterized by hyperglycemia. Hyperglycemia causes protein glycation and considerable pathogenesis of long term complications of diabetes. In particular, oxidative stress and AGEs formation induced by hyperglycemia are known to influence diabetic
renal changes and nephropathy. 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.28)

Many attempts have been made to improve the treatment of diabetes. Various kinds of hypoglycemic drugs or insulin are available now for the control of hyperglycemia, but there is no satisfactory therapy in modern medicine without undesirable side effects or contraindications. Considering these problems, herbal medicines have been focused on because of their comparably nontoxic and mild effects. Ginseng, one of the traditionally used anti-diabetic supplements, showed considerable effects in controlling hyperglycemia and obesity.11) The ginseng, a recently developed heat-processed ginseng, has been considered to have stronger biological activities than conventional WG and RG.17—21) However, the renal protective effect of SG in diabetic rats has not been reported yet, and there are no remarkable ameliorations in serum urea nitrogen and Cr levels. It was considered that the administration period, 15 d, was comparably too short to improve these parameters significantly and only early diabetic renal changes occurred, not advanced ones, in this study. Furthermore, the hyperglycemia condition, a chronic metabolic disorder of glucose, results in irreversible tissue damage by the protein glycation reaction, which leads to the formations of glycosylated protein and AGEs.32,33) The glycosylated serum protein level was increased in the present diabetic animal model, which implies that it stimulates the oxidation of sugars, enhancing damage to both sugars and proteins in circulation and the vascular wall, continuing and reinforcing the cycle of oxidative stress and damage. In addition, the accumulation of AGEs in the kidney was also observed. Excessive formation and accumulation of AGEs in tissues can alter the structure and function of tissue proteins. In people with diabetes and/or chronic renal failure, AGEs that accumulate in the kidney are responsible for pathological changes including increased kidney weight, glomerular hypertrophy, glomerular basement membrane thickening and progressive albuminuria.34) Moreover, AGEs stimulate free radical mechanisms and induce membrane peroxidation, which in turn increase membrane permeability. Therefore, AGEs accumulation in the kidney has been regarded as an index of progressive renal damage in diabetic nephropathy. SG decreased the levels of glycosylated serum protein and renal AGES significantly (Tables 2, 3), suggesting that it would inhibit oxidative damage and irreversible renal damage caused by the protein glycation reaction under diabetes.

A significant increase in TBA-reactive substance, an index of endogenous lipid peroxidation, has been shown under diabetic conditions.35,36) Therefore, the measurement of TBA-reactive substance is frequently used to determine the level of oxidative stress in diabetic patients. In addition, the increased lipid peroxidation in the kidney implies the level of suscepti-
bility to diabetic oxidative stress, leading to diabetic complications. From this viewpoint, prevention of lipid peroxidation resulting from oxidative stress is considered to play a crucial role in protection from disorders associated with diabetes. The administration of SG reduced the renal TBA-reactive substance level significantly and dose-dependently. These results suggest that SG may alleviate oxidative stress associated with diabetic pathological conditions through the inhibition of lipid peroxidation.

Continuing with considerations of hyperglycemia-induced renal function parameters and tissue damage, Western blot analyses concerning protein expressions related to oxidative stress and AGEs formation were performed. NF-κB is normally present in the cytoplasm of eukaryotic cells as an inactive complex with the inhibitory protein, IκB. When cells are exposed to various external stimuli, such as reactive oxygen species or AGEs, 1κB undergoes rapid phosphorylation with subsequent ubiquitination, leading to the proteosome mediated degradation of this inhibitor. The functionally active NF-κB exists mainly as a heterodimer consisting of subunits of the Rel family (e.g., Rel A or p65, p50, p52, c-Rel, v-Rel and Rel B) and translocates to the nucleus, where it binds to specific consensus sequences in the promoter or enhancer regions of target genes, thereby altering their expression. In addition, COX-2 and iNOS are known to be involved in the pathogenesis of many chronic diseases associated with oxidative stress. COX-2 and iNOS expressions are known to be significantly enhanced in the kidney of STZ-induced diabetic rats or mice. NF-κB is involved in the regulation of COX-2 and iNOS expressions. As shown in our results, NF-κBp65, COX-2 and iNOS were overexpressed in the diabetic rat kidney, and these overexpressions were concentration-dependently inhibited by SG administration (Fig. 2). These findings imply that SG inhibits COX-2 and iNOS expressions by the deactivation of NF-κB.

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. The 3-NT level was also significantly ameliorated by SG administrations (Fig. 3), suggesting that SG alleviates oxidative stress by inhibiting the generation of reactive-nitrogen species such as ONOO⁻. CML, a major AGE in human tissues, is known to be a marker of cumulative oxidative stress and is involved in the development of diabetic nephropathy. Moreover, activation of RAGE by CML results in the activation of NF-κB and the production of proinflammatory cytokines. Therefore, renal accumulation of CML and RAGE expression were investigated in this study. These levels in diabetic rats were 1.5 times higher than normal, but they were significantly ameliorated in SG administered groups (Fig. 4). These findings imply that SG can prevent diabetic nephropathy by inhibiting RAGE activation by AGEs formation.

In summary, our findings strongly suggest that the inhibition of oxidative stress and AGEs formation may improve and prevent diabetes-induced tissue damage and diabetic complications. SG showed a considerable protecting effect against hyperglycemia-induced renal damage such as diabetic nephropathy by inhibiting AGEs formation and oxidative stress. In particular, SG suppresses RAGE activation related to NF-κB activation by inhibiting AGEs formation. SG also protects the kidney against diabetic oxidative stress induced by expressions of COX-2 and iNOS via deactivation of NF-κB. Furthermore, SG alleviates oxidative stress under diabetic conditions through the inhibition of lipid peroxidation and the generation of reactive-nitrogen species such as ONOO⁻. However, the comparisons on the effects of WG, RG and SG were not confirmed in this study, and we want to clarify these subjects in a future study.

In conclusion, the present study provides scientific evidence of the preventive and therapeutic potential of SG against renal damage associated with diabetic oxidative stress.

REFERENCES