A THESIS FOR THE DEGREE OF

MASTER OF SCIENCE IN FOOD AND NUTRITION

Anti-diabetic Effects of Ginsenosides Rh2 and F1 in the Type 2 Diabetes Mellitus Mice Model

제 2형 당뇨 마우스 모델에서 진세노사이드 Rh2와 F1의 항 당뇨 활성 연구

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이 논문을 생활과학 석사학위논문으로 제출함

2012년 11월

서울대학교 대학원

식품영양학과

이 승 리

이 승 리의 생활과학 석사학위논문을 인준함

2013년 1월

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Abstract

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Rh2 and F1, compounds derived from *Panax ginseng*, are the final metabolites of panaxadiol and panaxatriol ginsenosides. Although ginseng is reported to have an anti-diabetic effect, the active components have yet to be clearly identified. In this study, the effects of ginsenosides Rh2 and F1 on type 2 diabetes mellitus (T2DM) were investigated in db/db mice. For nine weeks, animals were administered ginsenosides Rh2 and F1 orally at dosage levels of 5 and 20 mg/kg, respectively, in contrast to a vehicle for the control group. To evaluate the effects of Rh2 and F1, we assessed the

biochemical and histological parameters related to the control of the blood glucose level. Compared with the control group, Rh2 and F1 lowered the levels of fasting blood glucose, glycosylated hemoglobinA1c, and the amount of water intake. The Rh2 5 mg/kg group had lower levels of the area under the curve in an oral glucose tolerance test than that of the control group. F1 20 mg/kg group had lower levels of serum triglyceride and higher levels of serum adiponectin than those of the control group. In addition, Rh2 and F1 showed protective effects on the histology of pancreatic β-cells by suppressing the mRNA expressions of the pro-inflammatory cytokines tumor necrosis factor alpha and interleukin 6. Moreover, F1 activated the mRNA expression of peroxisome proliferator-activated receptor alpha. In conclusion, ginsenosides Rh2 and F1 may exert biological activities that suppress the severity of T2DM, showing their potential for use as new anti-diabetic compounds for the prevention of T2DM.

Keywords

Anti-diabetes, ginsenoside Rh2, ginsenoside F1, db/db mice, pancreatic β-cell

Student Number: 2011-21643

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List of Abbreviations

ELISA: Enzyme-linked immunosorbent assay

F1: Ginsenoside F1

IHC: Immunohistochemistry

IL-6: Interleukin 6

OGTT: Oral glucose tolerance test

Rh2: Ginsenoside Rh2

RT-PCR: Reverse transcription polymerase chain reaction

T2DM: Type 2 diabetes mellitus

TC: Total cholesterol

TG: Total triglyceride

TNF-α: Tumor necrosis factor α

INTRODUCTION

Type 2 Diabetes mellitus (T2DM) is one of the most common metabolic disorders worldwide induced by high caloric diets and insufficient exercise. One of the initial manifestations of T2DM is insulin resistance related to impaired glucose tolerance that results from decreased glucose metabolism in target tissues. Another is impaired insulin secretion due to decreasing pancreatic β-cell function, resulting in postprandial and subsequently fasting hyperglycemia. Finally, the combination of severe insulin resistance and pancreatic β-cell dysfunction results in the development of T2DM (Hajer *et al.*, 2008; Moller, 2001). Insulin resistance results in hyperinsulinemia, hyperglycemia, dyslipidemia, and pancreatic islet inflammation (Lebovits & Banerji, 2004). Pancreatic β-cell dysfunction occurs gradually and the function of the pancreas disappears eventually.

Therefore, dysfunction of β-cells is also an important risk factor for T2DM. In this regard, a current strategy to relieve T2DM has been associated with the amelioration of insulin resistance and the prevention of pancreatic β-cell dysfunction (Mokdad *et al.*, 2003; Moller, 2001). According to several studies, many anti-diabetic drugs such as sulfonylureas and thiazolidinediones can be used; however, they have various side effects (Moller, 2001; Modi, 2007).

Ginseng (*Panax ginseng,* C.A. Meyer) has been used as the herbal medicine for the improvement of immune function and various metabolic functions in Asia for several thousand years (Block & Mead, 2003; Van Kampen *et al.*, 2003). Various *in vivo* studies have demonstrated that ginseng and fermented ginseng had anti-diabetic activities (Attele *et al.*, 2002; Liu *et al.*, 2005; Jeon *et al.*, 2012). The active ingredients in ginseng are substances known as ginsenosides. Many studies have reported that ginsenosides exerted beneficial effects, including anti-diabetic, anti-inflammatory, anti-oxidant and anti-cancer effects (Han *et al.*, 2007; Lee *et al.*, 2006; Cho *et al.*, 2006). Ginsenosides F1, F2, Rg3, Rh1, Rh2 and compound K are minor ginsenosides because they exist in small amounts or are absent in ginseng. They can be produced by hydrolyzing the sugar moieties of the major ginsenosides such as Rb1, Rb2, Rc, Rd, Re and Rg1 using microbial enzymes. Deglycosylated minor ginsenosides are more rapidly absorbed into the bloodstream and act as active compounds compared to major ginsenosides (Park *et al.*, 2010). Therefore, minor ginsenosides are worthy of notice as potential pharmaceutical candidates with higher levels of bioavailability. Ginsenosides with more than forty structural derivatives are generally divided into three groups according to their structures. These are the oleanane, protopanaxadiol (PPD) and protopanaxatriol (PPT) types. Rh2 and F1 are known as the final metabolites of the PPD and PPT types of ginsenosides, respectively. In particular, Rh2 is produced by hydrolyzing a glucose unit from Rg3 by human intestinal bacteria (Park *et al.*, 2010). An anti-diabetic effect of Rh2 in an animal model by intravenous injection was reported (Lee *et al.*, 2006). However, an anti-diabetic effect of Rh2 orally

administrated in an *in vivo* model has not been reported. Moreover, there have been few studies on the biological activity of F1. Therefore, we had great interest in investigating the anti-diabetic activities of orally administered Rh2 and F1 by oral administration in a T2DM mice model to examine blood glucose levels. In addition, the present study assessed how Rh2 and F1 act on various target tissues in the body.

MATERIALS AND METHODS

2.1. Experimental animals

Five-week-old male diabetic db/db mice (n=50) and non-diabetic db/m littermates (n=10) were obtained from Central Lab Animal Incorporation (Seoul, Korea). The animals were maintained under specific pathogen-free conditions at 23±3℃ with 50±10% humidity and a 12h/12h light/dark cycle. All mice were fed a commercial diet and tap water ad libitum for two weeks to allow them to adapt to the new surroundings. The Db/db mice were divided into five groups of 10 mice in each group, a diabetic control group and four experimental groups. These were the diabetic control group (DC), the Rh2 5 and 20 mg/kg groups (Rh2 5 and Rh2 20), and the F1 5 and 20 mg/kg groups (F1 5 and F1 20). All mice were provided AIN-93G diets (Research Diets, Inc., USA). All animal processes and protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University.

2.2. Oral administration and experimental setup

Ginsenosides Rh2 (S-) (> 98 %) and F1 (> 98 %) powder were purchased from Chengdu Cogon Bio-tech Co., Ltd (China). Distilled water (200 ㎕ with 3 % Tween 80 (Yacukuri Pure Chemicals, Japan)) was orally administered daily as the vehicle to

the non-diabetic db/m mice (ND) and to the DC mice. For the experimental groups, 200 ㎕ of dissolved Rh2 and F1 in vehicle were orally administered daily at a dosage of 5 and 20 mg/kg body weight/day, respectively. Oral administrations were provided for 9 weeks. The mice were assessed in terms of their fasting blood glucose level, body weight, food intake and water intake once a week during the feeding period. At weeks 0 and 3 and on the final day of the experiment, blood samples were collected via the retro-orbital plexus after fasting for 12 h. After the collection of whole blood, the serum was separated immediately by centrifugation at 3,000 X g for 10 min and stored at -70℃. These serum samples were used to measure the levels of fasting insulin, adiponectin, total triglyceride (TG), total cholesterol (TC), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Upon the eighth week of the experiment, an oral glucose tolerance test (OGTT) was performed, following a 12 h period of fasting. After one week, the mice were anaesthetized with Zoletil 50 (Virbac Lab., France). Zoletil 50 was dissolved in phosphate-buffered saline at 25 mg/kg and injected into the thigh muscle. Glycosylated hemoglobinA1c (HbA1c) was analyzed using whole blood collected in EDTA-treated tubes. The liver, epididymal fat pad and pancreas were removed and stored in -70℃ until they were analyzed. Partial pancreases were collected in formalin for histology and immunohistochemistry (IHC).

2.3. Analysis of fasting blood glucose levels and HbA1c levels

The fasting blood glucose levels of the tail vein bleeds were measured once a week after fasting for 6 h using a Gluco-Dr. plus (Allmedicus, Korea). Blood glucose concentration was determined by glucose oxidase method with Gluco Dr. plus and calculated in mg/dL blood. Percent HbA1c levels were measured with Tosoh glycohemoglobin analyzer (Tosoh Corp., Japan) at the end of the experiment.

2.4. Analysis of body weight, food intake, water intake and organs weights

The body weights, the amounts of food and water intake were recorded every week. At the end of experiment, amount of body weight gain and food efficiency ratio (FER) were calculated. The weights of organs such as liver, pancreas, epididymal fat pad were measured at termination of the experiment.

2.5. Analysis of serum parameters

Serum samples collected at 0, 3 and 9 week were used in the assays. Serum TG and TC concentrations were analyzed enzymatically by commercial Triglyceride kit (HBi Co., Korea) and Total cholesterol kit (Asan Pharmaceutical Co., Korea), respectively.

Serum AST and ALT levels were measured by using AST/ALT reagent (Asan pharmaceutical Co., Korea). All analyses were followed by the manufacturer's instructions. Insulin and adiponectin concentrations in serum were determined by mouse insulin ELISA kit (Shibayagi Co., Japan) and mouse adiponectin ELISA kit (Shibayagi Co., Japan) using sandwich technique of enzyme immunoassay methods.

2.6. Oral glucose tolerance test (OGTT)

OGTT was performed at 8 week after the experimental ginsenosides were provided. Animals were fasted for 12 h prior to the test. The blood glucose levels were monitored from 0 to 120 min after glucose administration (1 g/kg glucose). Glucose levels were measured via tail vein with Gluco Dr. plus at the indicated intervals and the areas under the curve (AUCs) were calculated.

2.7. Quantitative analysis of relative mRNA expression levels in epididymal adipose tissue and liver tissue

Epididymal adipose tissue and liver tissue were isolated and stored at -80℃. Total RNA was extracted from collected tissues using RNAisoplus (Takara Bio Inc., Japan) according to manufacturer's instructions. Epididymal and liver tissue were homogenized in 1mL RNAisoplus reagent using a homogenizer (PowerGen 125, Fisher Scientific, UK). The homogenized samples were incubated at room temperature for 5 min and mixed with 200 ㎕ chloroform. After 5min of incubation at room temperature, the samples were centrifuged at 12,000 X g for 15min at 4℃. After separation of phase, 400 ㎕ of upper aqueous phase of each tube was transferred to fresh tubes and 400 ㎕ of isopropyl alcohol was added to the each tube and incubated at room temperature for 10 min. Then, the tubes were centrifuged 12,000 X g, 10min, 4℃ and supernatant was removed and 400 ㎕ of 75% ethanol was added to each tube for washing the RNA pellet. The tubes were centrifuged at 7,500 X g for 5 min at 4℃ and discard the supernatants. After drying in air for 10 to 20 min, RNA pellets were resuspended in 100 ㎕ of DEPC water and incubated at 56℃ for 20 min. After total RNA quantification, 4 μg of total RNA dissolved in DEPC water of each sample was reverse transcribed with oligo (dT) primers using PrimeScript 1st strand cDNA synthesis kit (Takara Bio Inc., Japan) following the condition of 42℃ for 50 min, 95℃ for 5 min and 4℃ for 10 min. 1 μg of cDNA samples were amplified using SYBR Premix Ex

Taq (Takara Bio Inc., Japan) and all analyses of quantitative real-time polymerase chain reaction (PCR) were performed by StepOne Real-Time PCR System (Applied Biosystems , USA). All the real-time PCR procedures were followed by the protocol of 95℃ for 10 sec, 95℃ for 5 sec and 60℃ for 30 sec up to 40 cycles. The results data were expressed as relative quantification (RQ) calculated using the ΔΔCt method (Schmittgen & Livak, 2008). β-Actin was used as an endogenous control. The primers used in real-time PCR were shown in Table 1.

Table 1. Primer sequences for real-time PCR

Target	Sequence (5'→3')
GLUT-2	(forward) TTG GAA GGA TCA AAG CAA TGT TG
	(reverse) CAT CAA GAG GGC TCC AGT CAA T
GLUT-4	(forward) TTC ATT GTC GGC ATG GGT TT
	(reverse) GGC AAA TAG AAG GAA GAC GTA AAG A
TNF-α	(forward) AGG GTC CAA CTC TGT GCT CAG A
	(reverse) CCA GGT CAC TGT CCC AGC AT
IL-6	(forward) ACC ACG GCC TTC CCT ACT TC
	(reverse) TCT GTT GGG AGT GGT ATC CTC TGT
PPAR-γ	(forward) CCA TGA GAT CAT CTA CAC GAT GCT
	(reverse) CCC TCT GAG ATG AGG ACT CCA T
UCP3	(forward) CCA CCT TAG GGC AAG AAC GA
	(reverse) AGA TGA GAA AAC CTC CGA GAG AGA
C/EBPα	(forward) GAC CAT TAG CCT TGT GTG TAC TGT ATG
	(reverse) TGG ATC GAT TGT GCT TCA AGT T
LPL	(forward) GGT CGA AGT ATT GGA ATC CAG AAA
	(reverse) CTG GAA AGT GCC TCC ATT GG
G6Pase	(forward) GGA CAA CGC CCG TAT TGG T
	(reverse) TTA TAG GCA CGG AGC TGT TGC T
PPAR-α	(forward) TGG CAG CAA TAT CAG AGG TAG ATT C
	(reverse) TCA TAT CAA AGG AGC TGC CAA A
β-actin	(forward) AAA TCG TGC GTG ACA TCA AAG A
	(reverse) GCC ATC TCC TGC TCG AAG TCT

2.8. Histology of pancreas

The pancreas of each mouse was removed and fixed in 10 % formalin. Fixed samples were embedded in paraffin and sectioned. The sections of pancreases were stained with hematoxylin-eosin. Histological scores were graded by severity of pancreatic langerhans islets β-cell lesion (score : 0, no significant lesion ; 1, slight lesion ; 2, mild lesion ; 3, moderate lesion ; 4, severe lesion with slight inflammatory cell infiltration ; 5, marked lesion with severe inflammatory cell infiltration) (Xpath, Korea).

2.9. Anti-insulin immunostaining of pancreas

The paraffin-embedded pancreatic sections were deparaffinized by clearing agent Histochoice (Amresco, U.S.A.) and rehydrated through graded ethanols and distilled water. Then, endogenous peroxidase activities were blocked by 3 % hydrogen peroxide. After rinsing with distilled water, the sections were heat-treated at 121℃ for 10 min to expose the antigenic epitope. After washing in phosphate buffered saline, the sections were incubated with 3 % bovine serum albumin solution for 1 h to block nonspecific binding of immunoglobulin. Insulin staining was performed by incubation for overnight with anti-insulin polyclonal antibody (Santa Cruz, USA), followed by incubation with horse radish peroxidase-conjugated second antibody. The color was

revealed using chromogen DAB (3,3'-diaminobenzidine) system. These peroxidase staining was performed by using IHC Polink-2 plus HRP DAB kit (GBI, USA) following the manufacturer's instructions. The stained sections were conterstained with hematoxyiln.

2.10. Statistical analysis

The results were presented as means ± standard error of means (S.E.M.). Statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by the Duncan post hoc test for multiple range tests and by non-parametric Kruskal-Wallis H test, followed by the Mann-Whitney U test using SPSS ver. 19. P values of < 0.05 were considered significantly different.

RESULTS

3.1. Effects of Rh2 and F1 on fasting blood glucose levels and HbA1c levels

The Rh2 and F1 groups showed lower fasting glucose levels than the DC group. In the Rh2 groups, the fasting blood glucose level of the low-dosage group was significantly lower from the fifth week (Fig. 1A). In the F1 groups, during the experimental period the fasting blood glucose level of F1 20 was lower than that of F1 5. The fasting glucose levels were significantly lower in the F1 20 group from the sixth week and in the F1 5 group upon the sixth and eighth week (Fig. 1B). The fasting glucose levels of the ND group were significantly lower than those of the DC group during the experimental period (data not shown). The concentrations of HbA1c in the Rh2 5 and F1 20 groups were significantly lower than that of the DC group upon the ninth week (Fig. 2).

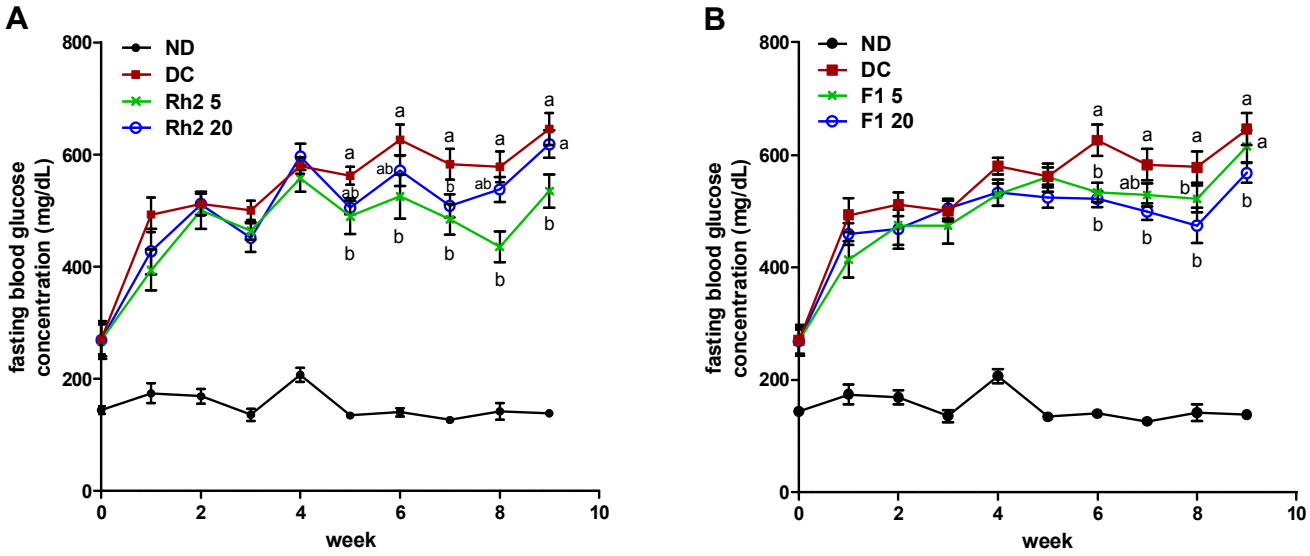


Fig. 1. Effects of ginsenosides on fasting blood glucose levels for nine weeks

A, Ginsenoside Rh2 groups; B, Ginsenoside F1 groups. The data are presented as Mean ± S.E.M. (n=9~10). A statistical analysis was performed using an ANOVA ($p < 0.05$) for comparison with the DC group. The different letters denote significant differences at $p < 0.05$.

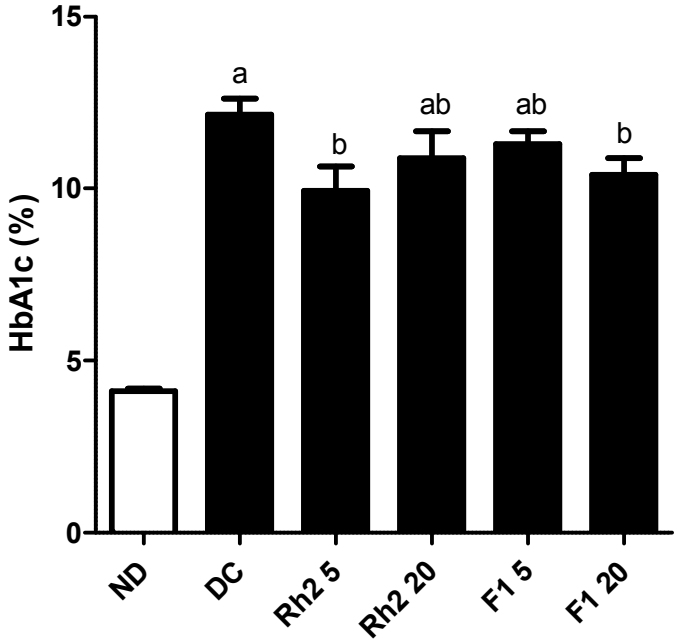


Fig. 2. Effects of ginsenosides Rh2 and F1 on HbA1c levels at 9 week

The data are presented as Mean ± S.E.M. (n=9~10). A statistical analysis was performed using an ANOVA ($p < 0.05$) for comparison with the DC group. The different letters denote significant differences at $p < 0.05$.

3.2. Effects of Rh2 and F1 on body weight, food intake, water intake and organs weights

Table 2 shows the changes in the body weight, the amounts of food and water intake, organ weights. In the present experiment, there were no significant differences in the amounts of body weight gain in the Rh2 and F1 groups compared to the DC group. Although the total amounts of food intake for the Rh2 5, F1 5 and 20 groups were significantly lower than that of the DC group, FER did not show any significant differences. The Rh2 5 and F1 groups showed significantly suppressed total water intake levels compared with the DC group. At the end of the experiment, the weights of the organs, specifically the liver, epididymal fat pads and pancreases, were not significantly different from those of the DC group.

Table 2. Effects of ginsenosides Rh2 and F1 on body weight, food intake, water intake and organs weights

Parameter	ND	DC	Rh2 5	Rh2 20	F1 5	F1 20
Body weight gain (g/9weeks)	9.0±0.6	11.1±1.4	12.2±1.7	12.3±2.2	11.4±1.6	11.8±1.7
Total food intake (g/9weeks)	201.8±3.3	480.8±17.1 [a]	429.3±24.7 [b]	423.3±9.6 [b]	451.7±10.4 [ab]	430.7±13.6 [b]
FER	4.5±0.3	2.3±0.3	2.9±0.5	2.9±0.5	2.5±0.4	2.7±0.4
Total water intake (mL/9weeks)	261.0±32.6	2601.4±173.6 [a]	2124.2±266.4 [b]	2115.4±169.0 [b]	2387.5±196.3 [b]	2147.9±120.5 [b]
Organ weights						
Epididymal fat (g)	1.58±0.16	2.50±0.10	2.48±0.08	2.41±0.13	2.39±0.10	2.66±0.13
Liver (g)	1.16±0.05	2.90±0.17	3.06±0.20	3.02±0.25	2.73±0.13	2.91±0.21
Pancreas (g)	0.27±0.01	0.30±0.03	0.29±0.01	0.28±0.02	0.26±0.02	0.27±0.02

Each data is presented as means ± S.E.M. FER (Food efficiency ratio) was calculated as [total body weight gain (g) / total food intake (g) X 100]. A statistical analysis was performed using an ANOVA followed by Duncan's multiple range test ($p < 0.05$) for comparison with the DC group. Values within the same row with different letters stand for significant difference at $p < 0.05$.

3.3. Effects of Rh2 and F1 on serum analysis

Fig. 3 and Fig. 4 show the changes in the serum lipid profiles for nine weeks and the levels of AST and ALT at ninth week, respectively. The serum TG level of the F1 20 group was significantly lower than that of the DC group at ninth week. However, there were no significant differences between the treated groups and the DC group in terms of the TC, AST, and ALT levels. Fig. 5 shows the results of serum insulin, adiponectin analyses and the values from the homeostasis model assessment of insulin resistance (HOMA-IR) at ninth week. The level of serum adiponectin of the F1 20 group was significantly higher than that of the DC group.

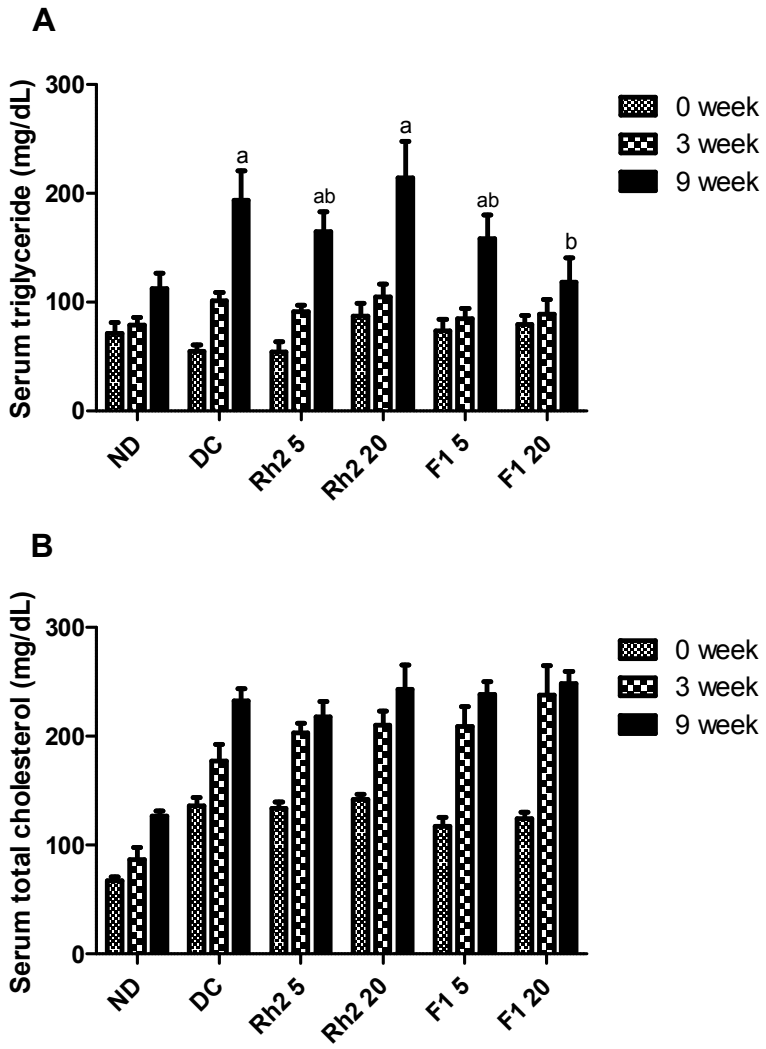


Fig. 3. Effects of ginsenosides Rh2 and F1 on serum lipid profile

A, Serum triglyceride levels; B, Serum total cholesterol levels. Each data is presented as means ± S.E.M. A statistical analysis was performed using an ANOVA followed by Duncan's multiple range test ($p < 0.05$) for comparison with the DC group. Values within the same row with different letters stand for significant difference at $p < 0.05$.

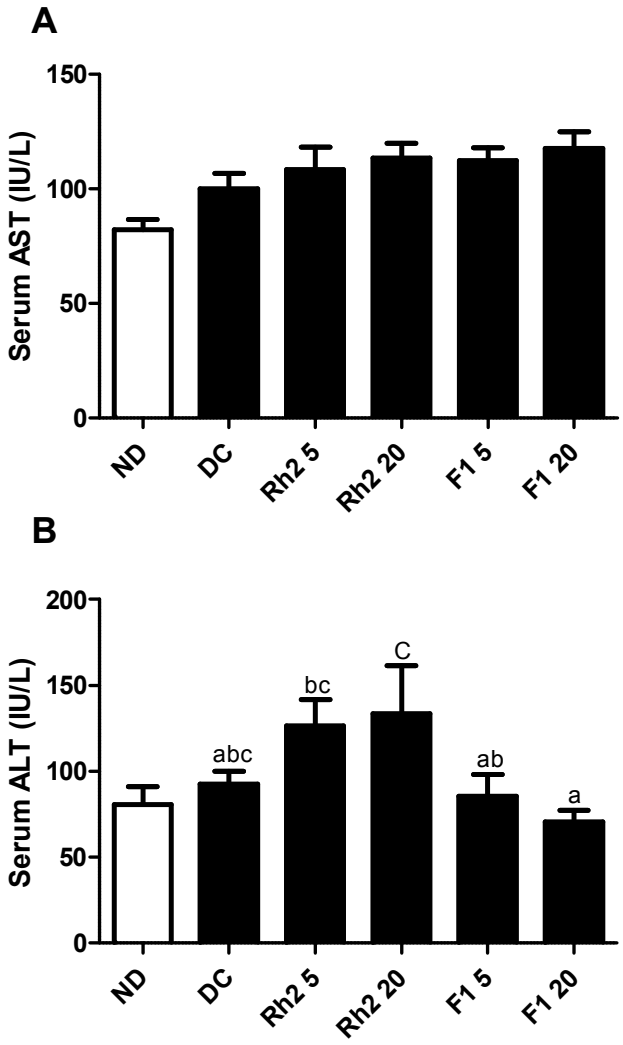


Fig. 4. Effects of ginsenosides Rh2 and F1 on serum AST and ALT levels

A, Serum AST levels; B, Serum ALT levels. Each data is presented as means ± S.E.M. A statistical analysis was performed using an ANOVA followed by Duncan's multiple range test ($p < 0.05$) for comparison with the DC group. Values within the same row with different letters stand for significant difference at $p < 0.05$.

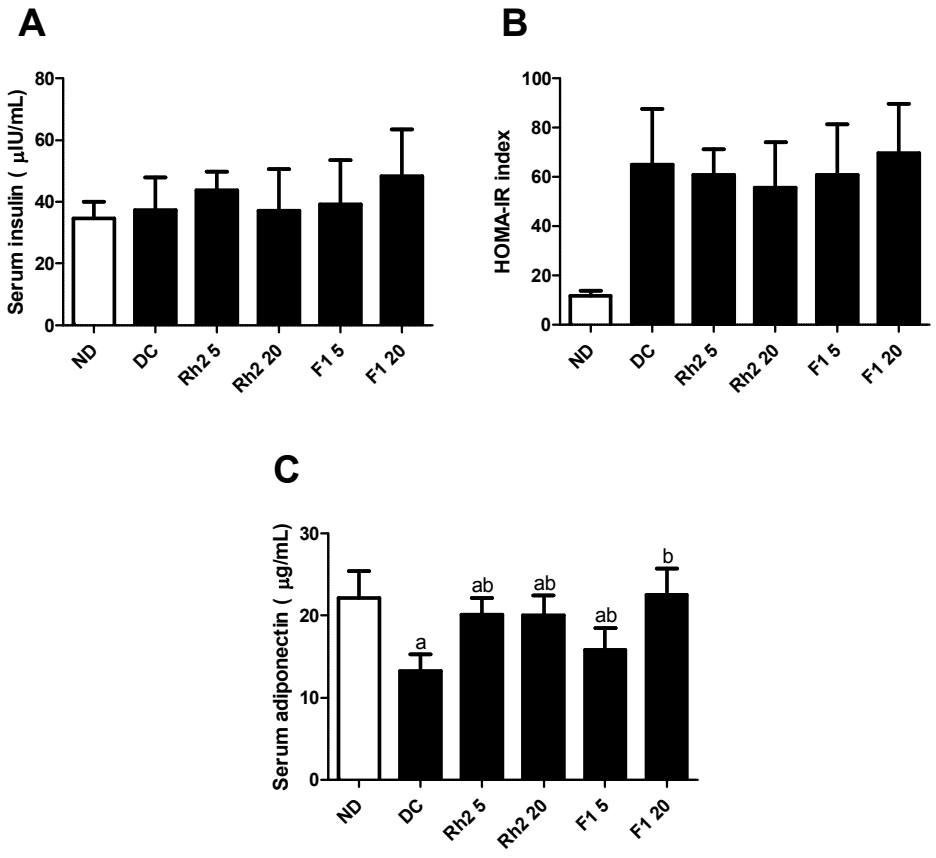


Fig. 5. Effects of ginsenosides Rh2 and F1 on serum insulin, HOMA-IR and adiponectin levels

A, Serum insulin levels; B, HOMA-IR index; C, Serum adiponectin levels. Each data is presented as means ± S.E.M. HOMA-IR was calculated as an insulin resistance index as [insulin (μIU/mL) X glucose (mM) / 22.5]. A statistical analysis was performed using an ANOVA followed by Duncan's multiple range test ($p < 0.05$) for comparison with the DC group. Values within the same row with different letters stand for significant difference at $p < 0.05$.

3.4. Effects of Rh2 and F1 on an oral glucose tolerance test

OGTT was performed 8 week after the experiment. The blood glucose levels of all groups were elevated 30 minutes after the glucose injection and gradually decreased over 120 min (Fig. 6A). After the OGTT, the AUCs were calculated by the excel program. The AUC level of the Rh2 5 group was significantly lower than that of the DC group, whereas those of the other treated groups were not (Fig. 6B). The AUC levels of the ND group were significantly lower than that of the DC group (data not shown).

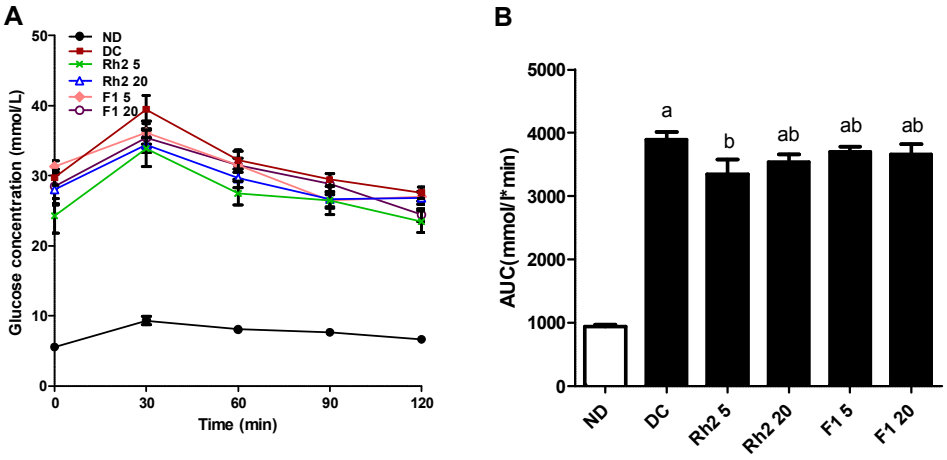


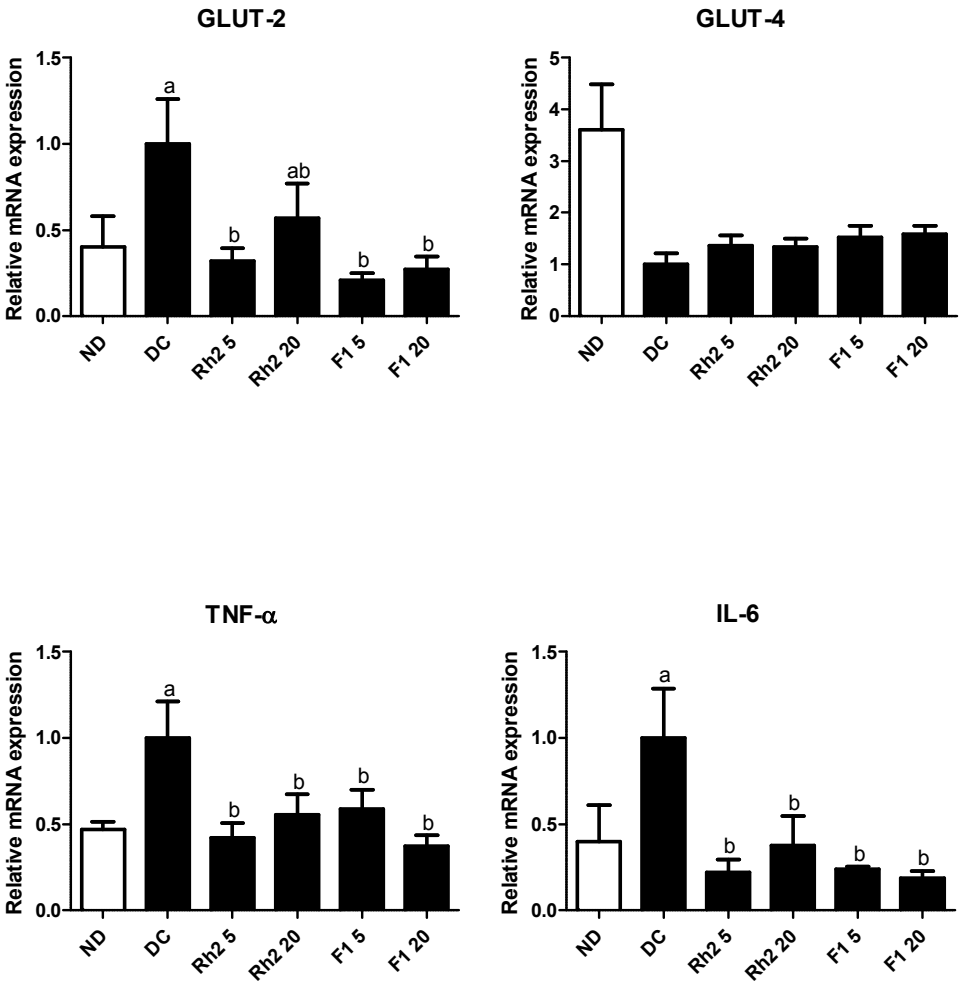
Fig. 6. Effects of ginsenosides Rh2 and F1 in OGTT

A, Glucose levels (mmmol/L) in OGTT; B, Calculated AUCs in OGTT. The data are presented as means ± S.E.M. A statistical analysis was performed using an ANOVA ($p < 0.05$) for comparison with the DC group. The different letters denote significant differences at $p < 0.05$.

3.5. Effects of Rh2 and F1 on the mRNA expression levels in adipose tissue and liver tissue

We compared the gene expression profiles in the liver and adipose tissue of each db/db mouse using real time PCR. Fig. 7 shows the mRNA expression levels of glucose transporter protein type 2 (GLUT-2), glucose transporter protein type 4 (GLUT-4), tumor necrosis factor (TNF-α), interleukin 6 (IL-6), peroxisome proliferator-activated receptor gamma (PPAR-γ), uncoupling protein 3 (UCP3), CCAAT/enhancer-binding protein alpha (C/EBPα) and lipoprotein lipase (LPL) in epididymal adipose tissue. Among the eight genes in adipose tissue, the expression levels of TNF-α and IL-6 of all treated groups were significantly lower than those of the DC group. Moreover, UCP3 expression levels of F1 groups were significantly lower than that of DC group and the C/EBPα expression level of the F1 20 group was significantly higher than that of the DC group. The mRNA expressions of LPL of the Rh2 5 and F1 groups were higher than that of the DC group, but not at a significant level.

In liver tissue, the relative mRNA expression levels of GLUT-2, peroxisome proliferator-activated receptor alpha (PPAR-α) and glucose-6-phosphatase (G6Pase) were analyzed. The expression levels of GLUT-2 of all treated group were significantly lower than that of DC group and PPAR-α expression level of the F1 20 group was significantly higher than that of the DC group.


GLUT-2
Relative mRNA expression
1.5
1.0
0.5
0.0
a
ab
b
b
b
b
ND
DC
Rh2 5
Rh2 20
F1 5
F1 20
GLUT-4
Relative mRNA expression
5
4
3
2
1
0
ND
DC
Rh2 5
Rh2 20
F1 5
F1 20
TNF-α
Relative mRNA expression
1.5
1.0
0.5
0.0
a
b
b
b
b
ND
DC
Rh2 5
Rh2 20
F1 5
F1 20
IL-6
Relative mRNA expression
1.5
1.0
0.5
0.0
a
b
b
b
b
ND
DC
Rh2 5
Rh2 20
F1 5
F1 20

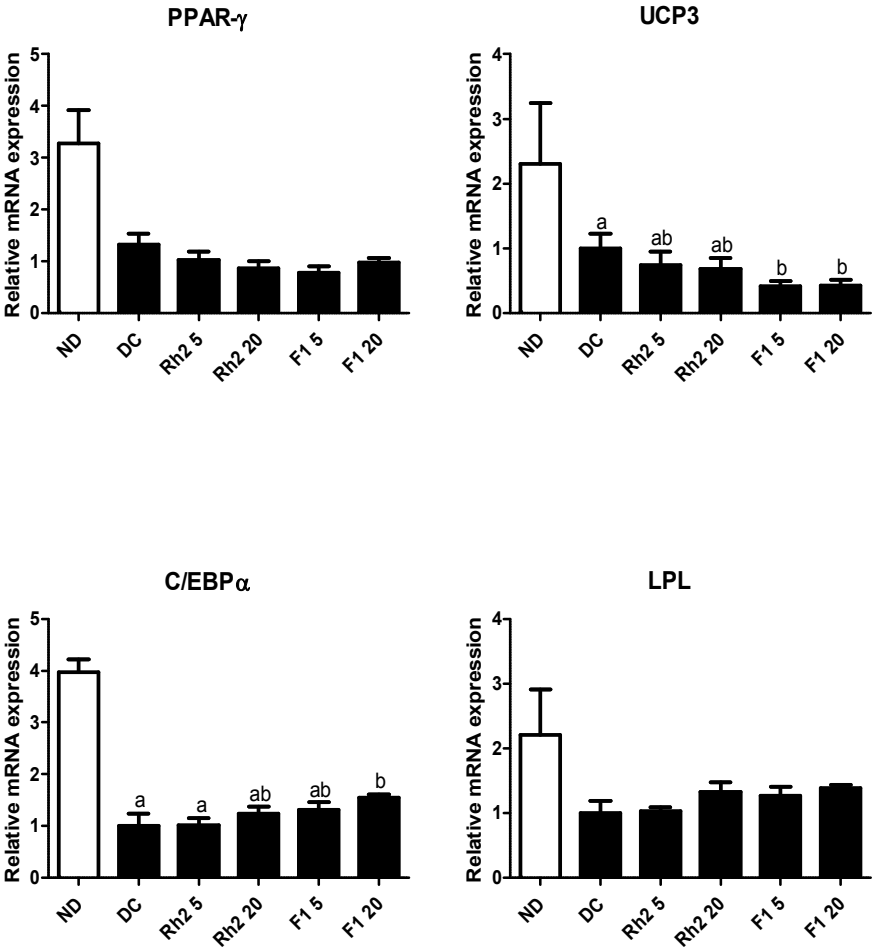


Fig 7. Effects of ginsenosides Rh2 and F1 on relative mRNA expressions of several genes in epidydimal adipose tissue

Each data is presented as means ± S.E.M. A statistical analysis was performed using an ANOVA followed by Duncan's multiple range test ($p < 0.05$) for comparison with the DC group. Values within the same row with different letters stand for significant difference at $p < 0.05$.

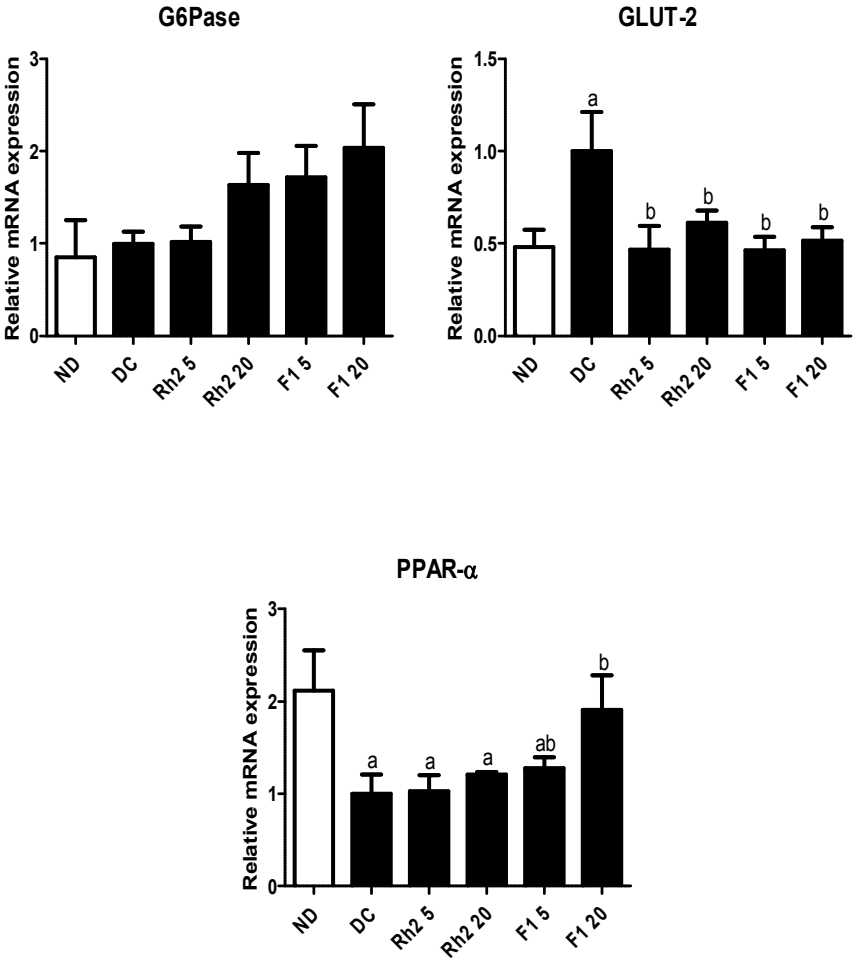


Fig 8. Effects of ginsenosides Rh2 and F1 on relative mRNA expressions of several genes in liver tissue

Each data is presented as means ± S.E.M. A statistical analysis was performed using an ANOVA followed by Duncan's multiple range test ($p < 0.05$) for comparison with the DC group. Values within the same row with different letters stand for significant difference at $p < 0.05$.

3.6. Effects of Rh2 and F1 on the histology of the pancreas

Fig. 9 and Table 3 show the degrees of each pancreatic beta cell lesion. Db/db mice generally have hypertrophied pancreatic islets of various sizes and morphologies, as well as a mixture of beta cells and non-beta cells in the islets. In the present experiment, necrosis of the pancreas islets was observed in all groups except for the ND group. However, the degree of necrosis differed for each group. Compared with the ND group, DC group mice revealed extensively degenerated islet tissue with proliferated and infiltrated exocrine cells, indicating beta cell damage. An indistinct boundary between the endocrine and exocrine regions was observed. Some of the islets of the DC group also showed inflammatory cell infiltration. The Rh2 20 group showed a level of beta cell lesions similar to that of the DC group, while the Rh2 5 group showed relatively less damage to the islet tissue and less inflammation than the DC group. Compared with the DC group, the pancreases from the F1 groups showed more distinguishable endocrine/exocrine boundary regions. This suggests that the F1 treatments prevented islet destruction and showed a nearly normal morphology in a dose-dependent manner ($p = 0.057$ in F1 5, $p = 0.029$ in F1 20).

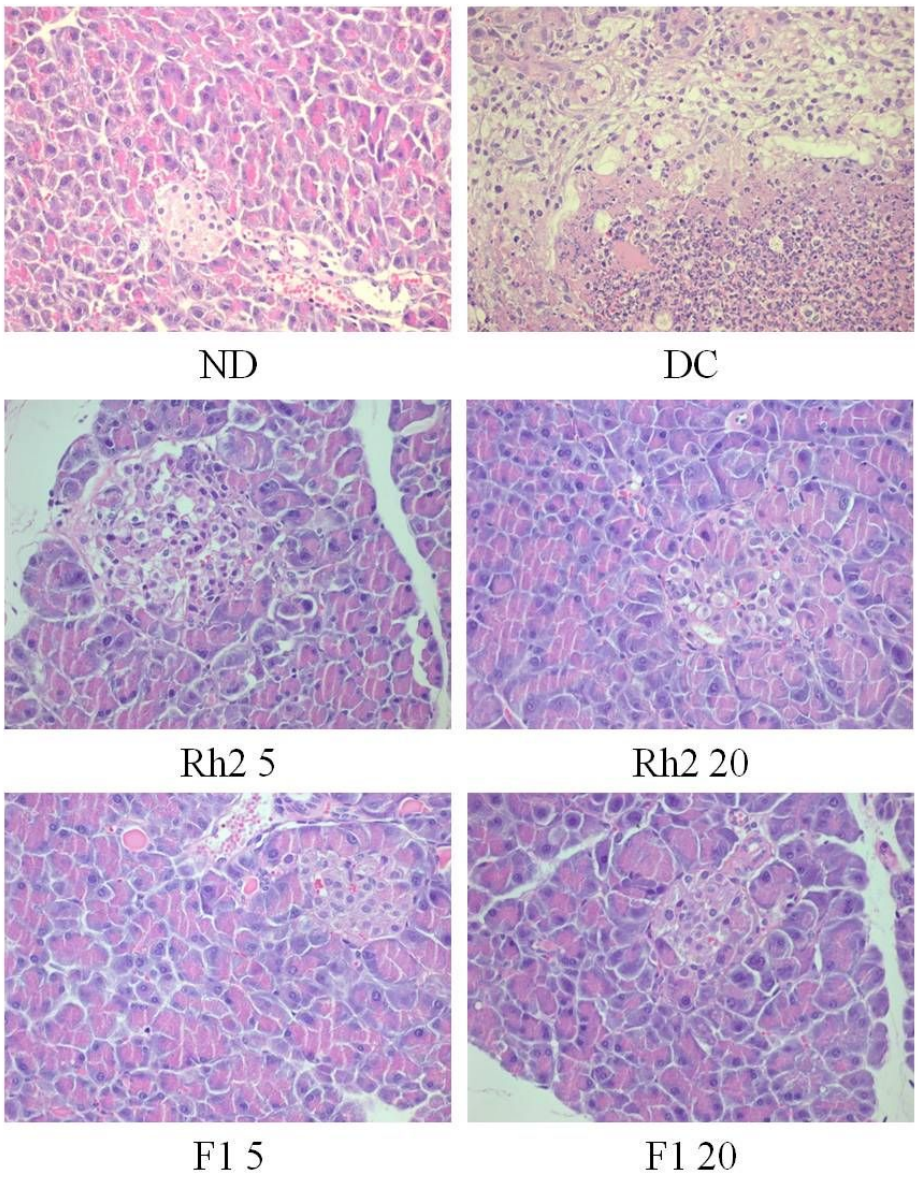


Fig 9. Histology of pancreatic β-cell in each db/db mouse fed ginsnenosides for nine weeks; Hematoxylin & Eosin staining ; Magnification X 400

Table 3. Histological scores of H&E stained pancreas

Group	Histological scores
ND	0.0 ± 0.0*
DC	3.5 ± 1.0
Rh2 5	3.0 ± 2.3
Rh2 20	3.5 ± 1.0
F1 5	1.5 ± 1.0
F1 20	1.0 ± 0.0*

H&E stained pancreas of each mice was scored by severity of pancreatic β-cell lesions. Each value represents means ± S.E.M. of four mice per group. A statistical analysis was performed by non-parametric Kruskal-Wallis H test, followed by Mann-Whitney U test to compare between DC group and each group (*$p < 0.05$).

3.7. Effects of Rh2 and F1 on the immunohistochemistry of the pancreas

Immunostaining with an anti-insulin antibody was performed to compare the insulin contents in the pancreases of each group. As shown in Fig. 10, insulin-positive islet cells of DC mice were scarcely detected. In contrast, the insulin-positive area of the Rh2 and F1-treated mice were larger than those of the DC group, suggesting that the Rh2 and F1-treated group mice maintained more insulin content levels than the DC group.

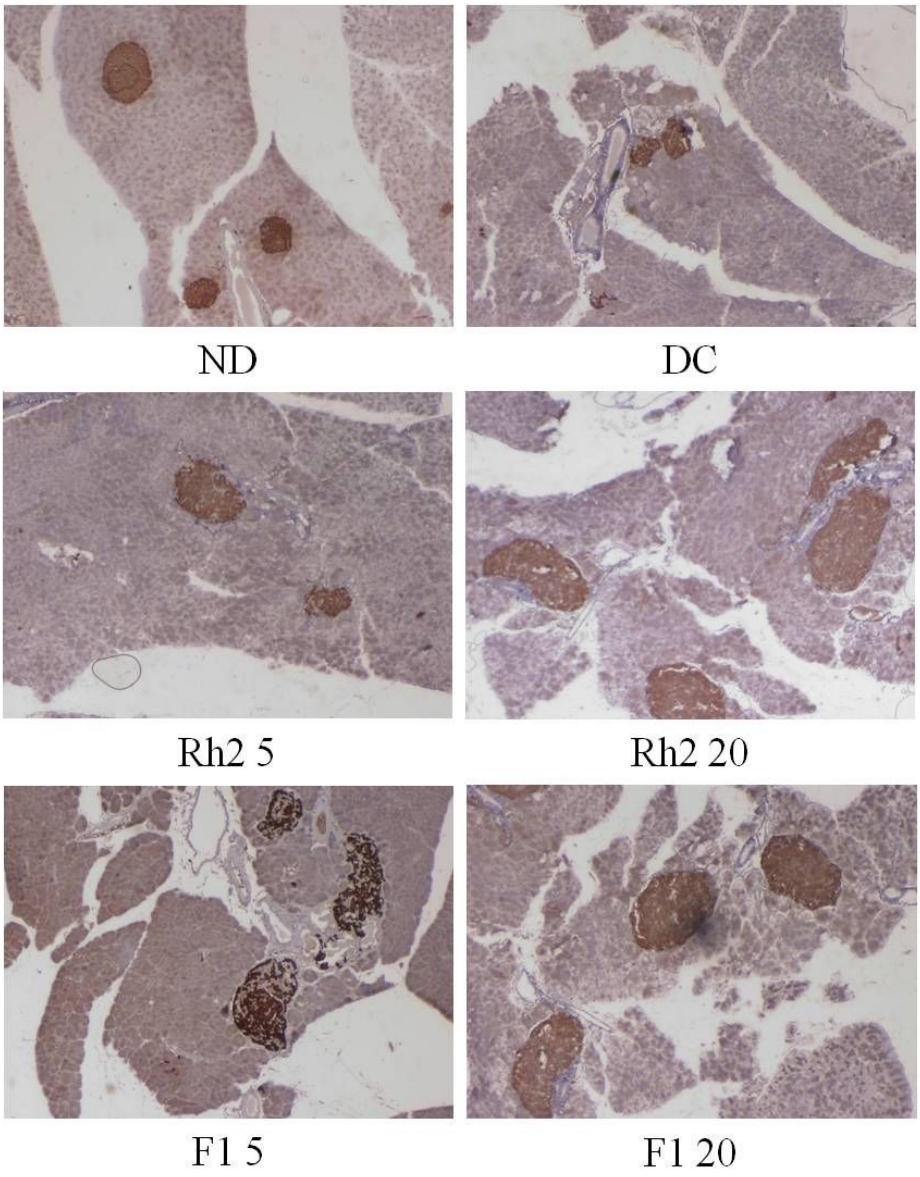


Fig 10. Anti-insulin immunostaining of pancreatic β-cell in each db/db mice fed ginsenosides for nine weeks; Magnification X 40

DISCUSSION

The present study showed that an oral administration of ginsenosides Rh2 and F1 was effective for the prevention of the development of T2DM. Rh2 and F1 significantly lowered fasting blood glucose levels and HbA1c levels, suggesting their potential for use in the alleviation of diabetes-associated symptoms. They also reduced the amount of total food intake and water intake. Moreover, Rh2 and F1 prevented pancreas islet destruction and lowered the mRNA expression of the pro-inflammatory cytokines of IL-6 and TNF-α.

During the development of insulin resistance in peripheral tissues, insulin production by pancreatic β-cells increases excessively in an attempt to control the elevated blood glucose level, consequently leading to β-cell failure of the pancreas, resulting in hyperglycemia. Db/db mice lack a functional leptin receptor; thus, they tend to overeat, become obese, and develop severe insulin resistance accompanied by marked hyperglycemia and hyperinsulinemia. Generally, the morphological features of the pancreas islet and the serum insulin levels of db/db mice are known to be age-dependent. At an early pathogenic stage of db/db mice, the initial adaptation to insulin resistance is hypertrophy of islet β-cells, resulting in hyperinsulinemia. However, with aging, degeneration of the β-cells and islet atrophy occur gradually, and hyperinsulinemia is diminished, resulting in insulin deficiency until the mouse's death

(Coleman, 1978; Lee *et al.*, 1996; Kawasaki *et al.*, 2005; Gapp *et al.*, 1983). Previous studies reported that prolonged exposure to high concentrations of glucose induced apoptotic β-cell death (Efanova *et al.*, 1998).

In the present study, we observed that F1 preserved the morphological integrity of β-cells relatively better compared with the DC group. The DC group exhibited atrophic islets and degenerated β-cells, indicating that degranulation of the insulin secretory vesicles had occurred, whereas the Rh2 and F1 groups showed hyperplastic islets. When β-cells were stained with anti-insulin antibodies, the lack of staining in the DC group demonstrated the degranulation of the insulin vesicles, while the strong staining in the Rh2 and F1 groups indicated the restoration of the insulin secretory capacity. In fact, some reports suggest that fermented ginseng, especially ginsenoside Rh2, can enhance insulin secretion (Jeon *et al.*, 2012; Kim & Kim, 2008; Su *et al.*, 2007; Lee *et al.*, 2006). Taken together, these data suggest that Rh2 and F1 slow the age-dependent insulin decline by preventing the reduction of the β-cell mass.

These protective actions on the β-cells of the Rh2 and F1 groups are likely associated with a marked suppression of the inflammatory cytokines in the adipose tissue. Adipose tissues play a role in the accumulation of fat cells as well as the secretion of various cytokines as an endocrine organ (Hotamisligil *et al.*, 1993). TNF-α and IL-6 contribute to the development of insulin resistance, as evidenced by the fact that an administration of recombinant TNF-α to animals impaired insulin action and

TNF-α knockout obese mice improved in terms of their insulin sensitivity compared with wild-type mice (Hotamisligil *et al.*, 1993; Uysal *et al.*, 1997). Moreover, IL-6 inhibited glucose-stimulated insulin secretion *in vitro* and *in vivo* (Choi *et al.*, 2004). Generally, TNF-α and IL-6 are known to promote insulin resistance by stimulating both c-Jun amino terminal kinase (JNK) and IκB kinase-β (IKK-β)/nuclear factor-κB (NF-κB) pathways, thereby reducing the ability of insulin receptor substrate-1 (IRS-1) and inhibiting downstream insulin signaling (Ruan *et al.*, 2002; Khan *et al.*, 2006). These defects in insulin signaling pathways have recently been found to disrupt insulin secretion by pancreatic β-cells, suggesting that insulin resistance may be responsible for the dysfunction of the β-cells (Greenberg & McDaniel, 2002). There is evidence that TNF-α and IL-6 accelerate the dysfunction and destruction of β-cells (Mandrup-Poulsen, 2001). The role of free radical nitric oxide (NO) in cytokine-induced β-cell destruction has been widely reported (Corbett *et al.*, 1993; Mandrup-Poulsen, 2001). TNF-α induces NO production, which breaks the pancreatic cellular DNA strand. Thereafter, DNA repair mechanisms, including the poly ADP-ribose polymerase (PARP) pathway, are activated, which can cause cell death through exhaustion of cellular nicotinamide adenine dinucleotide (NAD^+). NO-induced DNA strand breakage also causes apoptosis of β-cells through the activation of tumor suppressor protein p53 (Hajer *et al.*, 2008; Rabinovitch *et al.*, 1999; Mandrup-Poulsen, 2001). Along this line, the decreased mRNA expressions of TNF-α and IL-6 in adipose tissue in our study suggest that Rh2 and F1 suppress insulin resistance and protect against pancreatic β-cell dysfunction.

Moreover, the increased adiponectin levels in the Rh2 and F1 groups may be associated with the inhibition of TNF-α. TNF-α reduces the adiponectin level by suppressing its promoter activity (Maeda *et al.*, 2001). Adiponectin, an insulin-sensitizing hormone, is known to facilitate the β-oxidation of fatty acid and to ameliorate insulin sensitivity (Nedvidkova *et al.*, 2005).

PPAR-γ reportedly improves insulin sensitivity by inducing the differentiation of preadipocyte and stimulating the secretion of adiponectin (Kadowaki *et al.*, 2003). However, Rh2 and F1 did not show any effect on PPAR-γ mRNA expression in the present study. Moreover, UCP3 expression is up-regulated by PPAR-γ agonists in adipose tissue (Matsuda *et al.*, 1998), but the mRNA expression level of UCP3 was significantly decreased in the F1 groups, which suggests that F1 is not associated with PPAR-γ.

Liver tissue is another key target for glucose homeostasis and adequate lipid metabolism. In the liver, the activation of PPAR-α increases the uptake of fatty acids and their β-oxidation by stimulating the expressions of fatty acid transport protein and acyl-CoA synthetase (Neve *et al.*, 2000). PPAR-α also improve lipoprotein metabolism and inhibits vascular inflammation. PPAR-α stimulates the expression of LPL, which hydrolyses TG in very low-density lipoprotein (VLDL) and stimulates the uptake of low-density lipoprotein (LDL). It also inhibits apolipoprotein C-III, which increase the

LPL activity (Neve *et al.*, 2000; Fruchart *et al.*, 1999). Bajaj et al. (2007) found that PPAR-α decreased serum triglycerides in humans. Similarly, in the present study, the activation of PPAR-α was involved in the significant reduction of the serum TG level in the F1 20 group. As an insulin sensitizer, adiponectin is known to be increased in a PPAR-α–dependent manner (Khan *et al.*, 2006) according to evidence that the adiponectin receptor AdipoR2 is up-regulated by PPAR-α agonists (Chinetti *et al.*, 2004). Based on this result, the high level of serum adiponectin in the F1 20 group may have been associated with the activated mRNA level of PPAR-α in liver tissue. However, F1 20 did not show a significant reduction in HOMA-IR despite the high level of serum adiponectin accompanied by the suppressed TNF-α and activated PPAR-α.

According to Hwang et al. (2007), Rh2 at a high dose exerts cellular toxicity *in vitro* and increases DNA fragmentation. In accordance with this result, in our study, the concentrations of fasting blood glucose and HbA1c in the Rh2 20 group was higher than that of the Rh2 5 group, and more pancreatic β-cell destruction was observed in Rh2 20 than in Rh2 5.

In conclusion, we demonstrate that the ginsenosides Rh2 and F1 have beneficial effects in that they decrease blood glucose and HbA1c levels in the db/db mice model. It was especially found that Rh2 and F1 have protective effects with pancreatic β-cell preservation via the inhibition of TNF-α and IL-6 and that F1 plays a role as a PPAR-α

agonist to improve various metabolic disorders by activating PPAR-α. However, further studies are essential to find the detailed mechanism of Rh2 and F1 on the maintenance of glucose homeostasis and on lipid metabolism. Considering the results of this study, we suggest that Rh2 and F1 are potential components that prevent T2DM, providing the basis for the development of new anti-diabetic compounds for T2DM patients.

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국문초록

제 2형 당뇨병은 조직에 대한 인슐린 작용성의 감소로 인해 심각한 내분비 장애를 유발하는 대사증후군 중의 하나로서, 최근 육류위주의 식습관 및 운동부족 현상이 증가하면서 전 세계적으로 제 2형 당뇨환자의 발생률이 급격히 증가하고 있다. 인슐린 저항성 증가에 따른 췌장 베타세포의 인슐린 과다 생성은 췌장 베타세포의 기능을 저하시키고 결국 췌장의 인슐린 분비 기능 장애를 야기시킨다. 그러므로 인슐린 저항성 증가와 췌장 베타세포의 기능 장애는 제 2형 당뇨의 위험인자로 인식되고 있으며, 이를 조절하기 위한 약물과 천연물에 대한 연구가 활발히 진행되고 있다. 그 중, 인삼과 홍삼은 여러 연구들을 통해 항 당뇨 효과를 지닌다는 사실이 입증되었지만, 그 효과가 인삼이나 홍삼 속의 어떤 활성물질에 의해 나타나는지에 대해서는 그 연구가 미미한 상태이다. 진세노사이드는 인삼에 들어있는 활성물질로서, 그 중 진세노사이드 Rh2와 F1은 각각 panaxadiol과 panaxatriol 계열 진세노사이드의 최종 대사산물로 알려져 있다. 이들은 인삼 속에는 미량 함유되어 있지만, 주요 진세노사이드로부터 장내 미생물에 의해 전환되어 생성될 수 있으며, 체내 흡수가 빨라 더 높은 생리활성을 나타낼 수 있다고 알려져 있다. 그러므로 본 연구에서는 제 2형 당뇨 마우스 모델에서 진세노사이드 Rh2와 F1의 항 당뇨 활성을 연구하였다. 실험동물은 당뇨 대조군, Rh2 와

F1을 각각 5 mg/kg, 20 mg/kg의 농도로 투여한 군으로 나누었으며, 9주 동안 매일 하루에 한 번 해당 물질을 경구투여하였다. 매주 공복 혈당과 체중, 식이섭취량, 음수섭취량을 측정하였고, 혈청 분석을 비롯한 혈당 조절과 관련된 다양한 생화학적 지표 분석과 조직학적 분석을 실시하였다. 혈청 인슐린과 아디포넥틴 농도는 ELISA를 통해 측정하였으며, 혈청 지질 대사 분석을 위해 혈청 중성지질, 혈청 콜레스테롤 농도를 측정하였다. 실험 종료 1주 전에는 포도당내성검사를 실시하였으며, 실험 종료 시 희생을 통해 사후 장기 무게, 당화혈색소 수치를 측정하고 간, 지방, 췌장 조직의 분석을 실시하였다. 그 결과, 대조군과 비교하여 Rh2 5 mg/kg 투여군과 F1 20 mg/kg 투여군은 유의적인 공복 혈당 강하 효과를 보였고, 장기적인 혈당 관리 지표인 당화혈색소가 유의적으로 감소하였으며, 음수섭취량 또한 감소하였다. Rh2 5 mg/kg 투여군은 포도당내성이 개선되었으며, F1 20 mg/kg 투여군은 혈청 중성지질 농도가 감소하였고, 인슐린 민감성 호르몬인 아디포넥틴의 분비가 증가하였다. 또한 F1은 간 조직에서 과산화소체 증식체 활성화 수용체 알파 (PPAR-α)의 mRNA 발현을 증가시켜 당 대사의 중요한 장소인 간에서 혈당 조절을 촉진시킨 것으로 보인다. Rh2와 F1의 투여는 염증성 사이토카인인 종양 괴사 인자 알파 (TNF-α)와 인터루킨 6 (IL-6)의 mRNA 발현을 억제하여 췌장 조직의 염증도를 감소시켰고, 이로 인해 췌장 베타 세포를 보호하는 효과를 나타내는 것으로 사료되었다. 결론적으로, 본 연구에서 Rh2와 F1은

염증인자의 발현을 억제하여 췌장을 보호하고 이로 인해 제2형 당뇨 관련 증상을 개선시키고, 당뇨의 심화를 억제하는 데에 긍정적인 효과를 보였다. 앞으로 더 심화된 연구를 통해 항 당뇨 효능 검증이 필요할 것이며, 향후 항 당뇨 건강기능식품 소재로의 사용을 기대해 볼 수 있을 것으로 사료된다.

주요어: 항 당뇨 활성, **진세노사이드** Rh2, **진세노사이드** F1, **제 2형 당뇨 마우스 모델**, **췌장 베타 세포**

학번: 2011-21643