Effects of Heat-Processed Ginseng and Its Active Component Ginsenoside 20(S)-Rg3 on the Progression of Renal Damage and Dysfunction in Type 2 Diabetic Otsuka Long-Evans Tokushima Fatty Rats

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The effects of heat-processed ginseng (HPG) and ginsenoside 20(S)-Rg3 on the progression of renal damage in type 2 diabetic rats were investigated. Twenty-two-week-old male Otsuka Long-Evans Tokushima Fatty (OLETF) rats were divided into 4 orally administered groups: vehicle (diabetic control), HPG water extract (100 mg/kg) and 20(S)-Rg3 (5, 10 mg/kg). Non-diabetic Long-Evans Tokushima Otsuka (LETO) rats were used as a normal group. OLETF rats showed markedly higher blood glucose, triglyceride, and total cholesterol levels than those of LETO rats. The elevated blood glucose level of OLETF rats was significantly lowered by 20(S)-Rg3 administration. The elevated serum triglyceride and total cholesterol levels were significantly reduced by the administrations of HPG and 20(S)-Rg3. The serum levels of thiobarbituric acid-reactive substance, an index of lipid peroxidation, were markedly increased in OLETF compared to LETO rats, but it was significantly reduced by HPG and 20(S)-Rg3 administrations. The urinary protein level, an indicator of advanced diabetic nephropathy, of OLETF rats was 4.4 times higher than in LETO rats, but it was reduced significantly by the administrations of HPG and 20(S)-Rg3. Creatinine clearance of OLETF rats was significantly increased after HPG and 20(S)-Rg3 administrations. The elevation of inducible nitric oxide synthase and Nε-(carboxymethyl)lysine protein expressions in renal tissues of OLETF rats was prevented by 20(S)-Rg3 administration. This study provides scientific evidence that 20(S)-Rg3 prevents the progression of renal damage and dysfunction in type 2 diabetic rats via inhibiting oxidative stress and advanced glycation endproduct formation.

Key words heat-processed ginseng; ginsenoside 20(S)-Rg3; renal damage; type 2 diabetic rat

Herbs have been used for centuries to treat illness and improve health, and still account for about 80% of medical treatment in the developing world, with approximately one third of drugs being derived from plant sources. 1–3 With the high prevalence of obesity and type 2 diabetes, abnormalities in carbohydrate metabolism are common in the general population, and achieving glycemic control is important in reducing the complications of diabetes. 4 Among the frequently mentioned herbal medicines to help manage blood glucose, ginseng extracts made from the root, rootlet, berry, and leaf of Panax quinquefolium (American ginseng) and Panax ginseng (Asian ginseng) have proven effects of anti-hyperglycemia, insulin sensitization, islet protection, anti-obesity, and anti-oxidation in many model systems. 5–7 Constituent ginseng-specific saponins (ginsenosides) are considered to be the major bioactive compounds responsible for the metabolic activities of ginseng. 5–7

Ginseng has a long history in folk medicine, and the heat-processing method to strengthen the efficacy of ginseng has been well-defined in Korea based on a long history of ethnopharmacological evidence. 7 A few years ago, heat-processed ginseng (HPG) which is enriched in the ginsenoside 20(S)-Rg3, (20(S)-Rg3) was developed 8 and we identified that the protective effects of HPG and 20(S)-Rg3 against streptozotocin-induced diabetic renal damage (type 1 diabetic rat model) was mediated by inhibiting oxidative stress and advanced glycation endproduct (AGE) formation. 7,9,10

The therapeutic potential of HPG and 20(S)-Rg3 against diabetic renal damage in our previous study was assessed using an animal model of insulin-dependent diabetes (type 1). Type 1 diabetes is caused by the autoimmune destruction of pancreatic β cells, and affects about 10% of patients with diabetes. On the other hand, insulin resistance and relative insulin deficiency are the causes of type 2 diabetes, which accounts for 90% of patients with diabetes. 11 In fact, the number of diabetic patients in Japan has reached seven million, with a marked increase in non-insulin-dependent (type 2) diabetes mellitus. Furthermore, diabetic nephropathy has been the major cause of patients needing chronic hemodialysis since 1998. 12 Prevention of the occurrence and progression of diabetic nephropathy has become a very important issue.

Therefore, much effort has been focused on traditional and herbal medicines without toxic effects to identify a novel therapeutic agent for diabetic nephropathy both by us and others. 13–15 The aim of the present study was to identify the effects of HPG and its active component 20(S)-Rg3 on the progression of renal damage in type 2 diabetic rats, which has not yet been reported. This study may also shed a mechanistic light on recent clinical observations suggesting that ginseng supplementation is beneficial for anti-diabetic therapy. 16

MATERIALS AND METHODS

Reagents Phenylmethylsulfonyl fluoride (PMSF), thiobarbituric acid (TBA), and β-actin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protease inhibitor mixture dimethyl sulfoxide (DMSO) solution was purchased from Wako Pure Chemical Industries (Osaka, Japan). Inducible nitric oxide synthase (iNOS) and goat anti-rabbit and/or goat anti-mouse immunoglobulin G (IgG) horse-

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radish peroxidase (HRP)-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, California, U.S.A.). The primary polyclonal antibody against N^c-(carboxymethyl)lysine (CML) was kindly provided by Dr. Nagai of Kumamoto University. ECL Western Blotting Detection Reagents were purchased from GE Health care (Piscataway, NJ, U.S.A.). The other chemicals and reagents used were of high quality and obtained from commercial sources.

**Preparation of HPG and Ginsenoside** Dried *Panax ginseng* (4 years old) was purchased from a local ginseng market in Seoul (Korea). Fifty grams of *Panax ginseng* were ground to pass an 80 mesh sieve and boiled gently in 1000 ml of water 3 times for 1 h. The solvent was evaporated in vacuo to give a water extract with a yield of about 20%, by weight, of the original ginseng powder. *Panax ginseng* water extract was autoclaved at 120 °C and 0.11 MPa for 3 h, and the product was dried in an oven at 50 °C for 3 d to produce HPG.

HPG water extract was dissolved in MeOH (5 mg/ml), and analyzed with a Hitachi (Tokyo, Japan) L-7100 liquid chromatograph fitted with a C-18, reversed-phase column (5 μm, 25 cm×4 mm i.d.; YMC-Pack Pro) utilizing a solvent gradient system, as described previously. Briefly, the mobile phase consisted of water (solvent A) and acetonitrile (solvent B), and the flow rate was 1 ml/min. The detector was a SEDEX 55 ELSD (Sedere, France). The gradient elution was B, and the correlation coefficient was >0.99. The relative standard deviation value of intra-day repeatability was lower than 6%, representing satisfactory precision.

**Animals and Treatment** The Guidelines for Animal Experimentation approved by the University of Toyama were followed in the present study. Male Otsuka Long-Evans Tokushima Fatty (OLETF) and Long-Evans Tokushima Otsuka (LETO) rats (4 weeks old) were kindly supplied by the Tokushima Research Institute (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan). All rats were kept in plastic-bottomed cages with a controlled temperature (about 25 °C) and humidity (about 60%), and a 12-h light : 12-h dark cycle, and were given free access to laboratory pellet chow (CE-2, CLEA Japan Inc., Tokyo, Japan, comprising 24.0% protein, 3.5% lipids, and 60.5% carbohydrates) and water. When the rats were 22 weeks old, we determined blood glucose without fasting, urinary protein excretion, and creatinine clearance (Ccr) levels, and allocated the OLETF rats to four groups of 9 rats, avoiding any significant differences of these levels among the groups. Urine (24-h) was collected from each animal using the metabolic cages. Over the experimental period of 50 d, the non-diabetic LETO group and one diabetic OLETF group received water, and the other three OLETF groups received a solution of HPG water extract (100 mg/kg) and 20(S)-Rg3 (5 or 10 mg/kg) orally by gavage once a day. The volume (ml) of oral administration was calculated as 1/200 of each body weight (g).

Group 1: LETO rats (non-diabetic control, *n*=5) received water (no sample treatment)

Group 2: OLETF rats (diabetic control, *n*=9) received water (no sample treatment)

Group 3: OLETF rats (*n*=9) treated with HPG water extract (100 mg/kg) in aqueous solution orally for 50 d

Group 4: OLETF rats (*n*=9) treated with 20(S)-Rg3 (5 mg/kg) in aqueous solution orally for 50 d

Group 5: OLETF rats (*n*=9) treated with 20(S)-Rg3 (10 mg/kg) in aqueous solution orally for 50 d

The animal experiment schedule and sample doses used in this study were determined based on previous studies on OLETF rats and ginseng components. During the last 2 d in administration period, cumulative 24-h food and water consumption and urine volume were checked, with rats being individually kept in metabolic cages. At the end of the experiment, all rats were sacrificed after an intraperitoneal pentobarbital injection. Blood samples were collected from the abdominal aorta. 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, triglyceride, total cholesterol, and creatinine (Cr) were determined using commercial reagents (Glucose CII-Test Wako, Triglyceride E-Test Wako and Cholesterol E-Test Wako obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan; CRE-EN Kainos obtained from Kainos Laboratories Inc., Tokyo, Japan). The serum TBA-reactive substance level was determined by employing the method of Naito and Yamakawa. Urine protein and Cr levels were determined using commercial reagents (Micro TP-Test Wako; CRE-EN Kainos). Ccr was calculated on the basis of the urinary Cr, serum Cr, urine volume, and body weight using the following equation: Ccr = [urinary Cr (mg/dl)×urine volume (ml)/serum Cr (mg/dl)]×[1000/body weight (g)]×[1/1440 (min)].

**Western Blot Analyses** Renal cortical tissues (0.2 g) were homogenized with ice-cold lysis buffer (pH 7.5) containing 137 mM NaCl, 20 mM Tris–HCl, 1% Tween-20, 10% glycerol, 1 mM PMSF, and the protease inhibitor mixture DMSO. Samples were then centrifuged at 2000 g for 10 min at 4 °C. The protein concentration of tissue was determined using a Bio-Rad protein assay kit and bovine serum albumin as a standard, and then immunoblotting was carried out. For the determination of iNOS and CML protein levels in the kidney, 30 μg of protein of each sample was electrophoresed through 8 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electrophoretically transferred to a nitrocellulose membrane, blocked with 5% skim milk solution for 3 h at 4 °C, and then incubated with primary antibodies overnight at 4 °C. After the blots were washed, they were incubated with goat anti-rabbit and/or goat anti-mouse IgG HRP-conjugated secondary antibody for 90 min at room temperature. Each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (Fujifilm, Tokyo, Japan). Band densities were determined by Scion image software (Scion Corp., Frederick, MD, U.S.A.) and quantified as the ratio to β-actin.

**Statistical Analysis** The results are expressed as means±S.E. The effect on each parameter was examined...
using one-way analysis of variance. Individual differences among groups were analyzed employing Dunnett’s test. \( p < 0.05 \) was considered significant.

RESULTS

Contents of Ginsenosides in HPG Water Extract The contents of ginsenosides in HPG water extract were as follows: Re (0.05%), \( R_b_1 \) (0.16%), Rc (0.11%), \( R_b_2 \) (0.06%), Rd (0.02%), 20(S)-Rg3 (0.10%), 20(R)-Rg3 (0.05%), Rk1 (0.11%), and Rg5 (0.15%) (Fig. 1A). The total ginsenoside content of HPG water extract was 0.85%. Among them, 20(S)-Rg3 (Fig. 1B) was selected for further in vivo study based on its stronger in vitro antioxidant activity than other ginsenosides.7,9(10)

Changes in Physico-Metabolic Symptoms Table 1 shows the effects of HPG water extract and 20(S)-Rg3 on the changes in physico-metabolic symptoms during the experimental period. The body weight gain of OLETF rats was significantly higher than that of LETO rats. The initial, final, and gain in body weights of OLETF rats were not affected by the HPG water extract and 20(S)-Rg3 administrations throughout the experimental period. The levels of food intake and gain in body weight were markedly elevated compared to LETO rats, but there were no changes in the water intake and urine volume. After 50 consecutive days of HPG water extract and 20(S)-Rg3 administrations to OLETF rats, the volume of water intake was significantly reduced by the 10 mg/kg body weight of 20(S)-Rg3 administration, but no change was noted in the urine volume.

Biochemical Features of Serum Table 2 shows the effects of HPG water extract and 20(S)-Rg3 on serum glucose, triglyceride, total cholesterol, and TBA-reactive substance levels. When the rats were grouped avoiding any significant differences in the blood glucose levels, the diabetic OLETF rats showed a markedly higher blood glucose level compared to the LETO rats. The blood glucose level of LETO rats remained unchanged throughout the experiment. However, hyperglycemia was observed in OLETF rats at 50 d after grouping, whereas, in OLETF rats treated with 10 mg/kg body weight of 20(S)-Rg3, their blood glucose levels were significantly lower than in the water-treated groups. The serum triglyceride and total cholesterol levels in OLETF rats were significantly higher than in LETO rats, but these elevated serum triglyceride and total cholesterol levels were significantly reduced by the oral administration of HPG water extract (100 mg/kg body weight) and 20(S)-Rg3 (10 mg/kg body weight). The serum level of TBA-reactive substances, Glc-2-Glc, was reduced by the oral administration of 20(S)-Rg3.

Table 1. Physico-Metabolic Symptoms

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg body weight/d)</th>
<th>Body weight (Initial, g)</th>
<th>Body weight (Final, g)</th>
<th>Body weight (Gain, g)</th>
<th>Food intake (g/d)</th>
<th>Water intake (ml/d)</th>
<th>Urine volume (ml/d)</th>
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<tbody>
<tr>
<td>LETO rats</td>
<td>—</td>
<td>416.3±16.6</td>
<td>477.2±18.2</td>
<td>60.8±3.6</td>
<td>18.7±0.5</td>
<td>30.3±3.3</td>
<td>14.1±1.4</td>
</tr>
<tr>
<td>OLETF rats</td>
<td>—</td>
<td>485.9±8.5(d)</td>
<td>574.1±9.8(e)</td>
<td>88.2±4.8(e)</td>
<td>28.5±0.1(a)</td>
<td>32.1±2.2</td>
<td>16.1±1.2</td>
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<tr>
<td></td>
<td>Control</td>
<td>486.2±6.1(e)</td>
<td>579.9±9.3(e)</td>
<td>93.7±7.6(e)</td>
<td>29.1±0.8(a)</td>
<td>27.2±3.1</td>
<td>15.2±1.3</td>
</tr>
<tr>
<td></td>
<td>HPG</td>
<td>486.0±7.5(f)</td>
<td>579.0±6.2(f)</td>
<td>81.3±5.0(f)</td>
<td>28.1±0.5(a)</td>
<td>27.9±3.1</td>
<td>14.6±1.4</td>
</tr>
<tr>
<td></td>
<td>20(S)-Rg3 5</td>
<td>486.1±9.4(g)</td>
<td>580.5±18.1(e)</td>
<td>90.6±6.4(g)</td>
<td>29.8±0.5(a,b)</td>
<td>25.4±2.8(b)</td>
<td>14.5±1.5</td>
</tr>
<tr>
<td></td>
<td>20(S)-Rg3 10</td>
<td>486.2±2.8(h)</td>
<td>580.5±18.1(e)</td>
<td>90.6±6.4(g)</td>
<td>29.8±0.5(a,b)</td>
<td>25.4±2.8(b)</td>
<td>14.5±1.5</td>
</tr>
</tbody>
</table>

Data are expressed as the mean±S.E. \( a \) \( p < 0.05 \) compared with LETO rats. \( b \) \( p < 0.05 \) compared with OLETF control rats.

Table 2. Biochemical Features of Serum

<table>
<thead>
<tr>
<th>Item</th>
<th>LETO rats</th>
<th>OLETF rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HPG (100 mg)</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>104±7</td>
<td>200±8(e)</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>37.2±1.1</td>
<td>136.9±13.0(e)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>73.1±5.7</td>
<td>87.4±3.5(b)</td>
</tr>
<tr>
<td>TBA-reactive substance, nmol/ml</td>
<td>2.56±0.17</td>
<td>3.43±0.15(e)</td>
</tr>
</tbody>
</table>

Data are expressed as the mean±S.E. \( a \) \( p < 0.05 \) compared with LETO rats. \( b \) \( p < 0.05 \) compared with OLETF control rats.
an index of endogenous lipid peroxidation, was markedly increased in OLETF compared to LETO rats, but it was significantly reduced by the HPG water extract (100 mg/kg body weight) and 20(S)-Rg3 (10 mg/kg body weight) administrations.

Renal Function Parameters Figure 2 shows the effects of HPG water extract and 20(S)-Rg3 on the renal function parameters. The urinary protein level, an indicator of advanced diabetic nephropathy, of OLETF rats was 30.1 ± 3.7 mg/d, which is a 4.4 times higher value than in LETO rats, but it was significantly reduced by the administration of HPG water extract (100 mg/kg body weight) and 20(S)-Rg3 (10 mg/kg body weight). Although there were no significant changes in Ccr between the LETO and OLETF rats, it was significantly increased after HPG water extract (100 mg/kg body weight) and 20(S)-Rg3 (5 or 10 mg/kg body weight) administrations, indicating improvement of the renal function.

Western Blotting The protein expressions related to oxidative stress-induced damage in renal tissue are shown in Fig. 3. These protein band intensities were corrected by β-actin and graphed. There were significant increases in iNOS and CML protein expressions in diabetic OLETF compared to LETO rats. The elevated iNOS level, one of the commonly used markers for oxidative stress, was significantly reduced by 10 mg/kg body weight of 20(S)-Rg3 administration. CML is one of the major AGEs and involved in the development of diabetic nephropathy. No significant ameliorations were noted in the CML protein expression of diabetic OLETF rats by HPG water extract administration, but the increase of CML protein expression levels in 20(S)-Rg3-administered groups was not significantly elevated compared to that of LETO rats, which suggests the prevention of CML formation by 20(S)-Rg3 administration.

DISCUSSION

Increasing evidence from both experimental and clinical studies suggests that AGEs and oxidative stress play a major role in the pathogenesis and complications of both types of diabetes mellitus. AGEs can alter the structure and function of intra- and extracellular molecules, increase oxidative stress, and modulate cell activation, signal transduction, and the expression of cytokines and growth factors through receptor-dependent and -independent pathway. Direct high glucose-induced protein kinase and mitogen-activated protein kinase activation causes the increased production of oxidative stress in the diabetic kidney. In addition, oxidative stresses such as hydroxyl radicals and peroxynitrite are known to increase the formation of CML, a major in vivo AGE, by the oxidative cleavage of Amadori products. Therefore, the inhibition of AGEs and oxidative stress has received considerable interest because of their close association with the prevention of diabetic renal damage.

In the present study, we used OLETF rats to identify the effects of HPG and its active component 20(S)-Rg3, revealing them to show in vitro free-radical scavenging activities and in vivo renoprotective effects, on the progression of renal damage in type 2 diabetic rats. The OLETF rat is an animal model of spontaneously obese type 2 diabetes characterized by hyperglycemia, insulin resistance, hyperinsulinemia and hypertriglyceridemia and with complications such as nonalcoholic fatty liver and renal disorders, and these typical characteristics of OLETF rats are known to be useful for analyzing the complex forms of human diabetes.

The results of our present study showed that 20(S)-Rg3, the active component ginsenoside of HPG, prevents the progression of renal damage and dysfunction in type 2 diabetic rats via inhibiting oxidative stress and AGE formation. These critical effects of 20(S)-Rg3 on oxidative stress and AGE formation were confirmed and supported by the following lines of observation made in the present study: Firstly, the elevated oxidative stress and lipid peroxidation levels in diabetic OLETF rats were significantly reduced by the HPG water extract and 20(S)-Rg3 administrations, as shown by the significant decreases in serum TBA-reactive substance and renal cortical iNOS protein expression levels (Table 2, Fig. 3A). Secondly, the progression of renal dysfunction in OLETF rats, which is characterized by the increase of urinary protein and decrease of Ccr, were markedly ameliorated by the HPG water extract and 20(S)-Rg3 administrations (Fig. 2). Thirdly,
based on the renal cortical CML protein expression as a marker of AGE formation and also cellular oxidative stress, we also showed that there was a decrease in CML levels in the renal tissue of diabetic OLETF rats on 20(S)-Rg3 administrations (Fig. 3B). The effects of 20(S)-Rg3 on renal damage and dysfunction were stronger than its crude drug HPG water extract, suggesting that 20(S)-Rg3 is a major active component of HPG.

Our results also confirmed and extended recent observations on the beneficial effects of ginseng and 20(S)-Rg3 on hyperglycemia, hyperlipidemia, and insulin resistance as shown in the lowered glucose, triglyceride, and total cholesterol levels of OLETF rats by HPG water extract and 20(S)-Rg3 administrations (Table 2). Lee et al. reported that treatment with Korean red ginseng, steamed ginseng, improved insulin sensitivity and significantly preserved glucose tolerance compared with untreated control animals up to 50 weeks of age, implying that Korean red ginseng attenuated the development of overt diabetes. In addition, the Panax ginseng product prepared by vineyard extraction, which has an enriched content of 20(S)-Rg3, was reported to lower fasting and postprandial glucose concentrations and reduce the body weight of diabetic OLETF rats. These effects were associated with increased peroxisome proliferator-activated receptor γ expression and adenosine monophosphate-activated protein kinase phosphorylation in the liver and muscle.

Consistent with our earlier observation on the effect of 20(S)-Rg3 in the type 1 diabetic renal damage model, this study newly provides scientific evidence that 20(S)-Rg3 prevents the progression of renal damage and dysfunction in type 2 diabetic OLETF rats via inhibiting oxidative stress and AGE formation. This study identified the novel efficacy of 20(S)-Rg3 on renal damage in the type 2 diabetic animal model, and extended recent observations on the beneficial effects of ginseng products and components on hyperglycemia, hyperlipidemia, and insulin resistance.

REFERENCES