Anti-obesity effects of *Geranium thunbergii* extract via improvement of lipid metabolism in high-fat diet-induced obese mice

YOON-YOUNG SUNG, TAESOOK YOON, WON-KYUNG YANG, SEUNG JU KIM and HO KYOUNG KIM

Center of Herbal Resources Research, Korea Institute of Oriental Medicine,
Yuseong-gu, Daejeon 305-811, Republic of Korea

Abstract. This study investigated the anti-obesity properties of an extract of *Geranium thunbergii* (GTE) in high-fat diet-induced obese mice. GTE treatment significantly reduced body weight, adipose tissue mass, adipocyte size, as well as serum triglyceride, total cholesterol and low-density lipoprotein-cholesterol levels in obese mice compared to high-fat diet-fed mice. It also decreased serum leptin levels and increased adiponectin levels. The serum levels of aspartate transaminase, alanine transaminase, blood urea nitrogen and creatinine were not significantly changed in GTE-treated mice compared to serum levels in normal diet and high-fat diet-fed mice. Furthermore, GTE suppressed the mRNA levels of sterol regulatory element-binding protein 1c, peroxisome proliferator-activated receptor γ, adipocyte fatty acid-binding protein and fatty acid synthase in the adipose tissues of obese mice. These results suggest that GTE ameliorated high-fat diet-induced obesity by altering the adipokine levels and downregulating the expression of transcription factors and lipogenic enzymes involved in lipid metabolism.

Correspondence to: Dr Ho Kyoung Kim, Center of Herbal Resources Research, Korea Institute of Oriental Medicine, 483 Exporo, Yuseong-gu, Daejeon 305-811, Republic of Korea
E-mail: hkkim@kiom.re.kr

Abbreviations: ALT, alanine transaminase; aP2, adipocyte fatty acid-binding protein; AST, aspartate transaminase; C/EBP, CCAAT/enhancer binding protein; FAS, fatty acid synthase; FER, food efficiency ratio; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GTE, Geranium thunbergii extract; HDL, High density lipoprotein; HFD, high-fat diet; LDL, low density lipoprotein; ND, normal diet; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein

Introduction

Obesity accelerates the accumulation of excess fat, which is caused by an imbalance between energy intake and expenditure (1). Obesity is associated with additional metabolic diseases, such as dyslipidemia, type 2 diabetes mellitus, hypertension, stroke and arteriosclerosis (2), and is characterized by increased adipose tissue mass, which results from increased adipose cell size and number (3). Adipocyte size is determined by the amount of lipid that accumulates in adipose tissue, which is regulated by various enzymes such as fatty acid synthase (FAS), adipocyte fatty acid-binding protein (aP2), and lipoprotein lipase (LPL). During adipose tissue development, three major transcription factors, peroxisome proliferator-activated receptor (PPAR) γ, CCAAT/enhancer binding protein (C/EBP) α, and sterol regulatory element-binding protein (SREBP) 1c, regulate the expression of these lipid-metabolizing enzymes (4).

To treat obesity, various pharmacological approaches, including drugs, have been suggested to control lipid uptake or appetite. However, these pharmacological agents have been reported to cause undesirable adverse effects such as constipation, insomnia, vomiting, headache, stomachache and heart failure (5-6). Therefore, there is a demand for therapeutically effective and safe anti-obesity agents. In previous studies, numerous oriental medicinal herbs were reported to be useful for controlling obesity and hyperlipidemia without significant side effects (7-8).

Geranium thunbergii, which belongs to the family of Geraniaceae, is a perennial plant that is found in Korea, China and Japan. The plant has traditionally been used as an anti-diarrhetic agent in East Asia (9). Moreover, it has been reported to exhibit anti-mutagenic, anti-inflammatory, and anti-oxidative effects (10-12). One of the representative tannin in Geraniaceae, geraniin, has been shown to have anti-hypertensive, anti-bacterial and anti-fungal properties (13-14). However, no studies are currently available regarding the anti-obesity effects of *Geranium thunbergii*. In the present study, the effects of a 70% ethanol extract of *Geranium thunbergii* (GTE) on body weight, fat mass and serum lipid levels in high fat-diet-induced obese mice were examined. Furthermore, the mRNA expression levels of lipid accumulation-related genes in adipose tissue were investigated.

Materials and methods

Preparation of GTE. *Geranium thunbergii* was purchased as a dried herb from Omniherb Co. (Yeoungcheon, Korea) and was authenticated based on its microscopic and macroscopic characteristics by the Classification and Identification Committee of the Korea Institute of Oriental Medicine (KIOM). The committee comprised nine experts in the fields of plant taxonomy, botany, pharmacognosy, and herbology. A voucher specimen (no. JA-109) was deposited at the herbarium of the Department of Herbal Resources Research in KIOM. The dried leaves of *Geranium thunbergii* (100 g) were extracted twice with 70% ethanol (with 2 h reflux) and the extract was then concentrated under reduced pressure. The decoction was filtered, lyophilized and serially stored at 4˚C. The yield of the dried extract from starting crude materials was approximately 22.08% (w/w).

Animals and diets. Male 4-week-old C57BL/6J mice were purchased from Daehan Biolink Co. (Eumsung, Korea) and were maintained for 1 week prior to the experiment. The animals were housed in an air-conditioned room with a 12-h light/dark cycle at a temperature of 22±1˚C and humidity of 50±10%. Mice were provided with a laboratory diet and water *ad libitum*. All experimental protocols involving the use of animals were conducted in accordance with the National Institutes of Health (NIH) guidelines and approved by the Committee on Animal Care of our institute. For induction of obesity, the mice were fed a high-fat diet (HFD) (Rodent diet D12492, Research diet, New Brunswick, NJ, USA) consisting of 60% of energy as fat, 20% as protein and 20% as carbohydrates, in accordance with previously published reports (15). Normal mice were fed a commercial standard chow diet (Orient Bio Inc., Seongnam, Korea) consisting of 14% of energy as fat, 21% as protein and 65% as carbohydrates. The mice were randomly divided into three groups (n=6) and respectively fed a normal diet (ND), HFD, and HFD plus GTE (HFD+GTE) for 6.5 weeks. GTE was dissolved in normal saline and was orally administered to the mice at a dose of 400 mg/kg/day for 6.5 weeks. The dose of GTE used in these experiments was determined from preliminary dose-range experiments and from previously published reports (16). By contrast, saline was orally administered to mice in the ND and HFD control groups. Body weight and food intake were measured twice a week.

Biochemical analysis of blood. At the end of the experimental period, the mice were fasted prior to being sacrificed. The mice were anesthetized with ether and then blood samples were obtained from the inferior vena cava of each mouse. Blood samples were centrifuged at 2,500 rpm for 15 min at 4˚C, and serum was obtained and stored at -70˚C prior to analysis. Serum levels of triglycerides, total cholesterol, low density lipoprotein (LDL)-cholesterol, high density lipoprotein (HDL)-cholesterol, aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen and creatinine were analyzed with an automatic analyzer (Hitachi 7080, Hitachi Co., Tokyo, Japan). The concentration of serum leptin and adiponectin was measured with mouse leptin and adiponectin ELISA kits (R&D Systems, Minneapolis, MN, USA), respectively, according to the manufacturer's instructions. The

absorbance was measured using a microplate spectrophotometer (BioRad, Hercules, CA, USA).

Adipose tissue weight and histologic analysis. Following blood collection, the white adipose tissues (subcutaneous, epididymal and retroperitoneal) were removed from mice and weighed immediately. For adipocyte staining, adipose tissues were fixed in 10% neutral formalin solution for 1 day and embedded in paraffin. The tissues were cut at a thickness of 6 μm and stained with hematoxylin and eosin. To quantitate adipocyte size, the stained sections were analyzed using light microscopy (Olympus BX51, Olympus Optical Co., Tokyo, Japan) and an image analysis program (Image pro plus 5.0, Media Cybernetics, Silver Spring, MD, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Tissue was homogenized, and total RNA was isolated with the easy-BLUE total RNA extraction kit (Intron, Seoul, Korea) according to the manufacturer's instructions. For cDNA synthesis, 1 μg of total RNA was mixed with premix containing oligo (dT) primer and DEPC-treated water at a final volume of 20 μl and incubated at 45˚C for 60 min. The reaction was stopped by heat inactivation at 95˚C for 5 min. cDNAs were then amplified with gene-specific primers using the Taq PCR master mix (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The 20 μl amplification mixture contained 1 μl of cDNA, 10 μl of 2X Taq PCR master mix containing 1.5 mM $MgCl_2$, 0.1 μM of each primer, and water. After a 15 min preincubation at 94˚C, PCR amplification was performed for 35 cycles with the following cycling conditions: 30 sec of denaturation at 94˚C, 30 sec of annealing at 60˚C and 1 min of extension at 72˚C. Primers were designed using Primer3 software, and the sequences are available in the GenBank database. Table I shows the sequences of the gene-specific PCR primers. The relative expression levels of target genes were normalized using GAPDH as an internal control.

Statistical analysis. Differences between groups were examined using analysis of variance (ANOVA) followed by Duncan's multiple range test. Data are presented as the means ± SE. Significant differences were accepted when p<0.05.

Results

Body weight and food intake. The average body weight, body weight gain and food intake of the mice in the three treatment groups are shown in Fig. 1A and Table II. The final body weight of the HFD group was significantly higher than that of the ND group. However, HFD+GTE mice had a 13% lower body weight than HFD-fed mice. Body weight gain was markedly lower in the GTE-treated group compared to the HFD group. Additionally, GTE did not affect food intake. The food intake was not decreased in the HFD+GTE group compared to the HFD group. These results indicate that the reduction of body weight gain by the treatment of GTE did not depend upon a decreased food intake, since no difference was observed between control and experimental groups in food intake.

Weights and histology of adipose tissue. To investigate whether GTE decreases adiposity, mice were sacrificed and

Table I. Primer sequences used in the RT-PCR analysis.

Genes	Forward	Reverse	Accession number	Length (bp)
PPARγ	CCCTGGCAAAGCATTTGTAT	GAAACTGGCACCCTTGAAAA	NM_011146	225
SREBP1c	AGCTCAAAGACCTGGTGGTG	TCATGCCCTCCATAGACACA	NM_011480	216
FAS	CGCACCGGCTACCAAGCCAA	GCTTCCCGGGTTGCCCTGTC	NM_007988	266
aP2	TGGAAGCTTGTCTCCAGTGA	GCTCTTCACCTTCCTGTCGT	NM_024406	225

Table II. Effects of *Geranium thunbergii* extract on body weight gain and food intake in high-fat diet-induced obese mice.

	ND	HFD	HFD+GTE
Initial body weight (g)	21.06±0.30	21.03±0.50	21.21±0.33
Final body weight (g)	24.90±0.30	30.85±0.95[b]	26.89±0.75[c]
Body weight gain[a] (g)	3.84±0.23	9.81±0.59[b]	5.69±0.80[c]
Food intake (g/day)	4.65±0.29	2.50±0.07[b]	2.41±0.08

[a]Body weight gain = final body weight - initial body weight. Values are expressed as the means ± SE (n=6). Significant differences between the ND and HFD groups: [b]$p<0.001$. Significant differences between the HFD and HFD+GTE groups: [c]$p<0.01$.
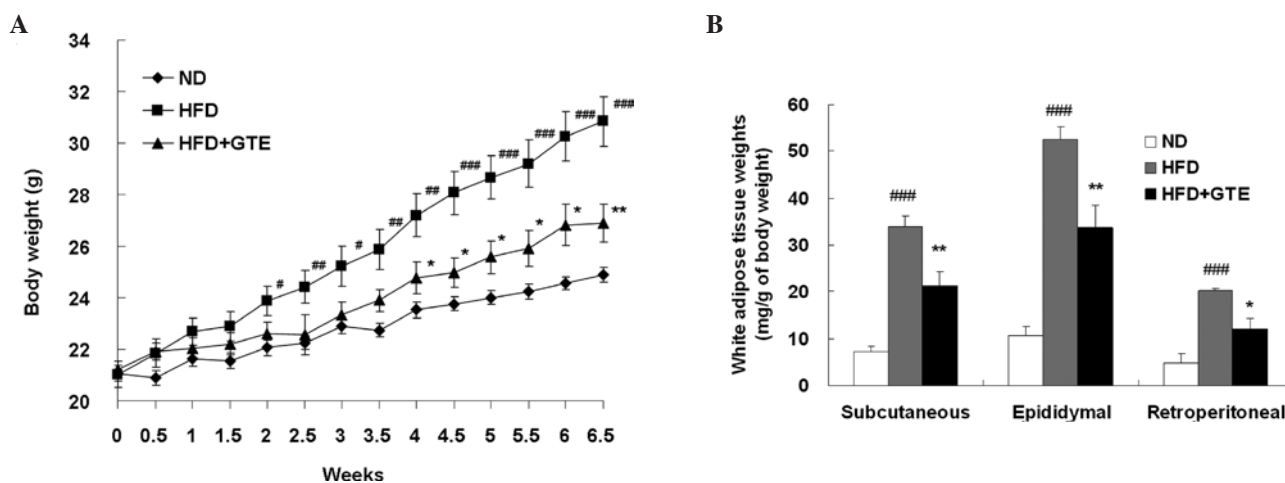

Figure 1. Effect of *Geranium thunbergii* extract on body weight and adipose tissue weight in obese mice fed a high-fat diet. GTE was administered orally to the mice at a dose of 400 mg/kg/day for 6.5 weeks. (A) Body weight and (B) white adipose tissue weight in high-fat diet-induced obese mice. ND, normal diet; HFD, high-fat diet; HFD+GTE, high fat diet + *Geranium thunbergii* extract. Values are expressed as the means ± SE (n=6). Significant differences between the ND and HFD groups: [#]$p<0.05$; [##]$p<0.01$; and [###]$p<0.001$. Significant differences between the HFD and HFD+GTE groups: [*]$p<0.05$ and [**]$p<0.01$.

adipose tissues were removed and weighed. The weight of various white adipose tissues, including subcutaneous, epididymal and retroperitoneal, were increased in the HFD-fed mice compared to the ND-fed mice, and the weight of white adipose tissues was significantly decreased by the administration of GTE (Fig. 1B). Adipose tissues were further examined histologically (Fig. 2A). Adipocytes in subcutaneous, epididymal and retroperitoneal adipose tissues was notably higher in the HFD than in the ND group. Compared to adipocytes in the HFD-fed mice, GTE treatment significantly decreased adipocyte size in obese mice (Fig. 2B).

Serum lipid parameters and leptin and adiponectin levels. GTE treatment in HFD-fed mice significantly inhibited

the HFD-induced increases in triglyceride, total cholesterol and LDL-cholesterol levels (Table III). However, HDL-cholesterol levels were not different between the HFD and HFD+GTE groups. HFD-fed mice had markedly higher serum leptin levels compared to the ND-fed mice. However, the GTE-treated group had decreased serum leptin levels compared to the HFD group (Fig. 3A). Conversely, GTE treatment significantly increased adiponectin levels in HFD-fed mice (Fig. 3B).

Assessment of potential toxicological effects. To evaluate potential toxic effects of ingesting GTE, serum toxicological markers, which indicate liver and kidney injury, were measured at the end of the experimental period. The levels of
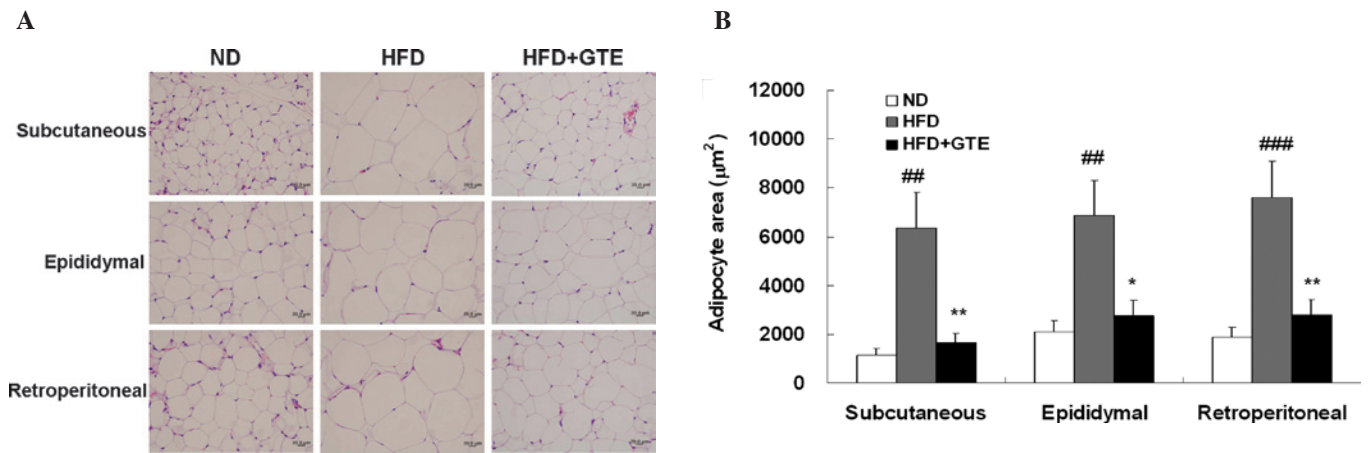
Figure 2. Histology of adipose tissues in high-fat diet-induced obese mice. (A) Representative images and (B) adipocyte area of hematoxylin and eosin-stained sections (magnification, x400). ND, normal diet; HFD, high-fat diet; HFD+GTE, high-fat diet + *Geranium thunbergii* extract. A total of 20 cells were measured for each mouse. Values are shown as the means ± SE (n=20). Significant differences between the ND and HFD groups: [##]p<0.01 and [###]p<0.001. Significant differences between the HFD and HFD+GTE groups: [*]p<0.05 and [**]p<0.01.

Table III. Effects of *Geranium thunbergii* extract on biochemical parameters in serum of high-fat diet-induced obese mice.

	ND	HFD	HFD+GTE
Triglyceride (mg/dl)	78.0±11.1	88.7±14.4	41.2±6.5[c]
Total cholesterol (mg/dl)	106.7±1.8	164.8±2.4[b]	140.0±7.0[c]
LDL-cholesterol (mg/dl)	4.8±0.1	6.2±0.2[a]	3.8±1.1[d]
HDL-cholesterol (mg/dl)	75.1±3.2	72.6±3.8	75.5±1.7
AST (U/l)	66.5±12.7	86.7±19.6	72.4±17.5
ALT (U/l)	36.5±2.5	24.7±0.8	23.0±2.2
Blood urea nitrogen (mg/dl)	32.5±0.9	22.0±1.1	25.4±2.0
Creatinine (mg/dl)	0.45±0.02	0.41±0.02	0.37±0.04

Values are expressed as the means ± SE (n=6). Significant differences between the ND and HFD groups: [a]p<0.01 and [b]p<0.001. Significant differences between the HFD and HFD+GTE groups: [c]p<0.05 and [d]p<0.01.
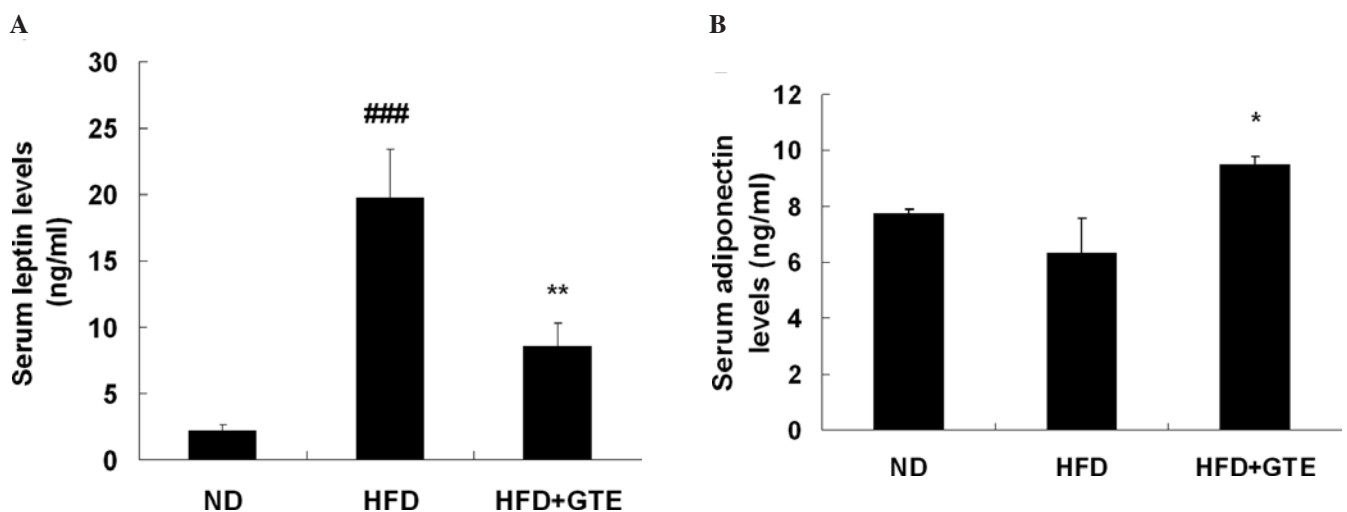

Figure 3. Effect of *Geranium thunbergii* extract on (A) leptin and (B) adiponectin serum levels in high-fat diet-induced obese mice. ND, normal diet; HFD, high-fat diet; HFD+GTE, high-fat diet + *Geranium thunbergii* extract. Values are expressed as the means ± SE (n=6). Significant differences between the ND and HFD groups: [###]p<0.001. Significant differences between the HFD and HFD+GTE groups: [*]p <0.05 and [**]p<0.01.
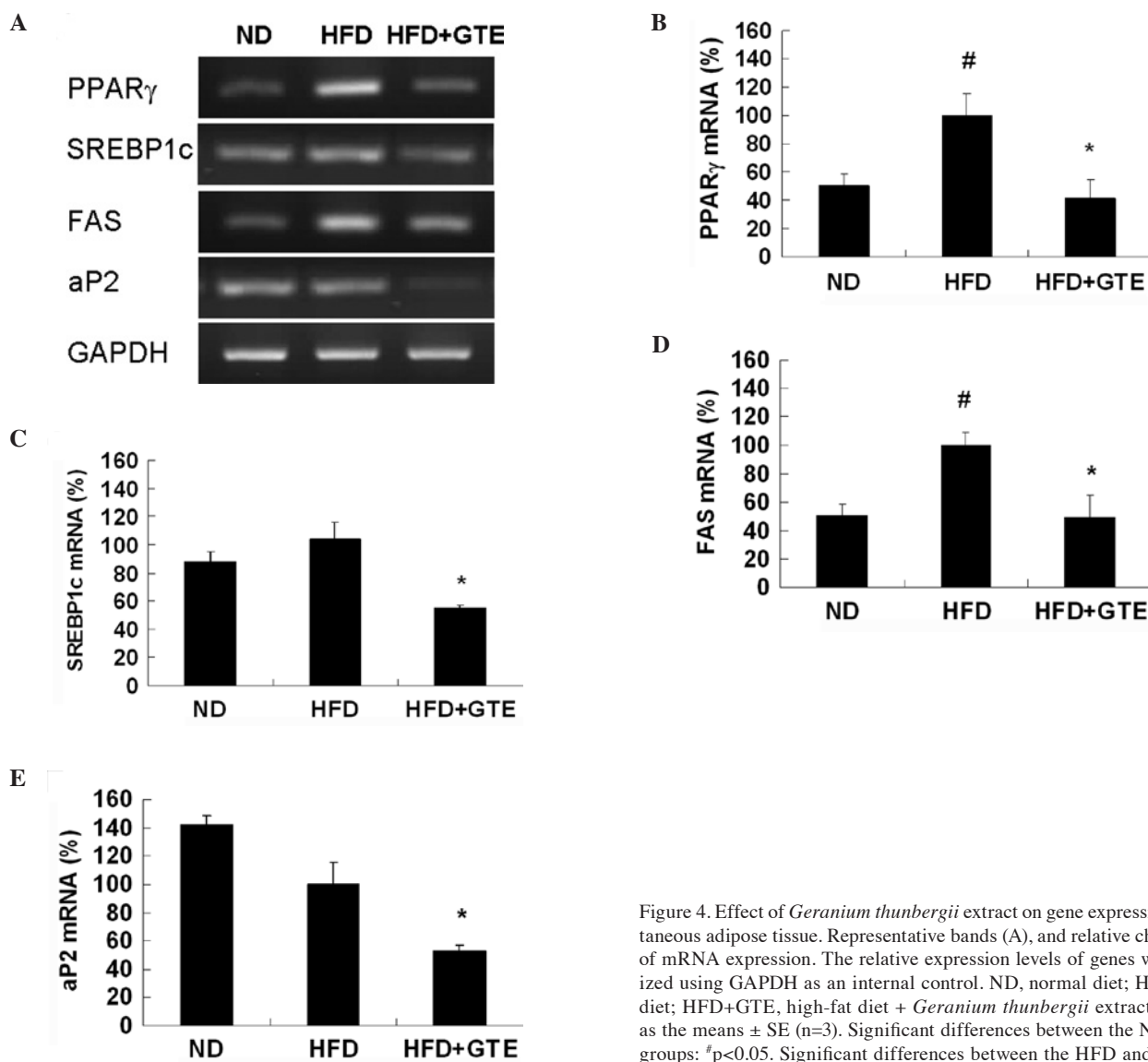
Figure 4. Effect of *Geranium thunbergii* extract on gene expression in subcutaneous adipose tissue. Representative bands (A), and relative changes (B-E) of mRNA expression. The relative expression levels of genes were normalized using GAPDH as an internal control. ND, normal diet; HFD, high-fat diet; HFD+GTE, high-fat diet + *Geranium thunbergii* extract. Values are as the means ± SE (n=3). Significant differences between the ND and HFD groups: [#]p<0.05. Significant differences between the HFD and HFD+GTE groups: [*]p<0.05.

AST, ALT, blood nitrogen urea and creatinine were not significantly changed in GTE-treated mice compared to HFD-fed mice (Table III). Serum levels were in the normal range in all groups according to previously published reports (17-18). Additionally, the GTE-treated mice did not induce significant changes in the weight of liver and spleen (data not shown). These data indicate that administration of 400 mg/kg/day of GTE for 6.5 weeks induced no detectable adverse toxic effects in the mice.

Expression of genes involved in lipid metabolism. To understand the mechanism involved in the effects of GTE on lipid metabolism, the expression of lipogenesis-related genes in adipose tissue was investigated (Fig. 4A). Compared to the normal mice, the HFD-induced obese mice had increased mRNA levels of adipocyte markers such as SREBP1c, PPARγ and FAS in the subcutaneous adipose tissue. By contrast, GTE treatment significantly decreased the mRNA expression of SREBP1c, PPARγ, FAS and aP2 compared to their levels in the HFD group (Fig. 4B-E).

Discussion

Obesity is characterized by increased fat mass due to an increase in the size and number of adipocytes that differentiated from preadipocytes (19). In obese conditions, adipocytes accumulate large amounts of lipids and become enlarged. In the present study, GTE treatment inhibited adipose tissue accumulation and adipocyte size as well as decreased body weight gain in HFD-induced obese mice. GTE also reduced triglyceride droplets in differentiated 3T3-L1 cells (data not shown). These results show that GTE may lower adipocyte size by reducing lipid accumulation in 3T3-L1 cells, resulting in decreased adipose tissue mass. PPARs are well-characterized transcription factors that are members of the nuclear hormone receptor superfamily (20). There are three subtypes of PPARs, i.e., α, β, γ, which have distinct tissue distributions. Specifically, PPARγ, which is predominantly expressed in adipose tissue, plays a significant role in the regulation of genes involved in adipocyte differentiation, lipid storage and glucose homeostasis (21). PPARγ is known to regulate the transcription of LPL, aP2 and phosphoenolpyruvate carboxykinase in adipose

tissue (22-23). aP2 is a member of a family of intracellular fatty acid-binding proteins that bind long chain fatty acids with high affinity (24). FAS is a key lipogenic enzyme that catalyzes the biosynthesis of long chain fatty acids from acetyl-CoA precursors (25). Expression of FAS is activated through binding to its promoter region by SREBP1 (26). It has also been reported that SREBP1c activates PPARγ through the production of PPARγ ligands (27). In the present study, GTE treatment in HFD-fed mice markedly decreased the mRNA expression of PPARγ, SREBP1c, aP2, and FAS. These results suggest that GTE downregulates the expression of the transcription factor SREBP1c, leading to a reduced expression of PPARγ, aP2 and FAS to inhibit lipogenesis in adipose tissue.

GTE treatment in the HFD-fed mice also reduced serum triglyceride, total cholesterol, and LDL-cholesterol levels and leptin concentrations. Adipocytes secrete a variety of proteins known as adipokines. The secreted proteins include tumor necrosis factor-α, interleukin-6, resistin, leptin and adiponectin (28). Leptin plays a crucial role in regulating body weight by controlling the size of adipose tissue. It was reported that plasma leptin concentrations and transcription are directly associated with adiposity and body weight changes in humans and rodents (29). The decreased mass of adipose tissue in GTE-treated mice could be attributed to reduced leptin levels. GTE treatment also increased serum adiponectin levels. Adiponectin is known to contribute to insulin sensitivity and fatty acid oxidation (30). Circulating concentrations of adiponectin are inversely correlated with body mass (31). Therefore, the increase in adiponectin levels following GTE treatment may have ameliorated insulin resistance in obese mice, resulting in decreased serum lipid levels and weight loss.

Chemically, *Geranium thunbergii* was found to contain tannins and flavonoids, such as geraniin, corilagin, ellagic acid, gallic acid, quercetin, kaempferol, and kaempferol-7-rhamnoside (32). Quercetin has been found to improve dyslipidemia and hyperinsulinemia in obese Zuker rats (33). In mice fed with a Western diet, chronic dietary intake of quercetin reduced visceral and liver fat accumulation and improved hyperglycemia, hyperinsulinemia, and dyslipidemia (34). However, further study is needed to clarify the pharmacological mechanisms of GTE and to identify the active substances responsible for its anti-obesity properties.

In conclusion, the administration of GTE significantly reduced body weight gain and adipose tissue accumulation and improved serum total cholesterol, triglyceride, and LDL-cholesterol levels in HFD-induced obese mice. The anti-obesity effects of GTE were mediated by altering serum adipokine levels and downregulating the expression of SREBP1c, PPARγ, aP2 and FAS in adipose tissue. These results suggest that *Geranium thunbergii* is useful for treating metabolic diseases such as obesity and hyperlipidemia.

Acknowledgements

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