Article

Anti-diabetic effects of wild soybean *Glycine soja* seed extract on Type 2 diabetic mice and human hepatocytes induced insulin resistance

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Abstract: Anti-diabetic effects of *Glycine soja* seed extract (GS) on Type 2 diabetes mellitus mouse model and human hepatocytes induced insulin resistance were investigated. 3 weeks old *db*/*db* mice were divided into 5 groups (n = 6) including two control groups and 3 GS treated groups with different doses. Oral administration of GS for 6 weeks to diabetic *db*/*db* mice reduced blood glucose level significantly in a dose dependent manner by 44.7% (300 mg/kg/day), 30.9% (150 mg/kg/day) and 21.1% (75 mg/kg/day). GS treatment also lowered significantly plasma level of HbA1c, insulin, IGF-1 and leptin, and increased that of adiponectin. GS treatment activated AMPK, and down-regulated GLUT2 in liver tissues of mice while up-regulated GLUT4 in muscle tissues of mice. In *in vitro* study with insulin resistance induced human hepatocyte, GS treatment increased glucose uptake and increased the activities of Akt and PPAR-γ in response to insulin. Treatment of GS appears to reduce blood glucose level by regulating energy metabolism positively through various metabolic pathways and reducing insulin resistance in Type 2 diabetes mellitus.

Keywords: *Glycine soja* seed, Type 2 diabetes mellitus, Antidiabetic, AMPK, Akt, PPAR-γ

1. Introduction

Many people suffer from a metabolic disorder, Type 2 Diabetes mellitus (T2DM) and its complications [1]. Insulin has a key role to maintain the blood glucose level within a narrow range. The main cause of T2DM has been reported to be insulin resistance though altered insulin secretion seems to be responsible to some extent [2]. IGF-1 has an almost 50% amino acid sequence homology with insulin and brings about nearly the same blood glucose lowering response [3]. It has been reported that the effect of IGF-1 on insulin sensitivity and its relation to T2DM [4, 5]. It has been reported that adiponectin [6] and leptin [7] concentrations in blood are functionally related to insulin resistance and T2DM.

Impaired energy metabolism often observed in T2DM, obesity, hyperlipidemia and cardiovascular diseases is closely related to biochemical malfunction in the cellular level [8]. Insulin resistance is partly responsible for impaired energy metabolism that causes in turn various pathological events [9]. AMPK acts as a sensor to maintain a balance of energy metabolism and has important biochemical functions [10, 11]. AMPK stimulates or inhibits many downstream effectors in energy metabolism. Thus, AMPK has been studied as a target for the development of drugs designed to correct impaired energy metabolism since it has a key role in regulating both lipid and glucose metabolism [12, 13].

Akt pathway is a signal transduction pathway in response to extracellular signals and activated Akt mediates many downstream responses by phosphorylating a range of intracellular proteins. In such a way, Akt plays a key role in multiple cellular processes including glucose metabolism. Akt affects glucose metabolism by increasing translocation of glucose transporters to the cell membrane, stimulating glycogen synthesis by activation of the related enzymes [14].

Currently available hypoglycemic agents for T2DM have limitations to use because of side effects and restrictions to treat the patients having kidney, liver, and heart problems [15-18]. Because PPAR-γ regulates fatty acid storage and glucose metabolism, PPAR-γ agonists have been used in the treatment of hyperglycemia as an insulin sensitizing agent [19]. PPAR-γ agonists also have side effects [20], therefore, hypoglycemic agents increasing insulin sensitivity with less side effects still need to be sought. It is desirable to find a safe herb from nature applicable for a long term treatment to control hyperglycemia caused by T2DM.

Glycine soja is an annual winder plant widely grows in Korea, Japan, China, and Russia [21]. It is known to be an original species of soybean (*Glycine max*). *Glycine soja* has been reported to have about 10% more protein and 10% less fat than soybean. *Glycine soja* has been reported to reduce blood glucose and total cholesterol in normal rats [22]. However, anti-diabetic effects of *Glycine soja* on T2DM and its relationship with energy metabolism and insulin resistance have not been reported yet.

2. Results

2.1. Flavonoids and pinitol contents of GS

Three flavonoids, epicatechin, daidzin, and genistin were identified in the sample of *Glycine soja* seed extract. The extract sample contained 41.2 ± 0.1 mg/g epicatechin, 8.1 ± 0.3 mg/g daidzin, and 6.5 ± 0.1 mg/g genistin(Fig. 1(A)). The pinitol content of the sample was 1.21 ± 0.01 mg/g(Fig. 1(B)).

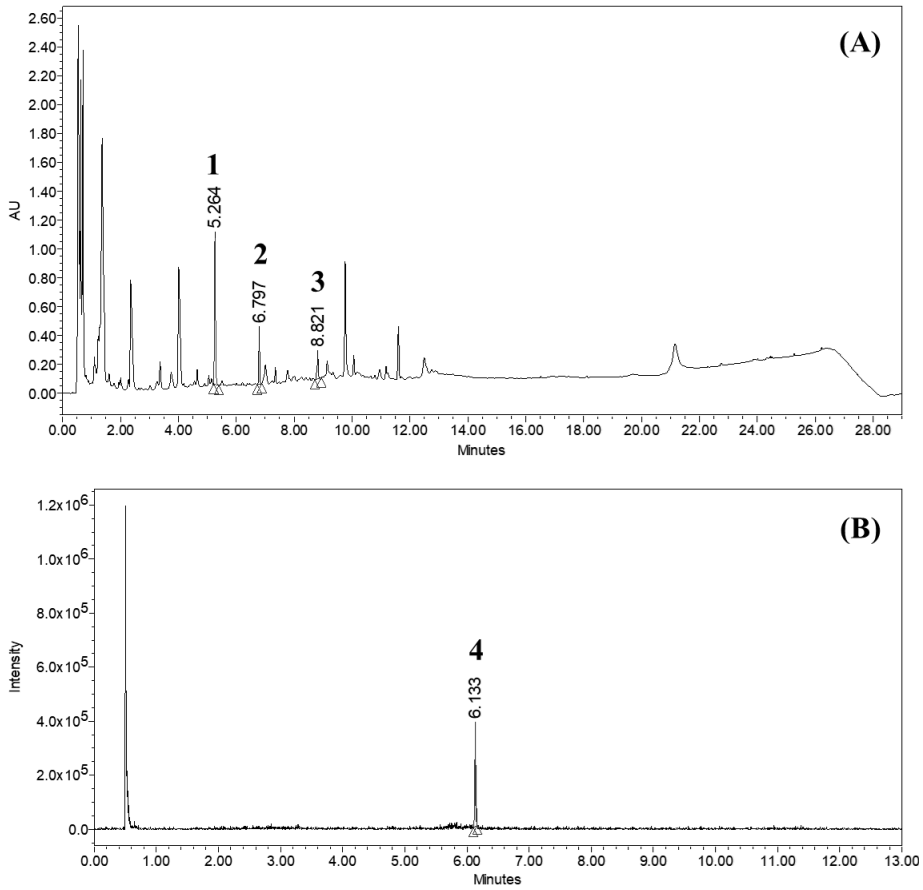

Figure 1. (A) Representative HPLC chromatogram of GS extract at 220 nm; 1) epicatechin, 2) daidzin, 3) genistin. (B) Representative total ion chromatogram of GS for pinitol quantification; 4) pinitol.

2.2. Animal study to evaluate effects of GS on T2DM

2.2.1. Effects of GS on weight change and FER

Weights of experimental mice were continuously gained during 6 weeks of treatment. No cases of weight loss were observed. As shown in Table 1, there are no significant differences in food intake, body weight gain and FER of *db*/*db* mice orally treated with 300 mg/kg/day (GS300), 150 mg/kg/day (GS150), and 75 mg/kg/day (GS75) of *Glycine soja* seed extract or 150 mg/kg/day of metformin (PC) for 6 weeks, compared to NC (negative control).

Table 1. Food intake, body weight gain, and food efficiency ratio of *db/db* mice treated with *Glycine soja* extract

Treatment	Food intake (g/day)	Body weight gain (g/day)	Food efficiency ratio (FER, %)
NC	6.32	0.753 ± 0.06	11.9 ± 1.00
PC	6.63	0.645 ± 0.03	9.73 ± 0.39

GS300	5.29	0.606 ± 0.03	11.4 ± 0.54
GS150	6.85	0.556 ± 0.05	8.11 ± 0.74
GS75	6.32	0.659 ± 0.04	10.4 ± 0.57

Oral doses of 300 mg/kg/day (GS300), 150 mg/kg/day (GS150), and 75 mg/kg/day (GS75) of *Glycine soja* extract or 150 mg/kg/day of metformin (PC) were administered for 6 weeks. Values are expressed as mean ± SEM (n = 6).

2.2.2. Effects of GS on plasma levels of AST, ALT, and BUN

To evaluate potential toxic effects of *Glycine soja* extract, plasma toxicity markers for liver (ALT, AST) and kidney (BUN) were assayed at the end of the experimental period. Plasma level of AST is shown to decrease significantly in PC compared to NC (Table 2). Plasma levels of AST and ALT are shown to decrease in GS groups compared to NC group as is shown in Table 2. However, the measurements were not statistically significant. Plasma level of BUN is not altered significantly in PC and GS groups compared to NC group. These data indicate that administration of 75 ~ 300 mg/kg/day of GS for 6 weeks has no significant adverse effects on experimental mice.

Table 2. Plasma levels of AST, ALT and BUN of *db/db* mice treated with *Glycine soja* extract

Treatment	AST (U/L)	ALT (U/L)	BUN (mg/dL)
NC	138.6 ± 30.5	77.6 ± 15.5	21.0 ± 3.3
GS300	101.1 ± 40.8	58.7 ± 12.7	22.2 ± 2.9
GS150	104.6 ± 22.2	66.3 ± 12.5	22.4 ± 4.4
GS75	122.0 ± 52.9	66.5 ± 9.7	25.9 ± 5.8
PC	92.4 ± 19.7*	57.0 ± 16.3	23.0 ± 2.1

Oral doses of 300 mg/kg/day (GS300), 150 mg/kg/day (GS150), and 75 mg/kg/day (GS75) of *Glycine soja* extract or 150 mg/kg/day of metformin (PC) were treated for 6 weeks. NC is a vehicle control. Values are expressed as mean ± SEM (n = 6). * $p < 0.05$ (compared to NC) express significant decrease as determined by one-way ANOVA.

2.2.3. Effects of GS on plasma lipid profile

The summary of plasma lipid profile is shown in Fig. 2. Plasma levels of triglyceride and LDL cholesterol are significantly decreased while HDL cholesterol level is significantly increased in PC group. LDL cholesterol level is significantly reduced in GS groups. However, triglyceride level is significantly reduced only in GS300 group.

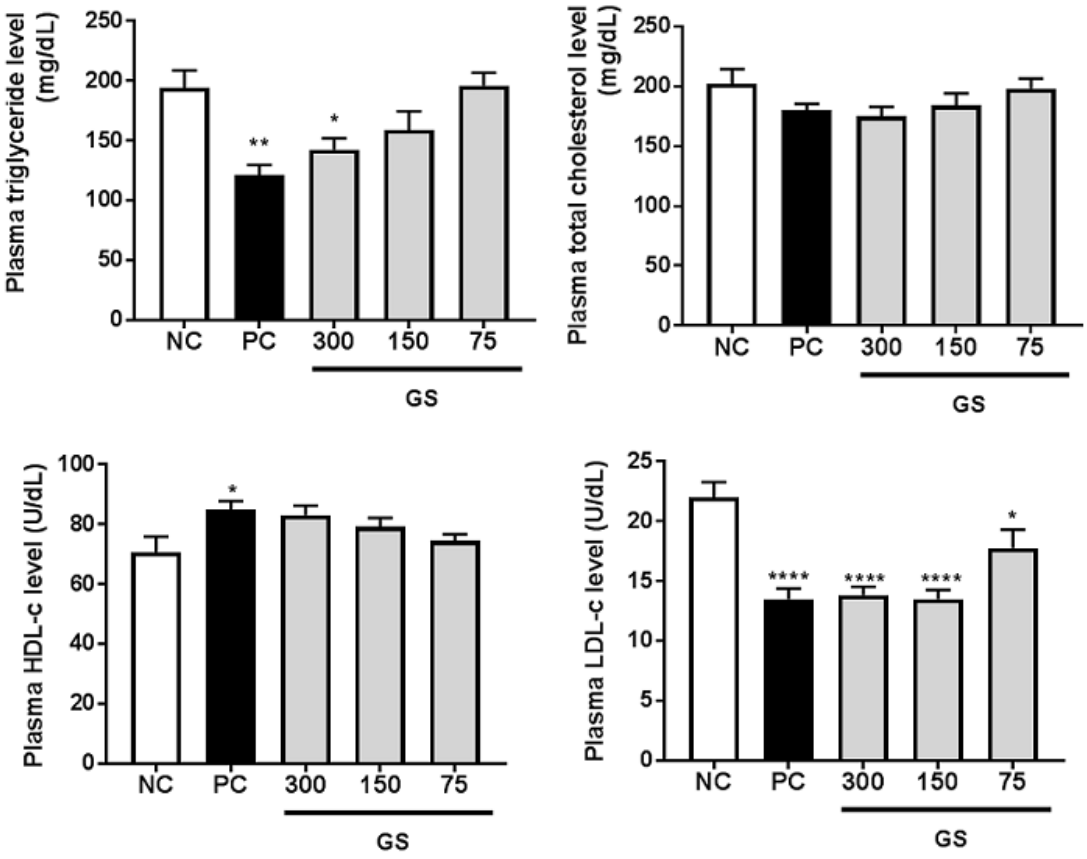

Figure 2. Plasma levels of triglyceride, total cholesterol, HDL-cholesterol, and LDL-cholesterol of *db*/*db* mice treated with 300 mg/kg/day (GS300), 150 mg/kg/day (GS150), and 75 mg/kg/day (GS75) of *Glycine soja* extract, or 150 mg/kg/day of metformin (PC) for 6 weeks. Values are expressed as mean ± SEM (n = 6). * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.001$ (compared to NC) express significant differences as determined by one-way ANOVA.

2.2.4. Effects of GS on blood level of glucose, HbA1c, insulin, IGF-1, adiponectin, and leptin

Blood glucose level of each experimental group was measured weekly from week 2 to week 6. Increment of blood glucose level is shown to decrease significantly from week 2 to week 6 in PC (increment of 118.2 mg/dL), GS300 (increment of 193.8 mg/dL), and GS150 (increment of 242.2 mg/dL), compared to NC (increment of 285.2 mg/dL) as shown in Fig. 3.

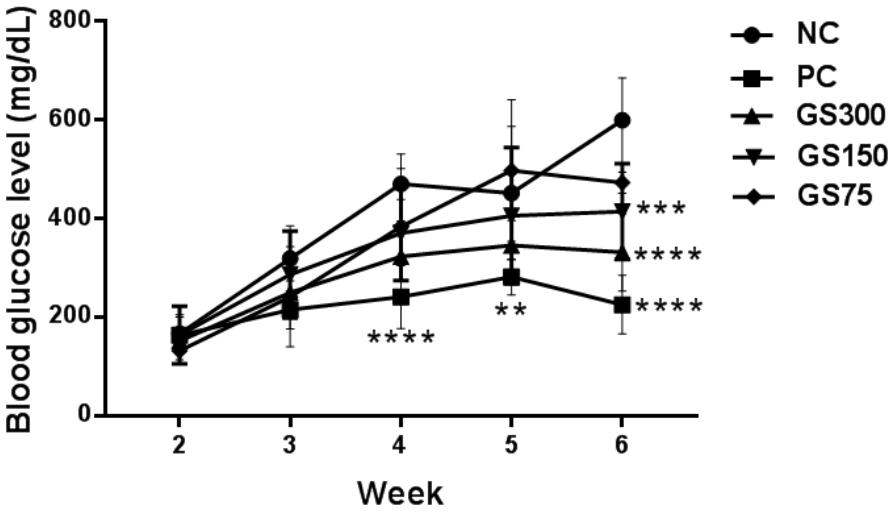

Figure 3. The blood glucose level of *db*/*db* mice treated with 300 mg/kg/day (GS300), 150 mg/kg/day (GS150), and 75 mg/kg/day (GS75) of *Glycine soja* extract, or 150 mg/kg/day of metformin (PC) for 6 weeks. Values are expressed as mean ± SEM (n = 6). ** $p < 0.01$, *** $p < 0.005$, and **** $p < 0.001$ (compared to NC) express significant decreases as determined by one-way ANOVA.

Blood glucose level measured after 6 weeks treatment is shown to decrease significantly in PC (225.2 ± 59.7 mg/dL), GS300 (331.3 ± 78.6 mg/dL), GS150 (414.3 ± 97.2 mg/dL), and GS75 (473.0 ± 21.4 mg/dL), compared to NC (599.3 ± 86.3 mg/dL) as shown in Fig. 4A. Plasma glucose level of GS groups is significantly decreased in a dose dependent manner.

Plasma HbA1c level is shown to decrease significantly in PC (3.8%) and GS300 (3.9%) compared to 8.2% in NC (Fig. 4B).

Plasma insulin level measured after 6 weeks treatment is shown to decrease significantly in PC (0.59 ± 0.28 ng/mL) and GS150 (1.59 ± 0.26 ng/mL), compared to NC (2.90 ± 0.33 ng/mL) as shown in Fig. 4C. Plasma IGF-1 level is significantly decreased in PC (110 ± 45 ng/mL), GS300 (174 ± 47 ng/mL), and GS150 (219.5 ± 28 ng/mL), compared to NC (380 ± 69 ng/mL) as shown in Fig. 3D. Plasma adiponectin level is significantly increased in PC (61.7 ± 8.3 μg/mL), GS300 (56.3 ± 7.8 μg/mL), and GS150 (71.2 ± 8.4 μg/mL), compared to NC (27.2 ± 8.2 μg/mL) as shown in Fig. 4E. Plasma leptin level is significantly decreased in PC (390.9 ± 76.4 ng/mL), GS300 (436.7 ± 39.6 ng/mL), and GS150 (448.4 ± 25.7 ng/mL), compared to NC (692.4 ± 148.2 ng/mL).

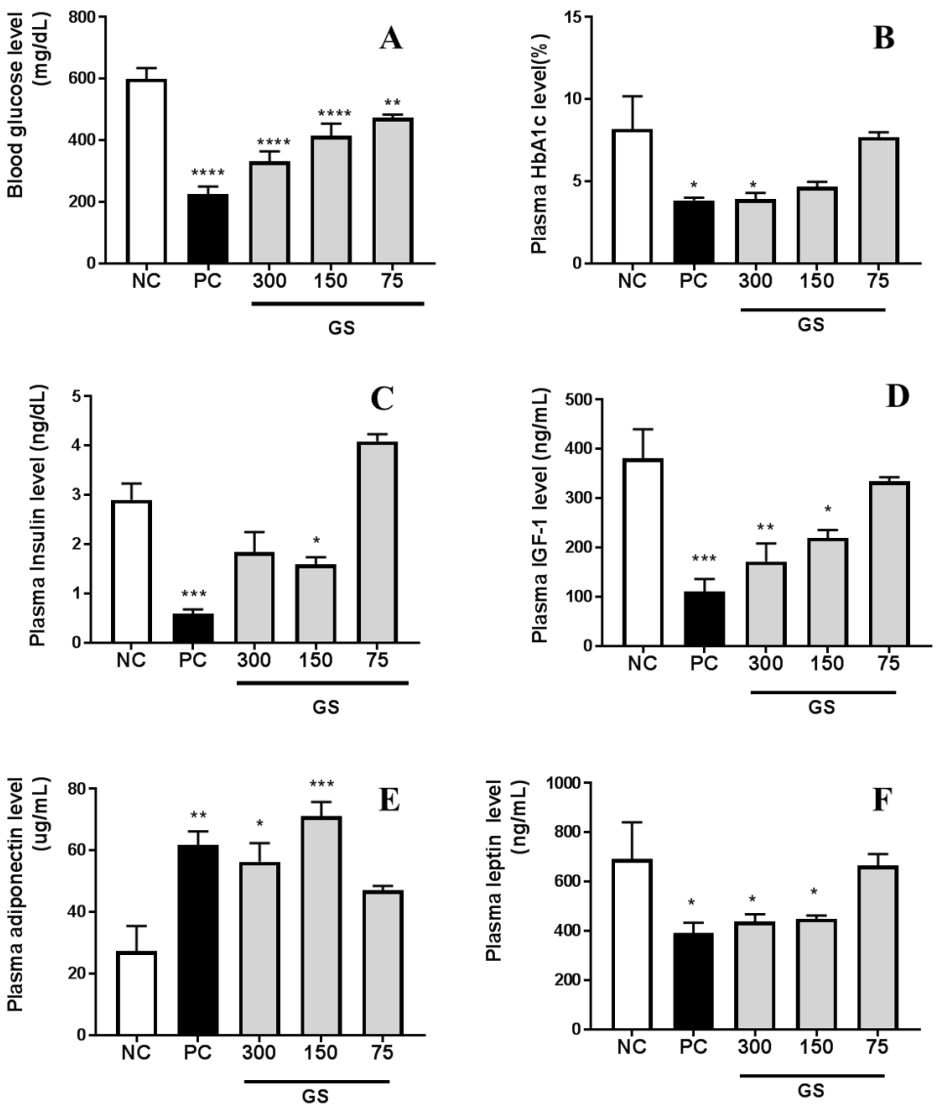

Figure 4. (A) Plasma glucose level, (B) plasma HbA1C level, (C) plasma insulin level, (D) plasma IGF-1 level, (E) plasma adiponectin level, and (F) plasma leptin level of *db*/*db* mice treated with 300 mg/kg/day (GS300), 150 mg/kg/day (GS150), and 75 mg/kg/day (GS75) of *Glycine soja* extract, or 150 mg/kg/day of metformin (PC) for 6 weeks. Values are expressed as mean ± SEM (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, and **** $p < 0.001$ significant decreases as determined by one-way ANOVA.

2.2.5. Effects of GS on expression of AMPK, GLUT-2, and GLUT-4

AMPK expression is significantly increased in liver tissues of PC and GS300, compared to NC as shown in Fig. 5A. GLUT-2 expression is significantly decreased in liver tissues of PC, GS300, and GS150, compared to NC as shown in Fig. 5B. GLUT-4 expression is significantly increased in muscle tissues of GS300 and GS150, compared to NC as shown in Fig. 5C.

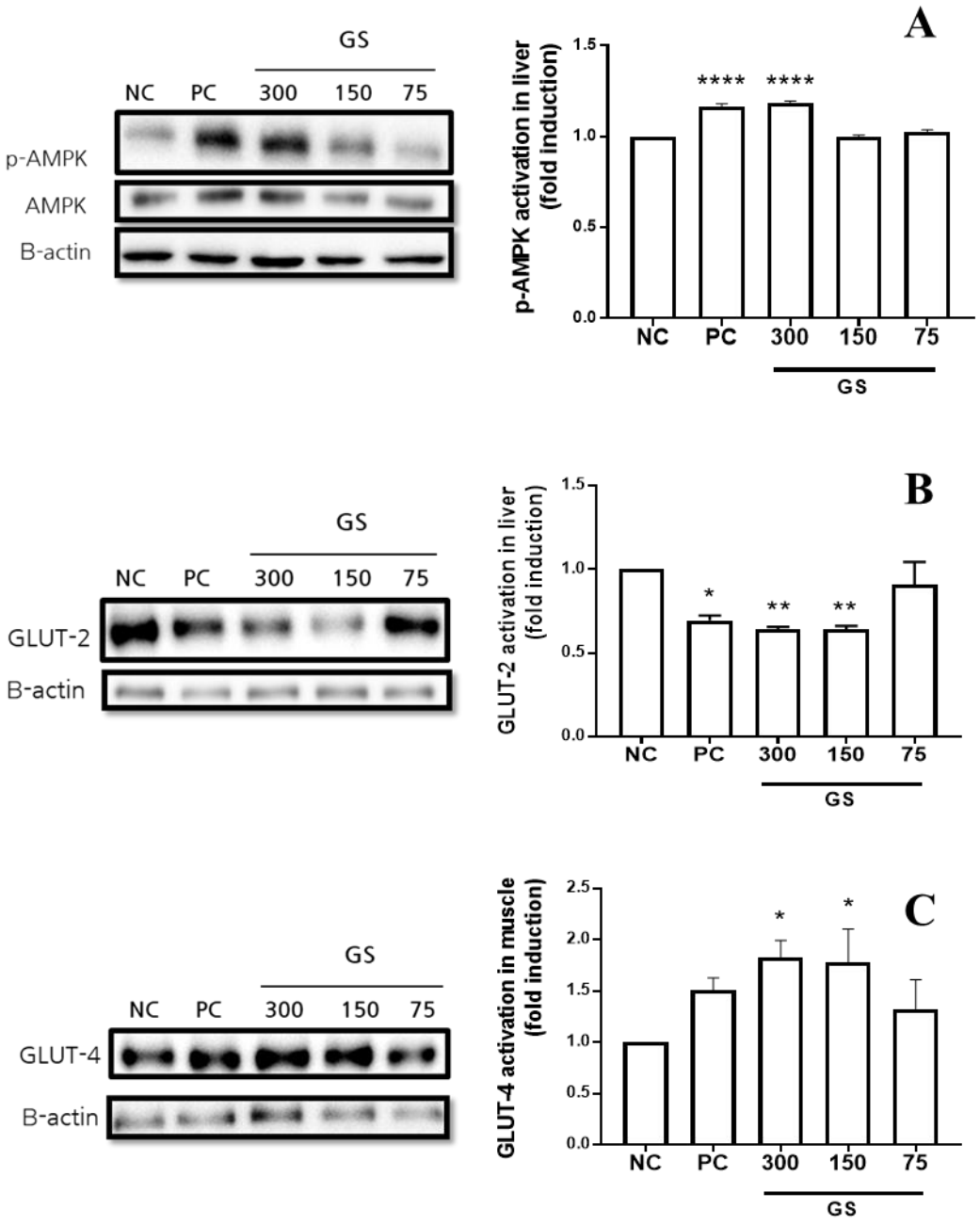

Figure 5. (A) p-AMPK expression in liver tissue, (B) GLUT-2 expression in liver tissue, and (C) GLUT-4 expression in muscle tissue of *db*/*db* mice treated with 300 mg/kg/day (GS300), 150 mg/kg/day (GS150), and 75 mg/kg/day (GS75) of *Glycine soja* extract, or 150 mg/kg/day of metformin (PC) for 6 weeks. Values are expressed as mean ± SEM (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, and **** $p < 0.001$ (compared to NC) express significant increase as determined by one-way ANOVA.

2.2.6. Histological observations

Histological observation reveals (Fig. 6) that lipid droplets are less accumulated and smaller in size in liver tissues of PC and GS300, compared to NC. Fig. 6 shows that the size of adipocytes is smaller in subcutaneous fat tissues of PC and GS300, compared to NC.

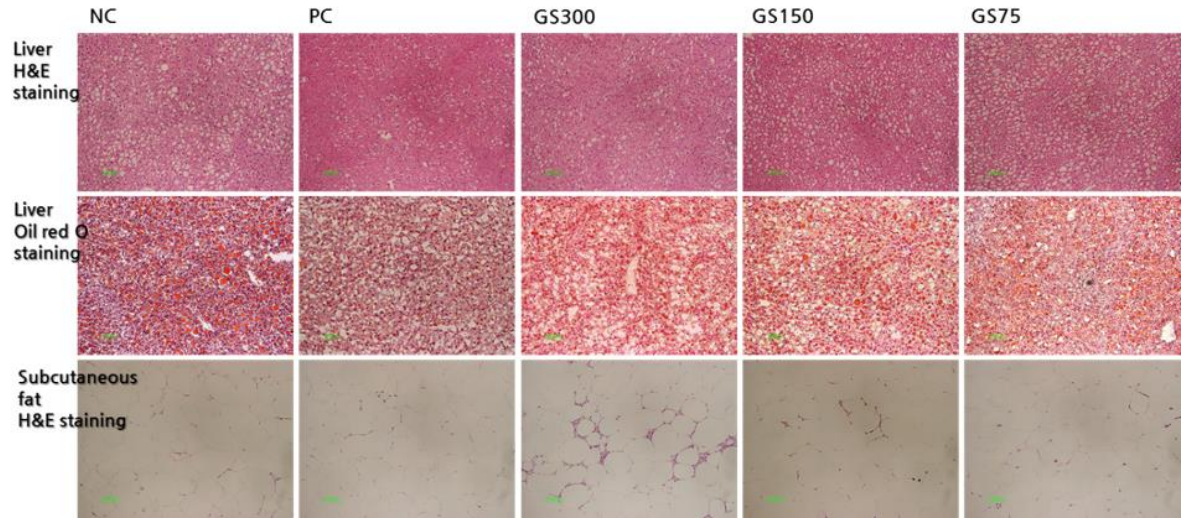

Figure 6. Stained tissues of liver and subcutaneous fat of *db*/*db* mice treated with 300 mg/kg/day (GS300), 150 mg/kg/day (GS150), and 75 mg/kg/day (GS75) of *Glycine soja* extract, or 150 mg/kg/day (PC) of metformin for 6 weeks.

2.3. In vitro study to evaluate effects of GS on T2DM

2.3.1. Effects of GS on glucose uptake, Akt activity, and PPAR-γ activity in hepatocytes

Human HepG2 cell line was used to evaluate the anti-diabetic effects of GS on glucose uptake by hepatocytes. The concentration level for GS treatment was validated by MTT assay that demonstrated no cytotoxicity throughout all the concentrations applied for the experiments (Fig. 7A). GS treatment significantly increased glucose uptake by HepG2 cells approximately 60% at the concentrations of 0.1, 1, and 10 μg/mL (Fig. 7B). The level of glucose uptake was determined by measuring intracellular level of 2-deoxyglucose.

The effects of GS on insulin responsiveness were investigated. It is known that the responsiveness to insulin signaling with respect to Akt activity is decreased in T2DM [23]. To mimic T2DM in this experiment, palmitate was treated to reduce insulin sensitivity, leading to the suppression of insulin-induced Akt phosphorylation. In Fig. 6C, GS treatments show significant recovery effects against the attenuated Akt phosphorylation caused by palmitate.

As shown in Fig. 6D, palmitate treatment attenuates insulin-induced transcriptional activity of PPAR-γ whilst GS treatment (10 μg/mL) significantly recovers from the attenuated transcriptional activity of PPAR-γ.

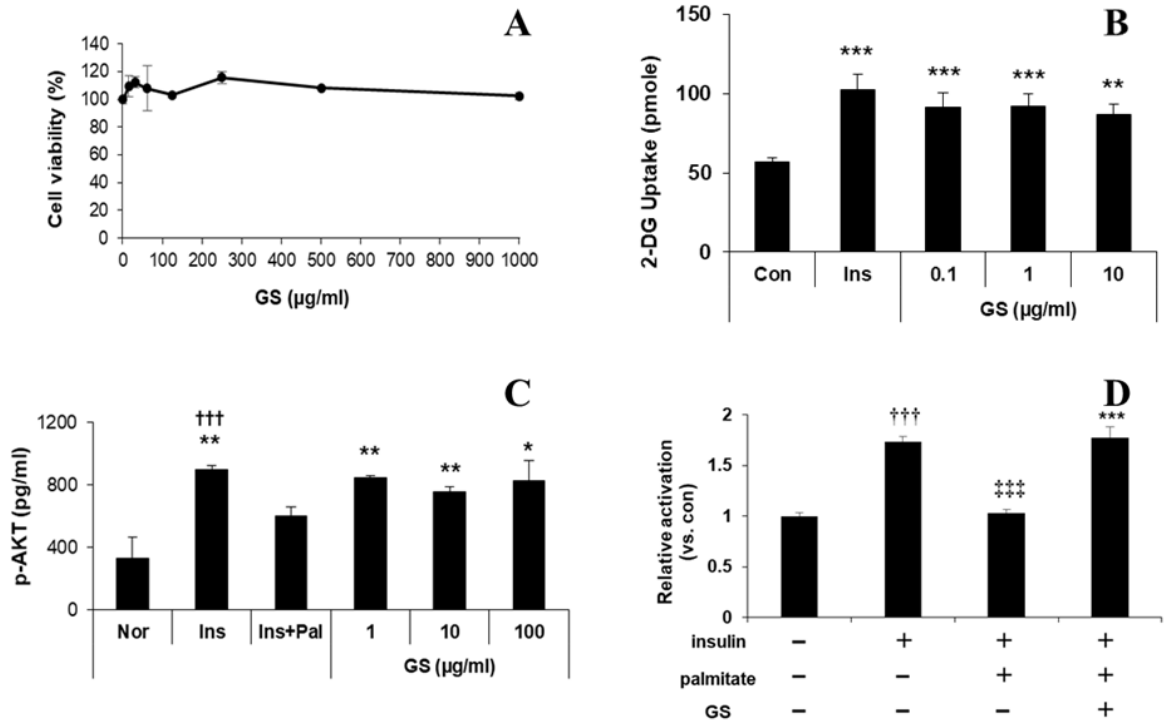

Figure 7. (A) Cytotoxicity test for GS treatment to HepG2 cells. (B) Effects of GS on glucose uptake in HepG2 cells. **$p < 0.01$, ***$p < 0.001$ vs. Con. 2-DG; 2-deoxyglucose, Con; control, Ins; insulin, GS; *Glycine soja* extract. (C) Effects of GS on insulin-induced Akt signaling pathway in HepG2 cells. †††$p < 0.001$ vs. Nor; *$p < 0.05$, **$p < 0.01$ vs. Ins + Pal. Nor; no treatment, Ins; insulin only, Ins + Pal; insulin with palmitate, GS; *Glycine soja* extract with palmitate and insulin. (D) Effects of GS on PPAR-γ activity in HepG2 cells. †††$p < 0.001$ vs. control, ‡‡‡ $p < 0.001$ vs. insulin only, ***$p < 0.001$ vs. insulin with palmitate, GS; *Glycine soja* extract.

3. Discussion

This study was designed to evaluate anti-diabetic effects of GS on T2DM animal model of *db*/*db* (BKS.Cg-m+/+*Lepr*^{db}/*J*) mice. During the weeks study, treatment of GS showed no significant adverse effects on the liver and kidney of experimental mice. Administration of GS is regarded to be safe considering observations on food intake, body weight gain and behavior of experimental mice.

T2DM is due to insulin resistance and inadequate insulin production by β-cells, though the causes are not completely understood so far. Two distinctive pathways are important in the regulation of blood glucose level. One is AMPK dependent mechanisms, and the other is insulin-dependent pathway that upregulates serine/threonine protein kinase Akt [23]. Metformin, used to treat T2DM by AMPK, increases peripheral glucose intake while reducing glucose production by liver. [24]. Akt signaling plays a key role in insulin-stimulated glucose uptake by both skeletal muscle and adipose tissue while inhibiting the release of hepatic glucose [25]. Insulin activates Akt phosphorylation which in turn promotes GLUT-4 translocation and glucose uptake in skeletal muscle [26]. In T2DM, the responsiveness to insulin signaling with respect to Akt activity is decreased [27].

In T2DM, hyperinsulinemia increases blood level of IGF-1 [28]. In this study, GS treatment reduced blood levels of glucose and HbA1c in T2DM animal model. However, GS treatment also reduced blood levels of insulin and IGF-1. This outcome seems to be achieved by increasing insulin (and IGF-1) responsiveness against insulin resistance of T2DM. Activation of AMPK by GS treatment is believed to assist sensitizing insulin considering that activated AMPK, in turn, inhibits GLUT-2 expression in liver and activates GLUT-4 expression in muscle as demonstrated in this study.

Though glucose disposal occurs only a small fraction by adipose tissue, physiological role of adipose tissue is crucial in regulating glucose metabolism [29]. Leptin is an adipokine mostly

produced by adipocytes. Blood glucose level is regulated by leptin as well as by insulin. Leptin has positive effects on energy metabolism resulting in weakening of hyperglycemia, hyperinsulinemia, and insulin resistance [30]. Adiponectin is known to modulate a number of metabolic processes, including glucose regulation and fatty acid oxidation [31]. Adiponectin has also been reported to mediate insulin-sensitizing effect to improve hyperglycemic conditions and hyperinsulinemia [32]. It has been reported that the reduction of adiponectin is associated with insulin resistance [33]. Physiological relationship between adipokines released from adipocytes and activation of AMPK has been reported [34].

In our study, blood level of adiponectin was increased while that of leptin was decreased by GS treatment. This outcome suggests that GS treatment increases leptin sensitivity as well as insulin sensitivity. And elevated adiponectin seems to regulate energy metabolism positively to improve hyperglycemic conditions, possibly via insulin-sensitizing effect.

Our *in vitro* study demonstrated that GS treatment increased glucose uptake by hepatocytes. GS treatment also activated Akt acting as a key effector of insulin signaling pathway in an induced insulin resistance model, and recovered from the attenuated insulin-induced transcriptional activity of PPAR-γ. This outcome supports the evidences of our animal study that GS treatment works positively on energy metabolism regulating blood glucose level and on insulin sensitivity.

Treatment of GS is thought to have effects on regulating energy metabolism to reduce blood glucose level and increasing sensitivities of insulin, IGF-1, and leptin, and activating AMPK and Akt in T2DM.

4. Materials and Methods

4.1. Preparation of wild Glycine soja seed extract

The seeds of wild *Glycine soja* were collected in Moonkyung, Korea. After ten days of natural dry, the seeds were pulverized to powder. To make GS extract sample, 1 kg of the powder was extracted with 10 L of water at 80°C for 4 hrs. The extracts were filtered, and then concentrated under reduced pressure in a rotary evaporator. Water extract sample was prepared by lyophilization of the extract.

4.2. Quantitative analysis of extract samples for flavonoids and pinitol

Flavonoids in samples were analyzed by reverse phase-high performance liquid chromatography of Waters Alliance 2695 system (Waters Co., Milford, MA, USA) coupled with 2996 photodiode array detector. Acquity UPLC® BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters, Milford, MA, USA) was used for the stationary phase. The mobile phase was composed of 0.1% (*v*/*v*) formic acid in water (A) and 0.1% (*v*/*v*) formic acid in acetonitrile (B). At zero time, the mobile phase consisted of 95% A and 5% B and was held for 20 min. From 20 to 25 min, a gradient was applied to 65% A and 35% B, and from 25 to 27 min, to 0% A and 100% B, then after 27 min, to 95% A and 10% B. For separation, 0.4 mL/min of flow rate and 2 μL of injection volume were kept throughout analyses that performed at 40°C.

Pinitol in samples was analysed by reverse phase-high performance liquid chromatography of Waters Alliance 2695 system (Waters Co., Milford, MA, USA) coupled with 2996 photodiode array detector. Acquity UPLC® HSS T3 column (2.1 × 100 mm, 1.8 μm; Waters, Milford, MA, USA) was used for the stationary phase. The mobile phase was composed of 0.1% (*v*/*v*) ammonium hydroxide in water (A) and acetonitrile (B). At zero time, the mobile phase consisted of 98% A and 2% B and was held for 10 min. From 10 to 12 min, a gradient was applied to 30% A and 70% B, and from 12 to 13 min, to 98% A and 2% B, then after 13 min, to 98% A and 2% B. For separation, 0.5 mL/min of flow rate and 5 μL of injection volume were kept throughout analyses that performed at 40oC. Positive electrospray ionization mode (ESI+) was used for the detection and pinitol (m/z 194.2) ion was monitored in the single ion-monitoring (SIM) mode. The following MS conditions were applied. The capillary voltage and probe temperature were set to 0.8 kV and 600°C, respectively. And nitrogen was used as dissolving and nebulizer gas. The cone voltage was set to 5 V.

Identification of flavonoids was made by comparing retention times and UV spectra for the peaks of HPLC/PDA chromatogram to those of commercially available standards. Quantification of sample flavonoids was made in comparison to the mixture of external standards of known concentration. Peak area of chromatograms was determined at 220 nm. Identification of pinitol was made by comparing retention times and mass spectra for the peaks of HPLC/QDa TIC with those of reference standard (Sigma, St. Louis, MO, USA). Quantification of sample pinitol was made in comparison to the external standard with selected ion chromatography mode.

4.3. Animal study to evaluate effects of GS on T2DM

4.3.1. Animals and experimental treatments

3 weeks old *db*/*db* (BKS.Cg-*m*+/+*Lepr*^{db}/*J*) mice (19 ~ 22 g) were purchased from Daehan Biolink Co., Eumsung, Korea and allowed to adapt for one week. The mice were housed in an air-conditioned SPF animal room having a 12 h light/12 h dark cycle at 23 ± 1°C temperature and 45 ± 11% humidity. They were allowed to access to diet and water ad libitum. Mice were divided into 5 groups of NC (negative control), PC (positive control), and 3 GS (*Glycine soja* seed extract treatment) groups of different doses. 6 mice were allocated to each group. NC group was administered orally with saline while PC group was administered with 150 mg/kg/day of metformin, a well-known drug being used widely to treat T2DM. 75 mg/kg/day (GS75), 150 mg/kg/day (GS150) and 300 mg/kg/day (GS300) of oral dosage were applied for GS groups. Oral administration to mice was made twice a day by dissolving a half of daily dosage in 0.2 mL of saline. The experiment was lasted for 6 weeks.

4.3.2. Measurement of weight change and food efficiency ratio (FER)

Body weight gain and amount of food intake were measured at the same time and the same day of a week during 6 weeks of experimental period after a week of adaptation period. Average body weight gain and average amount of food intake were calculated daily and recorded. FER was calculated by (total weight gain / total food intake) × 100.

4.3.3. Measurement of blood glucose level

Blood glucose level of mice was measured weekly. The mice were fasted for 6 hrs before collecting blood samples from the tail. The collected blood was immediately transferred to a portable kit (One TOUCH@Ultra, Johnson & Johnson, New Brunswick, NJ, USA) to measure the blood glucose level.

4.3.4. Plasma assays for biochemical parameters

At the end of 6 week experimental period, the mice were fasted for 16 hrs prior to sacrifice. Blood samples were collected from the tail and transferred to ethylene diamine tetra acetic acid vacuum tube and stirred to prevent blood clotting. They were then centrifuged at 3000 rpm for 15 min at 4 °C. The supernatants were collected and the separated plasma samples were stored at -80 °C. The plasma level of triglyceride, total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was analyzed by automatic biochemical analyzer (Hitachi-720, Hitachi Medical, Japan). The plasma concentration of HbA1C (Crystal Chem, IL, USA), insulin (Shibayagi, Shibukawa, Japan), IGF-1 (Insulin-like growth factor-1; R&D system, MN, USA), adiponectin (R&D system, MN, USA), and leptin (R&D system, MN, USA) were assayed with corresponding mouse ELISA (enzyme-linked immunosorbent assay) kits according to the protocols offered by the manufacturers. Plasma concentrations of the biomarkers were calculated by measuring absorbance with a microplate reader (Labsystems, Vantaa, Finland).

4.3.5. Assay for p-AMPK, GLUT-2, and GLUT-4 expressions

Tissue samples of mouse liver and muscle were added with PBS containing protease inhibitor and homogenized, and then centrifuged at 10,000 g for 5 min. Protein concentration was determined by

BCA assay (Pro-measure, Intron Biotechnology, Seoul, Republic of Korea). Quantified proteins were separated by 12% SDS-polyacrylamide gel (Bio-Rad, CA, USA) electrophoresis and transferred onto PVDF membrane (Bio-Rad, CA, USA). The membranes were blocked with 5% skimmed milk and then incubated at 4 ℃ overnight with primary antibodies (Cell Signaling Technology, MA, USA) against AMPK, p-AMPK, β-actin, GLUT-2, and GLUT-4. The membranes were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, TX, USA) for 1 hr at room temperature. Antibody binding was visualized by chemiluminescence detection system (Amersham Bioscience, NJ, USA) and image analyzer (LAS-3000, Fujifilm, Tokyo, Japan). Quantification of the expressed proteins was estimated with ImageJ software (NIH, MD, USA).

4.3.6. Histological observations

After sacrificing mice, the tissues of liver and subcutaneous fat were fixed in 10% neutral formalin solution for one day and embedded in paraffin. All tissues were sliced to 10 μm in thickness and stained with H&E (hematoxylin and eosin). Histological examination was performed with the stained samples and photos were taken with light microscope (Olympus BX51, Olympus Optical Co., Japan). The differentiated cells were washed twice with PBS and then fixed with 10% formalin for 1 hr. The fixed cells were stained with 0.3% oil red O solution for 10 min. The cells were visualized with Olympus CKX41 microscope (Olympus, Tokyo, Japan) and photographed at 100× magnification using the Motic image Plus 2.0 program (Motic, Causeway Bay, Hong Kong).

4.4. In vitro experiments to evaluate effects of GS on T2DM

4.4.1. Cell culture

Human hepatocyte, HepG2 cell line was purchased from Korea cell line Bank (Seoul, Korea). The cells were cultured in Dulbecco's modified eagle's medium (DMEM; Lonza, Walkersville, MD, USA) with 10% fetal bovine serum (FBS; Lonza), 100 U/mL penicillin and 100 μg/mL streptomycin (Lonza) in a humidified incubator at 37 ℃.

4.4.2. MTT assay

HepG2 cells were seeded in a 96-well plate at the concentration of 2×10^5 cells/mL and treated with GS at the concentrations from 0.1 to 100 μg/mL for 24 hrs. After then, the cells were washed with PBS and incubated with MTT solution (5 mg/mL) for 4 h at 37 ℃. The crystal formazan was dissolved in dimethyl sulfoxide. The optical density was measured by microplate reader at 570 nm microwave length.

4.4.3. Glucose uptake assay

Glucose uptake assay was performed with Glucose Uptake Assay kit (Abcam) according to manufacturer's instruction. Briefly, HepG2 cells were seeded in a 96-well plate at the concentration of 2×10^5 cells/mL, and treated with GS (0.1, 1 and 10 μg/mL) for 24 hrs. After then, the cells were incubated with BSA-KRPH (Krebs ringer phosphate HEPES) for 40 min and treated with insulin (10 μg/mL) or GS (0.1, 1 and 10 μg/mL) for 20 min. The cells were, then, treated with 2-deoxyglucose (10 mM) for 20 min and washed with PBS. The optical density was measured by microplate reader at 405 nm.

4.4.4. Akt activity assay

HepG2 cells were seeded in a 96-well plate at the concentration of 2×10^5 cells/mL. The cells were pre-treated with GS for 1 hr prior to incubation in a cell culture media containing BSA-palmitate for 24 hrs. Then, the cells were incubated with insulin (10 μg/mL) for 20 min. The cell lysates were harvested to measure Akt activity by Phospho-Akt ELISA kit (R&D systems, Minneapolis, MN, USA) according to manufacturer's manual.

4.4.5. PPAR-γ activity assay

HepG2 cells were seeded on 60-mm culture dishes at the concentration of 2 × 10^5 cells/mL. The cells were pre-treated with GS for 1 hr prior to incubation in a cell culture media containing BSA-palmitate in the presence or absence of GS for 24 hrs. After then, the cells were treated with insulin (10 μg/mL) for 20 min before the cell nuclear extracts were harvested by nuclear extract kit (Active Motif, Carlsbad, CA, USA). The transcriptional activity of PPAR-γ was measured by TransAM PPAR-γ Transcription Factor Assay kit (Active Motif, California, USA) according to manufacturer's protocol.

4.5. Statistical analysis

Differences between groups were assessed by an analysis of variance (one way ANOVA) followed by Tukey's test for verification (GraphPad Prism 7.0, GraphPad software Inc., San Diego, USA). The data are presented as mean ± SEM (Standard Error of the Mean) or mean ± SD (Standard Deviation). Differences were considered significant when the p values were less than 0.05.

5. Conclusions

Oral administration of GS at the doses of 300 mg/kg/day and 150 mg/kg/day showed significant anti-diabetic effects on T2DM. However, administration of GS at the dose of 75 mg/kg/day fell into non-efficacious dosage region. During the 6 week mice study, GS treatments showed no significant adverse effects to experimental mice.

Treatment of *Glycine soja* seed extract appears to reduce blood glucose level by regulating energy metabolism positively through various metabolic pathways and reducing insulin resistance in T2DM.

With an appropriate standardization, GS can be developed as a hypoglycemic agent increasing insulin sensitivity having no adverse effects for a long term treatment of T2DM.

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