Protective and anti-angiopathy effects of ginsenoside Re against diabetes mellitus via the activation of p38 MAPK, ERK1/2 and JNK signaling

YAWEI SHI[1], XUESI WAN[2], NAN SHAO[1], RUNYI YE[1], NING ZHANG[1] and YUNJIAN ZHANG[1]

Departments of [1]Thyroid and Breast Surgery and [2]Endocrinology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510080, P.R. China

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Abstract. The present study aimed to determine the protective and anti-angiopathy effects of ginsenoside (GSS) on Wistar rats with diabetes mellitus (DM). Diabetic angiopathy occurs during the early stage of diabetes, and in type 1 DM (T1DM) and type 2 DM (T2DM). In the present study, early DM, T1DM and T2DM were induced by treatment with a high-sucrose-high-fat diet, alloxan monohydrate or streptozocin, respectively. The levels of blood glucose, insulin, lipid metabolism markers [total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) and lipoprotein(a) (Lp-a)], and endothelial cell function markers [endothelin, nitric oxide, vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6)] were determined following treatment with GSS. In addition, oral glucose tolerance test and insulin tolerance test were performed. The phosphorylation levels of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) were detected in aorta samples harvested from T2DM rats by western blot analysis. The present study determined that GSS treatment effectively decreased the levels of blood glucose, TC, TG, Lp-a, VEGF, IL-6, phosphorylated (p)-p38, p-ERK1/2 and p-JNK; however, treatment with GSS increased insulin and HDL levels. Therefore, it is possible that GSS exerts protective and anti-angiopathy effects against the early stage of diabetes, T1DM and T2DM *in vivo* via the activation of p38 MAPK, ERK1/2 and JNK signaling.

Correspondence to: Dr Yunjian Zhang, Department of Thyroid and Breast Surgery, The First Affiliated Hospital of Sun Yat-sen University, 58 Zhongshan Er Road, Yuexiu, Guangzhou, Guangdong 510080, P.R. China
E-mail: zhangyunjianmmr_15@126.com

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Introduction

Diabetes mellitus (DM) is a group of metabolic diseases, which is characterized by chronic hyperglycemia and glucose intolerance, and may result in multi-organ dysfunction (1). It is one of the most common chronic diseases worldwide, the incidence of which is increasing at an alarming rate due to various factors (2,3), including increased economic development, which often leads to changes in diet and lifestyle habits, and increased obesity (4,5). A previous study reported that >60% of the worldwide population with DM resides in Asia (6). The morbidity and mortality rate of patients with DM remains high, regardless of extensive investigations into potential treatments, and DM is considered a contemporary challenge for public health. The primary cause of morbidity and mortality in patients with DM is diabetic micro- and macroangiopathy complications, also termed diabetic angiopathy, this may lead to accelerated and aggravated forms of atherosclerosis, renal failure, retinopathy, neuropathy and amputation (7,8). Therefore, it is extremely important to determine the pathogenesis of diabetic angiopathy and develop targeted clinical treatment strategies for the future.

American ginseng (*Panax quinquefolius*) and Asian ginseng (*Panax ginseng*) have been used as medicinal plants for the treatment of hyperglycemia, diabetes, and their associated complications (9-13). *P. ginseng* has been officially approved in China as an important ingredient in herbal therapeutic agents used for treating DM (14). The major active components of ginseng are ginsenosides (GSS). The mechanism of action of ginseng and GSS in terms of DM treatment is complex. A previous study demonstrated that their positive therapeutic effects may be associated with the modulation of insulin secretion and glucose metabolism, or regulation of the inflammatory pathway in insulin-dependent and -independent processes (15). GSS Re is the predominant protopanaxatriol in ginseng. However, the efficacy of GSS Re treatment on diabetic angiopathy remains to be elucidated.

The present study aimed to investigate the protective and anti-angiopathy effects of GSS treatment in rats with early stage diabetes, type 1 DM (T1DM) and type 2 DM (T2DM). The levels of blood glucose, insulin, lipid metabolism markers, and endothelial cell function markers were determined,

and the expression of the mitogen-activated protein kinase (MAPK) signaling pathway proteins, including p38 MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK), were determined.

Materials and methods

Animals and drugs. A total of 72 adult male Wistar rats (age, 7-8 weeks; weight, 200-250 g), purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), were used in the current study. All of the rats were housed individually in ventilated cages, with *ad libitum* access to a standard diet and tap water, and were maintained at controlled temperature (25˚C) and humidity (50%) under a 12-h light/12-h dark cycle. The study was approved by the ethics committee of The First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) The animal care and use was monitored by Sun Yat-sen University animal care committee (Guangzhou, China) and was in accordance with National Institutes of Health guidelines (16). GSS Re was obtained from Sangong Pharmaceutical Co., Ltd. (Shanghai, China). Alloxan monohydrate and streptozocin (STZ) were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany).

Experimental design and diabetes induction. Diabetic angiopathy occurs during the early stage of diabetes, T1DM and T2DM. In the present study, early diabetes, T1DM and T2DM were induced by three different methods: The administration of a high-sucrose-high-fat diet, alloxan monohydrate or STZ, respectively. The rats were randomly assigned into four groups (n=6 in each model/group): i) Control; ii) control + GSS; iii) DM; and iv) DM + GSS. The rats in the control group were fed a standard chow diet for 8 weeks and received intragastric administration of normal saline (20 mg/kg) for an additional 8 weeks. In the control + GSS group, rats were fed a standard chow diet for 8 weeks and received intragastric administration of GSS (20 mg/kg) for an additional 8 weeks. The rats in the DM group (n=6 in each model) were fed a high-sucrose-high-fat diet for 8 weeks, followed by induction of T1DM or T2DM, and a further 8-week high-sucrose-high-fat diet. In the DM + GSS group (n=6 in each model), following DM induction, rats were treated by intragastric administration of GSS (20 mg/kg) for an additional 8 weeks.

For the induction of early stage diabetes, the rats received a high-sucrose-high-fat diet for 8 weeks. For the induction of T1DM and T2DM, the rats were initially fed a high-sucrose-high-fat diet for 8 weeks, and were then fasted for 18 h. In order to induce T1DM the rats received intraperitoneal injection with alloxan monohydrate (120 mg/kg dissolved in normal saline) every other day for 4 days. T2DM was induced by a single administration of STZ (50 mg/kg dissolved in 0.9% sterile sodium chloride; i.p). Due to acute hypoglycemia, the rats were supplied with 10% sucrose solution for 48 h in place of drinking water. To confirm that the induction of diabetes was successful, blood samples were collected from the tail-end part of each rat and the blood glucose levels were determined using a glucometer. The rats with a blood glucose level ≥300 mg/dl (16.7 mmol/l) were regarded as diabetic.

Plasma biochemistry. Blood glucose was determined using a glucose monitoring system (Medtronic MiniMed, Inc., Northridge, CA, USA). Serum levels of insulin (Rat Ins1/Insulin ELISA kit; Sigma-Aldrich; Merck Millipore), vascular endothelial growth factor (VEGF; Rat VEGF ELISA kit; Sigma-Aldrich; Merck Millipore), endothelin (ET; Endothelin 1 ELISA kit; Abcam, Cambridge, MA, USA), and interleukin-6 (IL-6; Rat IL-6 ELISA kit; Abcam) were determined by commercial enzyme-linked immunosorbent assay kits according to the manufacturer's protocols. Serum levels of nitric oxide (NO) were determined by the Griess reaction, according to the manufacturer's protocol of an NO kit (R&D Systems, Inc., Minneapolis, MN, USA). High-density lipoprotein (HDL), triglyceride (TG) and total cholesterol (TC) were quantified by enzymatic colorimetric analysis (Roche Diagnostics GmbH, Mannheim, Germany). The concentration of lipoprotein(a) (Lp-a) was determined using an immunonephelometric method with N Latex Lp (a) reagent, according to the manufacturer's protocol (Siemens Healthcare GmbH, Erlangen, Germany).

Metabolic analyses. The rats were fasted for 6 h prior to the oral glucose tolerance test (OGTT). Blood samples were collected from the lateral saphenous vein at baseline (0 min) and then at 15, 30, 60, 90 and 120 min following the oral administration of 2 g/kg glucose. Glucose levels were assessed using a 2300 Stat Plus glucose analyzer (YSI, Inc., Yellow Springs, OH, USA). In order to perform the insulin tolerance test (ITT), the rats were fasted for 4 h and an intraperitoneal injection of insulin (0.5 U/kg) (Humulin; Lilly USA, LLC, Indianapolis, IN, USA) was administered. Blood glucose was monitored at the indicated time points (baseline 0, 15, 30, 60, 90 and 120 min).

Tissue preparation and western blot analysis. The T2DM rats were sacrificed using ketamine/xylazine (160/24 mg/kg). The aortic tissues were immediately harvested and washed with cold saline. The tissues were stored at -80˚C for further protein assays. Total protein was extracted from the aortas using NP40 protein lysis buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and protein concentration was assessed using the DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (20 μg) were resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and were transferred on to a polyvinylidene difluoride membrane. Subsequently, the membrane was blocked with 5% fresh non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 solution for 2 h at room temperature, and was incubated with the following rabbit monoclonal primary antibodies overnight at 4˚C: Phosphorylated (p)-p38 MAPK (p-p38; 1:1,000; cat. no. 4511), p-ERK1/2 (1:1,000; cat. no. 4094) and p-JNK (1:1,000; cat. no. 4668; all Cell Signaling Technology Inc., Danvers, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase was used as an internal housekeeping control with a rabbit monoclonal anti-GAPDH antibody (1:1,000; Cell Signaling Technology, Inc.; cat. no. 5174. Following incubation for 1 h at room temperature (25˚C) with the goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5,000; Cell Signaling Technology, Inc.; cat. no. 7071), enhanced chemiluminescence (Pierce ECL Western Blotting

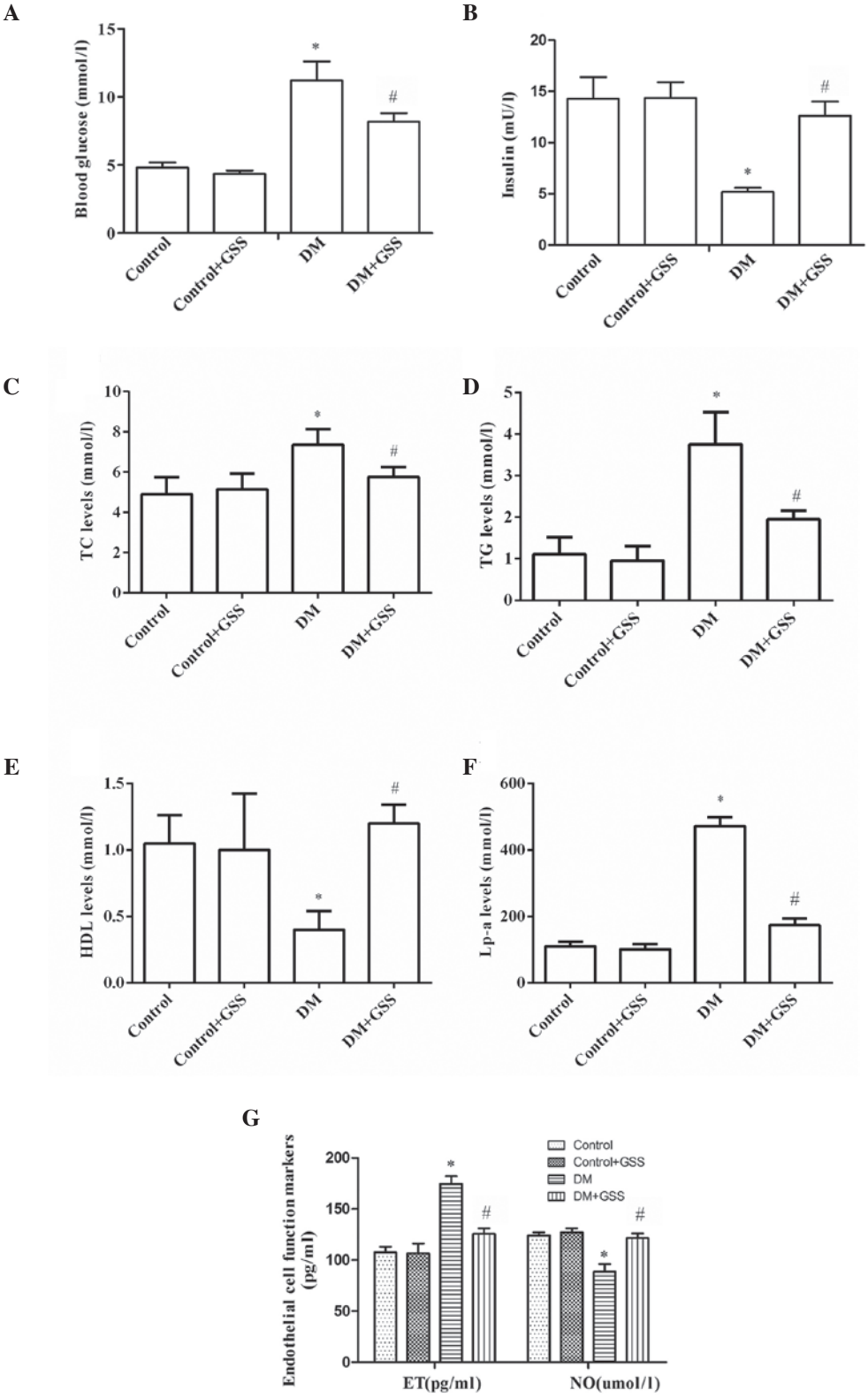

Figure 1. Effects of GSS on vascular injury caused by early stage diabetes. (A) Blood glucose levels in the four treatment groups. (B) Insulin levels in the four treatment groups. Levels of (C-F) lipid metabolism markers and (G) endothelial cell function markers. *P<0.05 vs. the control and control +GSS groups; #P<0.05 vs. the DM group. Data are presented as the mean ± standard deviation. A one-way analysis of variance with a post-hoc Tukey's test was used to identify significant differences among groups. GSS, ginsenoside; DM, diabetes mellitus; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; Lp-a, lipoprotein(a); ET, endothelin; NO, nitric oxide.

Substrate; Thermo Fisher Scientific, Inc.) and densitometric analysis were performed using ImageLab software (version 2.0.1; Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are presented as the mean ± standard deviation. Comparisons between groups were calculated using analysis of variance followed by a post-hoc Tukey's

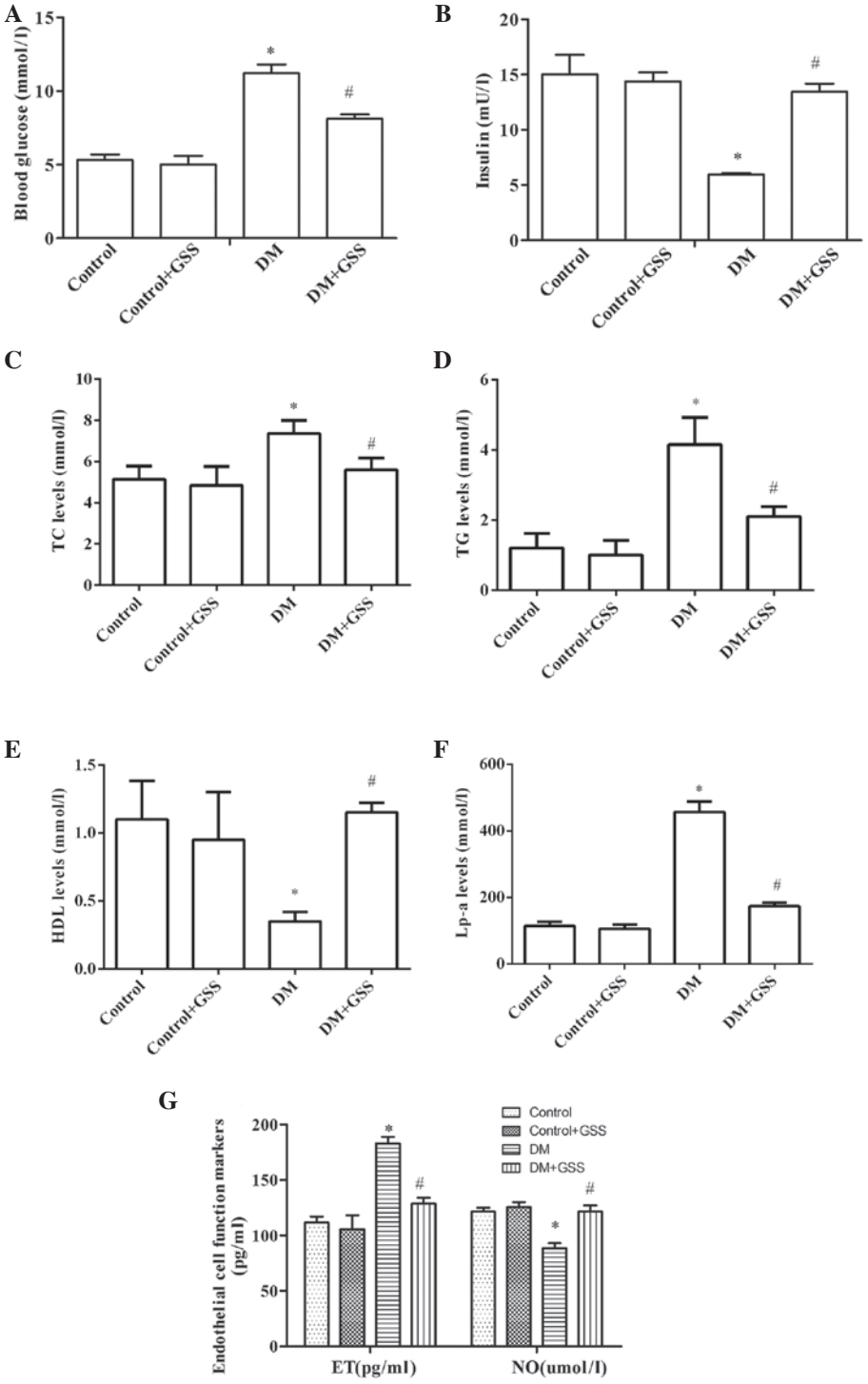

Figure 2. Effects of GSS on vascular injury caused by type 1 diabetes mellitus. (A) Blood glucose levels in the treatment groups. (B) Insulin levels in the treatment groups. Levels of (C-F) lipid metabolism markers and (G) endothelial cell function markers. *P<0.05 vs. the control and control +GSS groups; #P<0.05 vs. the DM group. Data are presented as the mean ± standard deviation. A one-way analysis of variance with a post-hoc Tukey's test was used to identify significant differences among groups. GSS, ginsenoside; DM, diabetes mellitus; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; Lp-a, lipoprotein(a); ET, endothelin; NO, nitric oxide.

test if data were parametric. If data were non-parametric a Kruskal-Wallis test was conducted followed by a Mann-Whitney U test. Statistical analyses were performed using statistical package for the social sciences (SPSS) version 16.0 (SPSS, Inc., Chicago, IL, USA) statistical software. P<0.05 was considered to indicate a statistically significant difference.

Results

GSS exerts a positive effect on vascular injury due to the onset of early stage diabetes. To determine the effects of GSS on vascular injury resulting from early stage diabetes, the levels of blood glucose, insulin, lipid metabolism markers (TC, TG, HDL, and Lp-a) and endothelial cell function markers (ET

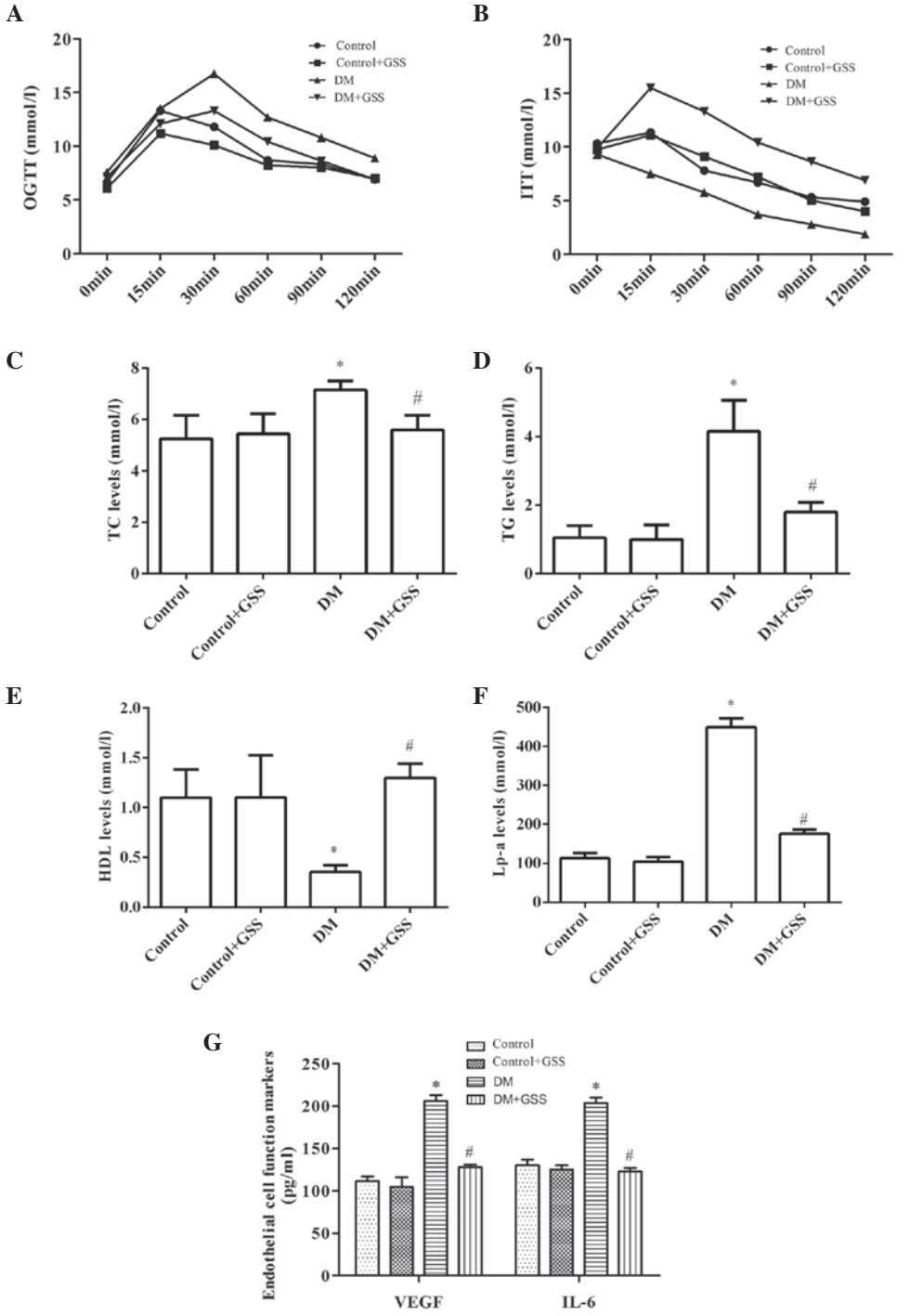

Figure 3. Effects of GSS on vascular injury caused by type 2 diabetes mellitus. (A) Different levels of blood glucose revealed by OGTT. (B) Different levels of insulin determined by ITT. Levels of (C-F) lipid metabolism markers and (G) endothelial cell function markers. *P<0.05 vs. the control and control +GSS groups; #P<0.05 vs. the DM group. Data are presented as the mean ± standard deviation. A one-way analysis of variance with a post-hoc Tukey's test was used to identify significant differences among groups (A and B). A Kruskal-Wallis test followed by a Mann-Whitney U test was performed to detect significant differences among groups (C-G). GSS, ginsenoside; DM, diabetes mellitus; OGTT, oral glucose tolerance test; ITT, insulin tolerance test; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; Lp-a, lipoprotein(a); VEGF, vascular endothelial growth factor; and IL-6, interleukin-6.

and NO) were determined following treatment with GSS. The levels of blood glucose were significantly higher in the DM group compared with the control and control + GSS groups (P<0.05; Fig. 1A). However, blood levels were significantly decreased in the DM + GSS group compared with the DM group (P<0.05; Fig. 1A). The blood insulin levels in the DM group were significantly lower when compared with the control and control + GSS groups (P<0.05; Fig. 1B). However, insulin levels were significantly increased in the DM + GSS group compared with the DM group (P<0.05; Fig. 1B). These results indicate that GSS may increase the levels of insulin and decrease the levels of blood glucose in rats during the early stage of diabetes. The levels of TC, TG and Lp-a were significantly increased in the DM group compared with the

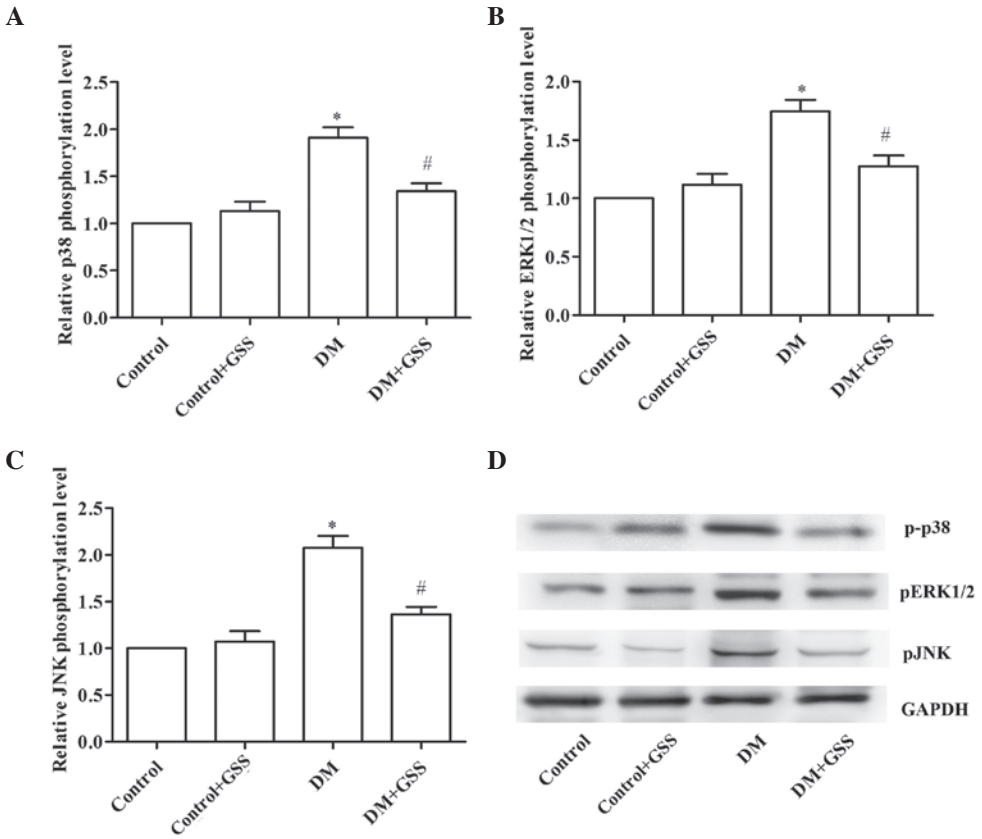

Figure 4. Effects of GSS on diabetic angiopathy. Relative phosphorylation levels of (A) p38 MAPK, (B) ERK1/2 and (C) JNK. (D) Western blotting images. *P<0.05 vs. the control and control +GSS groups; #P<0.05 vs. the DM group. Data are presented as the mean ± standard deviation. A one-way analysis of variance with a post-hoc Tukey's test was used to identify significant differences among groups. GSS, ginsenoside; DM, diabetes mellitus; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase.

control groups (P<0.05; Fig. 1C-F); however, following treatment with GSS they were significantly reduced compared with the DM group (P<0.05; Fig. 1C-F). Notably, the levels of HDL were significantly reduced in the DM group compared with the control groups (P<0.05; Fig. 1C-F). However, in the GSS-treated group HDL levels were higher compared with the DM group. Therefore, GSS treatment reduced the levels of TC, TG and Lp-a and increased the levels of HDL, thus indicating that GSS may effectively correct abnormal lipid metabolism. In addition, it was determined that GSS treatment significantly increased the levels of NO; however, the levels of ET were reduced compared with the DM group (P<0.05; Fig. 1G), thus suggesting that GSS may improve the endothelial cell dysfunction that frequently occurs during the progression of diabetes.

GSS reduces vascular injury resulting from the induction of T1DM. In order to determine the effects of GSS on vascular injury resulting from T1DM the levels of blood glucose, insulin, TC, TG, HDL, Lp-a, and ET and NO were determined following administration of GSS in T1DM-induced rats (Fig. 2). The levels of blood glucose (Fig. 2A), TC, TG, Lp-a (Fig. 2C-F) and ET (Fig. 2G) in the DM group were significantly increased when compared with the control groups (P<0.05). Furthermore, significantly decreased levels of insulin (Fig. 2B), HDL (Fig. 2C-F) and NO (Fig. 2G) were observed in the DM group compared with the controls (P<0.05). The opposite was observed for these markers in the group treated with GSS compared with the DM group (P<0.05; Fig. 2). These results indicate that GSS treatment may protect against vascular injury during the pathogenesis of T1DM.

GSS reduces vascular injury resulting from T2DM. In order to determine the effects of GSS on vascular injury caused by T2DM OGTT and ITT were performed, and the levels of TC, TG, HDL, Lp-a, VEGF and IL-6 were determined (Fig. 3). GSS was able to effectively reduce the levels of blood glucose (Fig. 3A), TC, TG, Lp-a (Fig. 3C-F), VEGF and IL-6 in the DM + GSS group compared with the DM group (Fig. 3G; P<0.05). Increased levels of insulin (Fig. 3B) and HDL (Fig. 3E) were also observed compared with the DM group (P<0.05), indicating that GSS treatment may protect against vascular injury due to T2DM.

Molecular mechanism underlying GSS effects on diabetic angiopathy. In order to determine the molecular mechanism, which allowed for GSS to improve vascular injury resulting from T2DM, the phosphorylation levels of p38 MAPK, ERK1/2 and JNK in aortic tissues were determined (Fig. 4). The phosphorylation levels of p38 MAPK (Fig. 4A), ERK1/2 (Fig. 4B) and JNK (Fig. 4C) were compared with total p38, ERK1/2 and JNK, respectively, and determined to be significantly reduced in the DM + GSS group compared with the DM group (P<0.05). These results suggest that during vascular injury GSS may activate p38 MAPK, ERK1/2 and JNK signaling.

Discussion

In the absence of effective intervention strategies for DM and diabetic angiopathy, targeted treatments may be a novel alternative. The use of *P. quinquefolius* or *P. ginseng* may be beneficial due to their anti-glycemic effects and lack of systemic toxicity (17). The present study demonstrated that GSS may exert protective and anti-angiopathy effects during DM, including the early stage of diabetes, T1DM and T2DM, via increased insulin levels, reduced blood glucose levels, corrected abnormal lipid metabolism and improved endothelial cell dysfunction. This may be achieved via activation of p38 MAPK, ERK1/2 and JNK signaling.

The early stage of diabetes is characterized by normal levels of fasting blood-glucose; however, over time insulin secretion decreases and fasting hyperglycemia develops. Progressive autoimmune destruction of pancreatic islet beta cells results in permanent insulin deficiency, which leads to T1DM (18). Environmental triggers in genetically susceptible individuals also contribute to T1DM progression (19). T2DM accounts for ~90% of all cases of diabetes and is prevalent in the general adult population; T2DM is also associated with genetic factors (20). The mechanism of T1DM and T2DM pathogenesis differs; however their symptoms and outcomes are similar, including hyperglycemia, insulin deficiency, lipid metabolic disorder and endothelial cell dysfunction. In addition, they are associated with a high risk of developing chronic diabetic angiopathy in various organs, including nephropathy, neuropathy, retinopathy and atherosclerosis, which often result in an unfavorable prognosis and may lead to a marked decline of life expectancy for patients (21).

However, the pathogenesis of diabetic angiopathy is complex and remains to be elucidated. Epidemiological studies and clinical trials have confirmed that alongside various factors, hyperglycemia and dyslipidemia initiate the pathology of the vessel wall (22-24). Hyperglycemia contributes to the microvascular pathology. Therefore, strict control and monitoring of blood glucose levels is critical in order to prevent or reverse diabetic complications, which may improve the quality of life and possibly prolong survival in patients with DM (25,26). In addition to hyperglycemia, impaired lipid metabolism also contributes to the pathology of T2DM and macroangiopathy (27,28). A previous study determined that individuals with DM frequently have dysfunctional lipid metabolism (29). This is often reflected by increased levels of TC, TG and very low-density lipoproteins, alongside reduced levels of circulating HDL (30,31). However, endothelial cells remain the most important active participant in DM pathogenesis. Dysfunction of endothelial cells has been considered a key factor in the pathogenesis and development of vascular disease in patients with DM (28,32,33). VEGF has been identified as an important survival factor for endothelial cells and may inhibit the apoptosis of endothelial cells (34). ET-1 is an effective vasoconstrictor, proinflammatory and proliferative endothelial cell-derived peptide, which is important for the modulation of vascular function. In conjunction with NO, ET is responsible for the progressive development of endothelial dysfunction. Overexpression of ET-1 and its receptors has been determined to contribute to the development of atherosclerosis and diabetic angiopathy (35). Furthermore, a previous study indicated that inflammatory mediators, including tumor necrosis factor α, IL-1β and IL-6 may be associated with diabetic angiopathy (36). Therefore, for effective treatment of DM and diabetic angiopathy the following measures should be taken: Control of blood glucose, regulation of insulin levels, correction of abnormal lipid metabolism and improvement of endothelial cell dysfunction.

Previous studies have confirmed that GSS, an active compound of ginseng, is important for the prevention and treatment of various diseases, including cancer, cardiovascular disease and diabetes (37-39). In addition, previous studies have suggested that GSS has a substantial anti-hyperglycemic effect (40,41), anti-inflammatory activity, and is able to reduce serum insulin and lipid levels (42-44). The effects of GSS on reduced insulin resistance have also been reported to be associated with JNK, nuclear factor-κB and peroxisome proliferator activated receptors γ (45,46). However, few studies have investigated the function of GSS in diabetic angiopathy. The present study used GSS Re to investigate its effect on diabetic angiopathy and the possible molecular mechanism. In conjunction with previous studies (47,48), the current study confirmed that GSS may reduce the levels of blood glucose, TC, TG, Lp-a and reverse the decreased levels of insulin and HDL in various types of diabetes, including early stage diabetes, T1DM and T2DM. In addition, it was determined that GSS may protect against diabetic angiopathy by decreasing levels of ET, VEGF, and IL-6, and increasing the levels of NO. In addition to JNK signaling, p38 MAPK and ERK1/2 signaling may also be involved in these effects.

In conclusion, the present study provided experimental evidence that GSS may exert protective and anti-angiopathy effects against vascular damage induced by early stage diabetes, T1DM and T2DM. These effects were the result of a reduction in blood glucose levels, increased insulin levels, improved lipid metabolism and reduced endothelial cell dysfunction. The underlying mechanism of these effects may be the activation of p38 MAPK, ERK1/2 and JNK signaling.

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