BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 20(2), 220-225 (2012)

Pectinase-Processed Ginseng Radix (GINST) Ameliorates Hyperglycemia and Hyperlipidemia in High Fat Diet-Fed ICR Mice

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Abstract

To develop a ginseng product possessing an efficacy for diabetes, ginseng radix ethanol extract was treated with pectinase and obtained the GINST. In the present study, we evaluate the beneficial effect of GINST on high fat diet (HFD)-induced hyperglycemia and hyperlipidemia and action mechanism(s) in ICR mice. The mice were randomly divided into five groups: regular diet group (RD), high fat diet group (HFD), HFD plus GINST at 75 mg/kg (GINST75), 150 mg/kg (GINST150), and 300 mg/kg (GINST300). Oral glucose tolerance test reveals that GINST improves the glucose tolerance after glucose challenge. Fasting plasma glucose and insulin levels were decreased by 4.3% and 4.2% in GINST75, 10.9% and 20.0% in GINST150, and 19.6% and 20.9% in GINST300 compared to those in HFD control group. Insulin resistance indices were also markedly decreased by 8.2% in GINST75, 28.7% in GINST150, and 36.4% in GINST300, compared to the HFD control group. Plasma triglyceride, total cholesterol and non-esterified fatty acid levels in the GINST300 group were decreased by 13.5%, 22.7% and 24.1%, respectively, compared to those in HFD control group. Enlarged adipocytes of HFD control group were markedly decreased in GINST-treated groups, and shrunken islets of HFD control mice were brought back to near normal shape in GINST300 group. Furthermore, GINST enhanced phosphorylation of AMP-activated protein kinase (AMPK) and glucose transporter 4 (GLUT4). In summary, GINST prevents HFD-induced hyperglycemia and hyperlipidemia through reducing insulin resistance via activating AMPK-GLUT4 pathways, and could be a potential therapeutic agent for type 2 diabetes.

Key Words: Pectinase-processed ginseng radix, High fat diet, Insulin resistance, AMP-activated protein kinase, Glucose transporter 4

INTRODUCTION

Diabetes mellitus (DM) is the most common endocrine disease (Kim *et al.*, 2007; Tuttle *et al.*, 2007). Worldwide, more than 194 million people are affected and this number is expected to grow further to approximately 333 million by 2025 (Liao *et al.*, 2010; Zhu *et al.*, 2010). High caloric diets and sedentary lifestyles in industrialized societies are fundamental causes of this fast-spread "epidemic". Obesity is a well-recognized risk factor for type 2 diabetes when combined with other known risk factors (Niswender, 2010). It has been an important therapeutic goal to reduce the risk of type 2 diabetes through weight management. Numerous epidemiological studies showed that hyperglycemia and hyperlipidemia are the principal cause of cardiovascular diseases (Cusi, 2010; Niswender, 2010; Yamamoto *et al.*, 2010). Therefore, effective blood glucose and lipid control are the key to preventing or reversing diabetes complications and improving quality of life in diabetic patients (Warren, 2004). Modern drugs, including insulin and other hypoglycemic agents such as biguanides, sulfonylureas etc. control the blood glucose level only when they are regularly administered, but these treatments are tedious and have several disadvantages (Kobayashi *et al.*, 2000; Stades *et al.*, 2004; Chiang *et al.*, 2007).

Ginseng is a well-known medicinal plant widely used in oriental societies as one of the most valuable medicines. The ginseng root has been used as a health product or natural remedy for a long time. To develop a ginseng product with an efficacy for hyperglycemia and hyperlipidemia, ginseng radix was processed with pectinase enzyme to give us a 'GINST'. Here, we investigate the anti-diabetic and anti-hyperlipidemic activities of GINST using high fat diet-fed ICR mice.

www.biomolther.org

Open Access **http://dx.doi.org/10.4062/biomolther.2012.20.2.220**

pISSN: 1976-9148 eISSN: 2005-4483

Received Sep 19, 2011 **Revised** Nov 21, 2011 **Accepted** Dec 12, 2011

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MATERIALS AND METHODS

Chemicals

Pectinase was purchased from the DSM food specialties (ZAE La Baume, Servian, France). Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK, acetyl-CoA carboxylase (ACC), phospho-ACC, glucose transporter 4 (GLUT4) were from Cell Signaling Technology (Beverly, MA, USA), and anti-actin, anti-insulin and anti-goat IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein extraction and western blot detection kits were from Intron Biotechnology Inc (Beverly, MA, USA). Bio-Rad protein assay kit was from Bio-Rad Laboratories (Hercules, CA, USA). Polyvinylidine difluoride membrane was from Millipore (Bedford, MA, USA). All other chemicals were of analytical grade.

Preparation of GINST

GINST was obtained from ILHWA Pharmaceutical Co. LTD. (Guri, Korea). Briefly, the ginseng radix (containing with 30-40% moisture) was extracted with 40-50% ethanol and concentrated with a speed-vac, then incubated with an enzyme solution containing pectinase at 55°C for 24 h.

Analysis of ginsenosides in GINST

An acquity liquid chromatograph (Waters, Milford, MA, USA) equipped with gradient pump, autosampler, and diode array detection was used. An Acquity UPLC BEH C18 reversed-phase column (100×1.0 mm, i.d., 1.7 μm) was used. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B). The gradient elution was used as follow: 0-3 min, 5% B; 10 min, 15% B; 12 min, 30% B; 15 min, 35% B; 20 min 60% B. The column temperature was kept constant at 35°C, and the flow rate was 0.5 ml/min.

Animals

Five-week-old ICR mice were purchased from ORIENT BIO (Seoul, Korea). All animals were acclimatized to the laboratory environment for 1 week before the experiment. Mice were allowed to freely access to drinking water and food under constant room temperature (22 ± 2°C) and humidity (50 ± 10%) conditions with an automatic 12 h light and dark cycle and experimental protocol was approved by the Institutional Animal Ethics Committee of the Kyung Hee University. Mice were randomly divided into five groups as following; group fed a regular diet (RD), group fed a high fat diet (HFD, Research diet, New Brunswick, NJ, USA), treatment groups fed a high fat diet plus GINST at 75 mg/kg (GINST75), 150 mg/kg (GINST150), and 300 mg/kg (GINST300). GINST was orally administered once a day for five weeks and body weight was measured once a week.

Oral glucose tolerance test (OGTT)

At the end of the experiment, the ICR mice were fasted for 12 h prior to the experiment. Glucose (1.5 mg/kg) was orally administered at 0 min, and the blood was withdrawn from the orbital venous plexus at 0, 30, 60 and 90 min after glucose administration. Plasma glucose was determined by the glucose oxidase method.

Determination of serum parameters

At the end of treatment, the mice were fasted for 12 h and anaesthetized by diethyl ether and blood samples were collected by cardiac puncture. Blood samples were centrifuged at 3,000 g for 15 min at 4°C, and plasma glucose, triglyceride (TG), total cholesterol (TC) and non-esterified fatty acid (NEFA) levels were determined using commercial kits (Stanbio Laboratory, Boerne, TX, USA) and automatic analyzer (SMARTLAB, Mannheim, Germany). The plasma insulin concentrations were determined using a mouse insulin enzyme immunoassay kit (Shibayagi, Gunma, Japan).

Histological analysis

For hematoxylin-eosin (HE) staining, the pancreas and epididymal fat were removed and fixed in 10% neutral buffered formalin. The tissues were subsequently embedded in paraffin and sectioned with 5 μm thickness (Leica, Wetzlar, Germany) and stained with hematoxylin-eosin for microscopic assessment (Olympus, Tokyo, Japan). The number of islet cells was measured as previously described (Liu *et al.*, 2009) with some minor modifications and graded as 1, 1-40 cells per section; 2, 41-100 cells per section; 3, 101-201 cells per section; 4, 201-400 cells per section; 5, >400 cells per section. To examine the insulin contents in pancreas, immunohistochemistry technique was used. The sections were deparaffinized in xylene and rehydrated through a graded ethanol series. Antigen retrieval was performed by 0.1% trypsin. To block nonspecific binding of immunoglobulin, the sections were incubated with normal serum blocking solution for 30 min at room temperature. Goat anti-insulin IgG (1:75) was applied overnight at 4°C, and then tissue sections were incubated with donkey anti-goat IgG-HRP (1:200) for 30 min at room temperature. Positive control was visualized DAB peroxide substrate solution for 5-10 min, and tissues were counterstained with hematoxylin.

Western blot analysis

To determine protein expression of AMPK and GLUT4 in skeletal muscle, femoral muscle was removed. Total protein extracts were prepared using a protein extraction kit and insoluble protein was removed by centrifugation at 13,000 g for 20 min. Protein concentrations in cell lysates were measured using a Bio-Rad protein assay kit. For Western blotting, 40 μg of protein was separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membrane was further incubated with the indicated primary antibody, followed by secondary antibody conjugated with horseradish peroxidase. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit and then exposed to X-ray film.

Statistical analysis

Data were presented as mean±SEM. Statistical analysis was performed using one-way ANOVA followed by student-newman keuls test. $p<0.05$ was considered significant.

RESULTS

UPLC analysis

UPLC chromatograms of untreated and pectinase-treated ginseng radix extracts are shown in Fig. 1. The saponin peaks in untreated ginseng radix (peak 3, 4, 5 and 6 represent ginsenoside Rb1, Rc, Rb2 and Rd, respectively) were decreased during the enzyme treatment. After 24 h of pectinase treatment, these four ginsenosides were difficult to identify in the

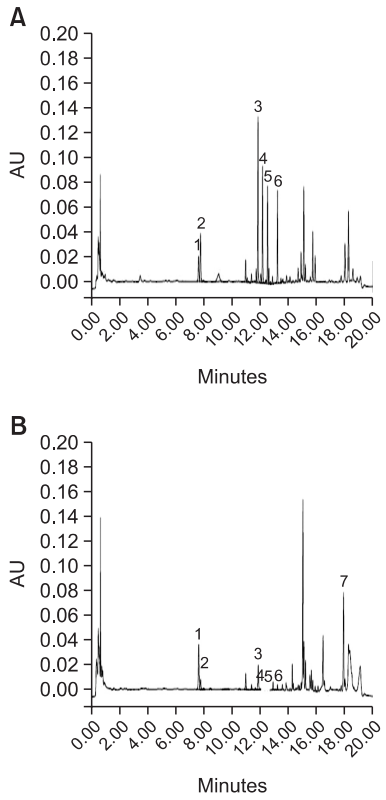

Fig. 1. UPLC profiles of ginseng radix (A) and pectinase-processed ginseng radix (GINST) (B). 1: Rg1, 2: Re, 3: Rb1, 4: Rc, 5: Rb2, 6: Rd, 7: IH-901.

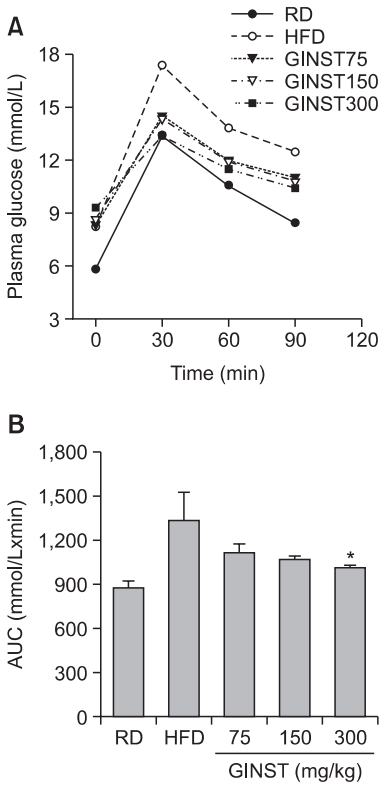

Fig. 2. Plasma glucose responses to an oral glucose challenge (1.5 g/kg) after 12 h food deprivation in ICR mice. Plasma glucose responses to an oral glucose challenge (A) and the area under the curve (AUC) of plasma glucose levels up to 90 min (B). Values are mean ± S.E. (n=5). *p<0.05 compared to HFD control group.

chromatogram (Fig. 1A). On the other hand, IH-901 (peak 7 in Fig. 1B) was appeared during the enzyme process. IH-901 is an intestinal metabolite of protopanaxadiol type ginsenoside and might be one of active components for pharmacological effects of GINST. Several groups have reported anti-cancer, anti-inflammation, and anti-diabetic activities of IH-901 (Lee *et al.*, 2000; Choi *et al.*, 2007; Yoon *et al.*, 2007). Having this chromatogram we attempt to explore whether GINST shows anti-hyperglycemic and anti-hyperlipidemic effects in high fat diet-induced ICR mice.

Effect of GINST on OGTT

One of the major characteristics of insulin resistance is the impaired glucose tolerance. To examine the effect of multiple oral administration of GINST on glucose tolerance, OGTT was carried out at the end of the experiment. As shown in Fig. 2, glucose challenge dramatically increased the blood glucose levels in the HFD group, whereas GINST-treated groups significantly prevented the blood glucose levels from rising during 90 min after glucose challenge (Fig. 2A). When the area under the curve (AUC) was compared between groups, GINST prevented the glucose absorption in a dose-dependent manner (Fig. 2B).

Effects of GINST on body weight and metabolic parameters

Body weight and metabolic parameters related to diabetes and hyperlipidemia are shown in Table 1. Weight gains in RD and HFD control groups during 5-week- period were 4.1±0.8 g and 8.3 ± 1.0 g, respectively. However, GINST-treated groups lowered weight gain when compared to the HFD control group. Compared to the HFD control group, weight gain was lowered by 9.6% in GINST75, 21.7% in GINST150, and 48.2% in GINST300. Fasting plasma glucose levels in GINST-treated groups showed a significant decrease by 19.6% (p<0.05) in GINST300 group, compared to the HFD control group. Plasma insulin levels were also significant decreased by 4.2% in GINST75, 20.0% (p<0.01) in GINST150, and 20.9% (p<0.01) in GINST300 group, compared to the HFD control group. With decreased plasma glucose and insulin levels, the insulin resistance index (HOMA-IR) values (Matthews *et al.*, 1985) for GINST-treated groups were markedly decreased by 8.2% in GINST75, 28.7% (p<0.01) in GINST150, and 36.4% (p<0.01) in GINST300 compared to the HFD control group. The plasma levels of TG, TC and NEFA levels were also decreased by 13.5% (p<0.001), 22.7% (p<0.01) and 24.1% (p<0.001), respectively, in the GINST300 group compared to those in the HFD control group.

Histological analysis

High fat diet produces enlarged adipocytes, but GINST prevented them from formation of large adipocytes dose-dependently (Fig. 3A, B). The pancreas islets of the HFD control group mice were degenerated, whereas mice treated with GINST preserved islet architecture (Fig. 3A, B). Shrunken of

Table 1. Effects of GINST on body weight gain and metabolic parameters

	RD	HFD	GINST (mg/kg)		
			75	150	300
Body weight (g)					
Initial	32.7 ± 1.6	32.7 ± 1.1	32.7 ± 0.7	32.8 ± 0.8	32.7 ± 2.4
Final	36.8 ± 1.5	41.1 ± 0.8$^{###}$	40.2 ± 1.0	39.2 ± 0.9	36.7 ± 2.5***
Weight gain	4.1 ± 0.8	8.3 ± 1.0$^{###}$	7.5 ± 0.9	6.5 ± 0.4**	4.0 ± 0.7***
Epididymal fat (g)	1.0 ± 0.1	2.4 ± 0.1$^{###}$	1.9 ± 0.3	1.8 ± 0.1*	1.5 ± 0.1**
Energy intake (Kcal/day)	13.1 ± 0.2	16.1 ± 0.9$^{###}$	15.7 ± 0.7	14.0 ± 0.6*	13.1 ± 0.4***
Glucose (mM)	6.5 ± 0.4	9.2 ± 1.0$^{##}$	8.8 ± 0.4	8.2 ± 0.4	7.4 ± 0.2*
Insulin (μU/ml)	31.8 ± 1.2	47.9 ± 3.5$^{###}$	45.9 ± 5.1	38.3 ± 5.4**	37.9 ± 4.7**
HOMA-IR	9.1 ± 0.5	19.5 ± 1.9$^{###}$	17.9 ± 0.5	13.9 ± 1.0**	12.4 ± 0.9***
Plasma lipids					
TG (mg/dl)	93 ± 11	114 ± 21$^{###}$	116 ± 14	104 ± 17*	99 ± 11***
TC (mg/dl)	136 ± 13	206 ± 25$^{###}$	187 ± 10	175 ± 18*	159 ± 16**
NEFA (μEq/L)	2,253 ± 9	2,961 ± 350$^{###}$	2,526 ± 274*	2,519 ± 244*	2,248 ± 53***

Data are mean ± standard error (n=5). Homeostasis model assessment (HOMA)-insulin resistance (IR) was used to calculate an index of insulin resistance as insulin (μU/ml)×glucose (mM)/22.5. *p<0.05, **p<0.01, and ***p<0.001 compared to high fat diet control (HFD) group. $^{##}p$<0.01, and $^{###}p$<0.001 compared to regular diet control (RD) group.

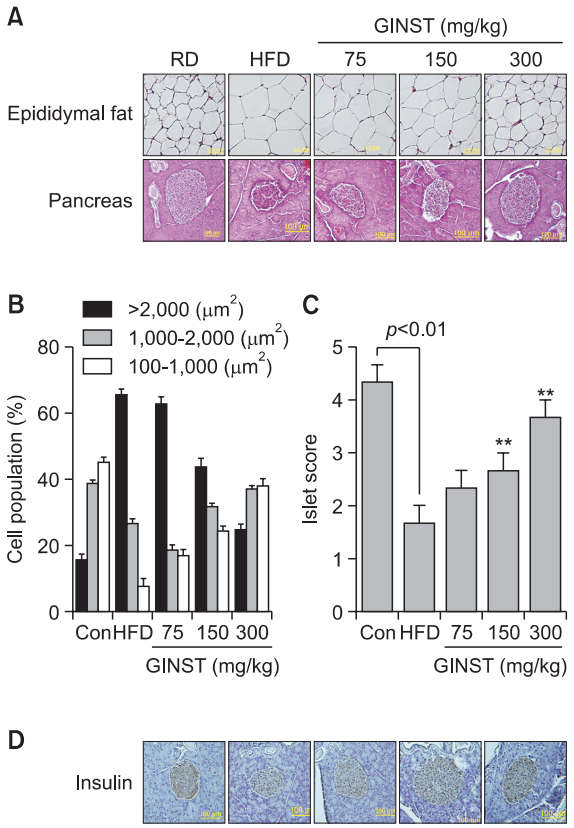

Fig. 3. Effects of GINST on tissue morphology. Magnification 200x. H&E stainings of epididymal fat pad and pancreas (A). Histomorphometric comparisons of epididymal fat cell size (B) and number of islet cells per section (C). Insulin immunostaining counterstained with hematoxylin (D). **p<0.01 vs. HFO.

islet architecture induced by high fat diet was protected by GINST treatment, and this result was confirmed by insulin immunostaining (counterstained with-hematoxylin) (Fig. 3C). Insulin contents in GINST-treated groups were dose-dependently restored when compared to the HFD control group.

Effects of GINST on AMPK activation

Numerous studies demonstrated that skeletal muscle AMPK is implicated in a variety of antidiabetic properties of exercise, including GLUT4 expression, glycogen regulation, fatty acid oxidation, and enhanced insulin sensitivity (Furugen *et al.*, 2011; Zhang *et al.*, 2011). Thus, we examined whether GINST activates AMPK through phophorylation in the femoral muscle. As shown in Fig. 4, GINST stimulates the phosphorylation of AMPK in a dose-dependent manner. ACC and GLUT4 (immediate targets of AMPK) protein expressions were also enhanced in GINST-treated groups.

DISCUSSION

DM is a metabolic disorder characterized by chronic hyperglycemia. The management of diabetes without any side effect is still a challenge to the medical practice. Clinical evidence has suggested that appropriate use of traditional Chinese medicines with modern Western medicinal or mainstream antidiabetic drugs can prevent or ameliorate the development of diabetic complications. Many diabetic patients choose alternative therapeutic approaches such as herbal or traditional Chinese medicine along with the mainstream antidiabetic drugs because of their effectiveness and fewer side effects.

Ginseng has been used as tonic and restorative remedies in traditional Chinese medicine for several thousand years. The pharmacological properties of ginseng are mainly attributed to ginsenosides, which are the active components found in the extracts of different species of ginseng. IH-901, an intestinal metabolite of panaxadiol ginsenosides, is considered as

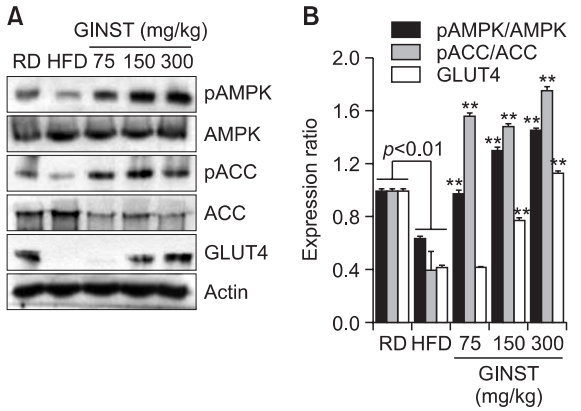

Fig. 4. Effects of GINST on phosphorylated AMPK and GLUT4 protein expression in femoral muscle. ***p*<0.01 vs. HFO.

the main active constituent in GINST (Fig. 1). Recently, IH-901 has received increasing attention because various pharmacologic actions including anti-cancer, anti-inflammation, and anti-diabetes were shown to be mediated by this compound (Lee *et al.*, 2000; Choi *et al.*, 2007; Yoon *et al.*, 2007). Previously, our group reported that IH-901 significantly decreased the fasting blood glucose levels in C57BL/KSJ *db/db* mice through insulin secretion and improved insulin resistance (Han *et al.*, 2007; Yuan *et al.*, 2011). In addition, IH-901 was shown to activate AMPK and affect lipid metabolism in insulin-resistant human HepG2 cells (Kim *et al.*, 2009). Therefore, we expect that GINST having IH-901 as an active constituent shows anti-diabetic activities in HFD fed ICR mice.

Impaired insulin action on whole-body glucose uptake is a hallmark feature of type 2 DM. Defects in insulin signal transduction through the insulin-receptor substrate-1/phosphatidylinositol 3-kinase pathway are associated with reduced insulin-stimulated GLUT4 translocation and glucose transport activity in type 2 diabetic skeletal muscle (Ryder *et al.*, 2001). Therefore, elucidation of the signaling pathways governing contraction-induced increases in skeletal muscle glucose uptake may provide new pharmacological targets for the treatment of individuals with type 2 diabetes. In this study, GLUT4 protein expression was dose-dependently enhanced in GINST-treated groups in the skeletal muscle. AMPK is emerging as a signaling intermediary that controls the use of glucose and fatty acids in skeletal muscle (Egawa *et al.*, 2011). Moreover, in skeletal muscle cells, AMPK may be activated by contraction or 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR), leading to increase in glucose uptake (Musi and Goodyear, 2003). In the present study, GINST induced the phosphorylation of AMPK in the femoral muscle. Being an AMPK activator, GINST dose-dependently decreased plasma glucose and insulin levels when compared to the HFD control group. With decreased plasma glucose and insulin levels, insulin resistance in the GINST-treated group were also significantly decreased compared to the HFD control group. Histological analyses for epididymal fat pad and pancreas were performed. As shown in Fig. 3A, size of adipocytes in the HFD control group was markedly decreased in a dose-dependent manner. This result was, at least in part, due to suppression in calorie intake in GINST-treated groups. In addition, islets number per section of pancreas was analyzed using insulin immunostaining counterstained with hematoxylin. Islets score of the HFD control group was lowered by more than 50% compared to that of normal diet-fed control group, whereas islets of GINST-treated groups was restored in a dose-dependent manner and islets score of 300 mg/kg GINST-treated group was recovered to a level comparable to the one observed in mice fed a normal diet (Fig. 3C). High fat diet caused the islets inflammation (evidently shown macrophages in islets) and GINST protects the pancreas from inflammation and consequently increased the number of islets per section.

Impaired activation of AMPK pathway is a hallmark of obesity associated insulin resistance and contributes to glucose intolerance and hyperglycemia (Steinberg and Jørgensen, 2007). In the present study, compared with the normal control group, the HFD group developed overt glucose intolerance in the OGTT. The AUC in the HFD group was increased by 51.8%, compared to the normal control group. Compared to the HFD group, GINST groups dose-dependently improved the glucose tolerance by 16.4%, 19.7%, and 23.8%. The plasma lipid (TG, total cholesterol, and NEFA) levels of GINST-treated group were all significantly lowered than those in the HFD control group. These results suggest that GINST may have a beneficial effect on the insulin resistance and hyperlipidemia induced by HFD.

In summary, we may conclude that GINST prevents HFD-induced hyperglycemia and hyperlipidemia *via* activating AMPK-GLUT4 signaling pathway in ICR mice and GINST may be a potential therapeutic agent for type 2 diabetes, although additional work is needed to further clarify underlying mechanisms.

ACKNOWLEDGMENTS

This work was supported by a grant from the Kyung Hee University post-doctoral fellowship in 2011 (KHU-20110691).

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