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RESEARCH ARTICLE

Ethanol Extracts of Unroasted *Coffea canephora* Robusta Beans Suppress Adipogenesis in Preadipocytes and Fat Accumulation in Rats Fed a High-fat Diet

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Abstract Anti-adipogenic and anti-obesity effects of a green coffee been extract (GCE) from unroasted *Coffea canephora* Robusta beans were investigated *in vitro* and *in vivo*. Insulin-induced adipogenesis in 3T3-L1 preadipocytes was inhibited by 25.6 and 87.9% following treatment with 100 and 500 μg/mL GCE, respectively. Expression of adipogenesis-specific genes was down-regulated. Body weight gains of C57BL/6 mice fed a high-fat diet (HFD) were suppressed in a concentration-dependent manner using GCE (0.3 or 1% in the diet), exhibiting a marked lower gain in 1% GCE-fed mice than in normal diet-fed animals. Decreases in body fat weights and the size of adipocytes were confirmed. GCE supplementation reduced concentrations of blood lipids and leptin, and attenuated hepatic steatosis. GCE inhibits adipogenesis by modulating cell signaling and improves dietary obesity by reducing lipid accumulation and the size of adipocytes.

Keywords: green coffee bean extract, adiopogenesis, obesity, hyperlipidemia

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Introduction

Overweight and obesity are emerging as a major cause of the metabolic syndrome, which is increasing rapidly in modern societies (1). Obesity, characterized as an excessive accumulation of fat in subcutaneous tissues and the abdominal cavity, is influential for cerebrovascular and cardiovascular disorders (2,3). In addition, high energy intake induces radical reactions in different tissues causing tissue injury and organ dysfunction (4,5). Abdominal obesity correlates with the occurrence of diabetes mellitus, increasing the morbidity risk by 40× and 60.9× in individuals with a body mass index (BMI) of 35 and >35 kg/m^2, respectively, and worsening diabetes in 80% of obese diabetics (6).

In spite of the many risk factors of obesity, including genetic background, endocrine dysfunction, and inappropriate dietary habituation and stress, excessive net intake of calories (energy) is the major cause of obesity (7). The feeding pattern affects the metabolic rate of lipids. For example, *ad libitum* and meal-based feedings lead to different accumulation rates of triglycerides (TG) in adipocytes through alteration of adipogenic and adipolytic processes (8). Since TG synthesis occurs in mature adipocytes, the differentiation and maturation of preadipocytes to adipocytes may be the rate-limiting factor for adipogenesis and fat accumulation. During lipid metabolism, very low-density lipoproteins (VLDL) containing a high concentration of TG are hydrolyzed by lipoprotein lipase (LPL) to intermediate-density lipoproteins (IDL) and low-density lipoproteins (LDL), which are removed by hepatic LDL receptors (9). Insulin resistance accompanying obesity inhibits LPL, causing abnormal lipid metabolism in type II diabetes (10). Therefore, preadipocytes have been

used for screening of candidate compounds based on anti-adipogenic activities during insulin-induced maturation of cells (11).

Although diverse factors induce obesity, a high-fat diet (HFD) is one of the most important causes (11). Because a HFD increases the amount of adipose tissue in animals and humans, long term procedures for HFD-feeding have been used for studies of weight control and mechanisms of obesity as a dietary obesity model (11-13).

Coffee is a favorite beverage worldwide. As a stimulant and diuretic, caffeine was found to facilitate lipolysis in human and animal adipocytes (14) and to decrease body fat amounts in HFD-fed rats (15). In comparison, polyphenols, including the chlorogenic acid (CA) that is abundant in coffee beans, have attracted attention for anti-obesity and hypotriglyceridemic properties (16). However, long-term ingestion of a high amount of roasted coffee beverages is required for reduction of blood concentrations and tissue accumulation of triglycerides, and for improvements in obesity (17). Notably, green (unroasted) coffee beans contain high concentrations of CA and related compounds that decrease body weight and blood and tissue lipid levels (18,19). Since the active ingredients CA and CA derivatives are degraded during roasting (20), extracts of green coffee beans should be superior to roasted extracts for improvement of lipidemia and obesity. In the present study, the anti-obesity activity of an ethanol extract of green coffee beans in a dietary obesity model was confirmed, and the action mechanisms *in vitro* cultured preadipocytes were suggested. In addition, a species difference in the anti-obesity activity between *Coffea canephora* and *C. arabica* extracts was demonstrated.

Materials and Methods

Test materials Green coffee bean extract (GCE) was prepared at Misuba RTech Co. (Asan, Korea). GCE was extracted from unroasted *C. canephora* Robusta beans at 30°C for 1 h using 70% ethanol in an ultrasound extraction device (Daihan Scientific, Chuncheon, Korea), then filtered through a filter paper (Whatman No. 1; GE Healthcare Life Sciences, Buckinghamshire, UK), vacuum-concentrated with a rotary evaporator (N-1000; Eyela, Tokyo, Japan), and dried with a freeze dryer (Lyop-Pride; Ilshinbiobase, Dongducheon, Korea). Extracts were pooled from 3 different extractions, leading to a yield of 18-22%. *C. arabica* beans were subjected to extraction following the same procedures to produce extracts (GCE-A).

Based on HPLC analysis (ACME 9000; Younglin, Anyang, Korea), the contents of CA and caffeine in GCE were higher than in GCE-A (28.0 and 17.6% for CA and 9.3 and 5.2% for caffeine, respectively). In addition, the total contents of caffeic acid, CA, and the derivative quinic acids were higher in GCE (82.3%) than in GCE-A (60.2%).

Cell culture and cytotoxicity 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were seeded in wells of a 96-well plate (1×10^6 cells/mL), treated with GCE (20-500 μg/mL), and incubated ad 37°C for 24 h (11,21). Cytotoxicity was assessed using a MTT assay (22).

Differentiation of preadipocytes and adipogenesis 3T3-L1 cells were treated using GCE (20-500 μg/mL). Differentiation was stimulated using a hormone sensitizer composed of insulin (5 μg/mL), dexamethasone (0.25 μM), and isobutyl methylxanthine (IBMX 0.5 mM) for 2 days, followed by insulin alone for an additional 4 days to stimulate maturation (11,21). Cells were fixed in a phosphate buffer containing 7% formaldehyde for 1 h, and stained using 99% isopropanol containing 1% Oil red O for 10 min. The amount of synthesized fat was quantified using an adipogenesis kit (#10006908; Cayman, Ann Arbor, MI, USA) at 490 nm after extraction of the Oil red O from cells using isopropanol (11,21).

Gene expression and protein production 3T3-L1 cells in wells of a 6-well plate (1×10^6 cells/mL) were treated with GCE (100-500 μg/mL) and insulin for differentiation and maturation, and then mRNA expression of adipogenesis-related genes, such as Acrp30 (adiponectin) and peroxisome proliferator-activated receptor-γ (PPAR-γ), was analyzed using reverse transcriptase-polymerase chain reaction (RT-PCR) system (Rotor-Gene 6000; Corbett Research, Sydney, Australia) with optimal primers (Bioneer, Daejeon, Korea) (11,21).

Animals and treatment ICR mice (6-week-old, male, mean body weight of 30 g) and C57BL/6 mice (mean body weight of 22 g) were procured from Daehan Biolink (Eumseong, Korea). Animals were housed in an environmentally controlled room with a temperature of 23±2°C, a relative humidity of 55±5%, and an alternating 12 h light (300 lx)/dark cycle, with pellet feed and purified water available *ad libitum*.

GCE and GCE-A were dissolved in purified water and administered orally to ICR mice at a dose of 330 mg/kg in a preliminary study. The animals were administered GCE or GCE-A once a day between 17:00-18:00, given normal pellet feed, and body weights were recorded using a digital balance (Cubis; Sartorius, Goettingen, Germany) every day for 25 days.

GCE was used for further study based on body weight gains and epididymal fat weights for examination of *in vitro* and *in vivo* anti-adipogenic and anti-obesity activities. GCE was mixed into a powdered HFD (D12451; Research Diets Inc., New Brunswick, NJ, USA) containing 20.69% lard. Casein matching the amount of added GCE (0.3 or 1%) was removed from the diet by ordering from the manufacturer. Control animals received a basal diet of D12450B for 10 weeks *ad libitum*. Study protocols met the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Laboratory Animal Research Center at Chungbuk National University, Korea (CBNUR-284-11).

Food intake and body and fat weights The daily food intake of mice was recorded once a week for 10 weeks. Food consumption was calculated by subtracting the amount of feed remaining from the amount provided every 24 h. Body weights of animals were recorded every day, and epididymal, perirenal, and mesenteric adipose tissues were weighed after sacrifice under deep anesthesia with diethyl ether following the 10-week treatment period.

Size of adipocytes After sacrifice, fat tissues were fixed in 4% formaldehyde and paraffin-embedded sections were stained using hematoxylin-eosin. The mean size of 10 adipocytes was calculated using a digital image analyzer (Image Inside; Focus, Seoul, Korea) under light microscopy (200× magnification) (11).

Hepatic lipid accumulation Liver tissue sections were stained using hematoxylin-eosin and examined for lipid accumulation under a light microscope (DM2700 M; Leica, Wetzlar, Germany).

Serum biochemical analyses Serum was analyzed for TG, total cholesterol (TC), LDL, and high-density lipoproteins (HDL) levels using a blood chemistry analyzer (Hitachi Medical, Tokyo, Japan). The serum leptin concentration was measured using a radio-immunoassay (RIA) system (Dream Gamma-10; Shinjin Medics, Seoul, Korea) with an animal leptin RIA kit (Linco Research, New York, NY, USA) (10,23,24).

Statistical analysis Results were presented as mean± standard error and the significance of differences was analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test at a level of $p<0.05$. All analysis was performed using SPSS statistical software version 13.0 for Windows (SPSS, Chicago, IL, USA).

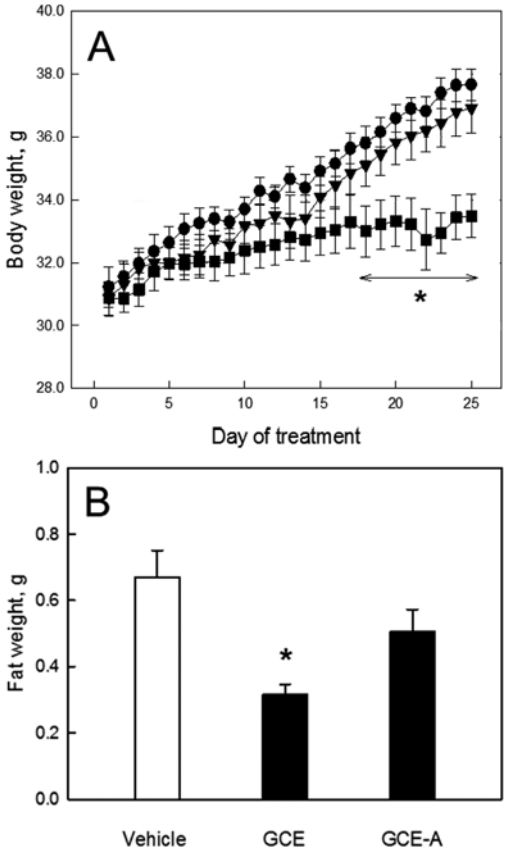

Fig. 1. Effects of green coffee bean extracts from *Coffea canephora* Robusta (GCE) and from *C. arabica* (GCE-A) on the body weight gain (A) and epididymal fat weights (B) of ICR mice. ●, vehicle; ■, 330 mg/kg GCE; ▼, 330 mg/kg GCE-A. *Significantly different from vehicle ($p<0.05$)

Results and Discussion

Effects of GCE and GCE-A on body and fat weights In ICR mice, GCE significantly ($p<0.05$) suppressed the body weight gain, whereas GCE-A did not show a significant effect (Fig. 1A). In parallel with the decrease in body weight gain, epididymal fat weights were significantly ($p<0.05$) reduced by GCE (Fig. 1B) compared with the vehicle control. GCE was superior to GCE-A for reduction of the amount of body fat. Therefore, GCE was used for further study to examine *in vitro* anti-adipogenic and *in vivo* anti-obesity activities.

Obesity is a major risk factor for the degenerative disorders diabetes mellitus and hypertension, and cardiovascular and inflammatory diseases (25). The distribution of body fat affects disease morbidity differently (26). Abdominal adipose tissue decreases insulin tolerance and enhances the incidence of atherosclerosis and cardiovascular disease

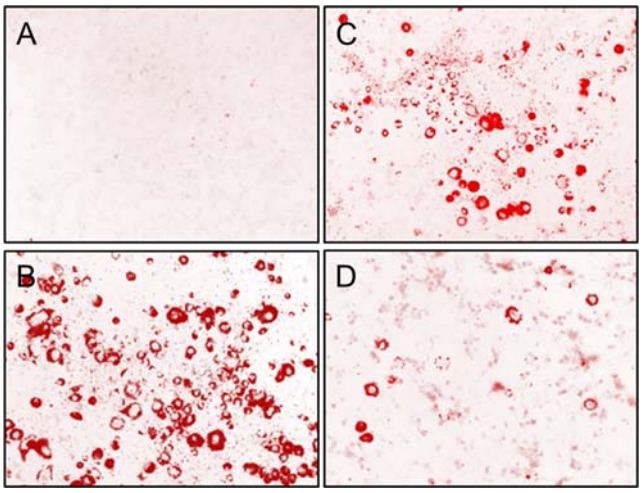

Fig. 2. Representative adipogenesis in 3T3-L1 preadipocytes exposed to insulin and GCE. 3T3-L1 cells were treated with GCE (100 or 500 μg/mL) and a hormone sensitizer (insulin, 5 μg/mL), and stained with Oil red O. A, control; B, insulin alone; C, insulin+100 μg/mL GCE; D, insulin+500 μg/mL GCE

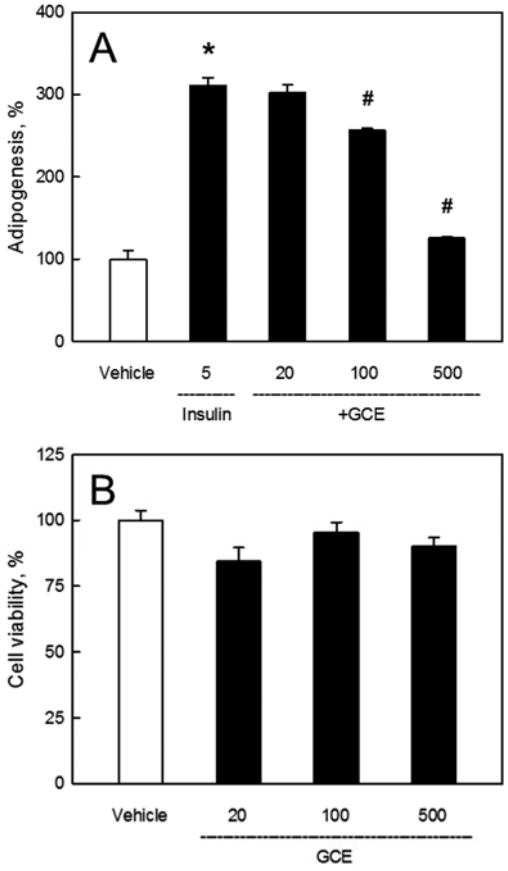

Fig. 3. Cytotoxicity and anti-adipogenic activities of GCE in 3T3-L1 cells. 3T3-L1 cells were treated with GCE (20, 100, or 500 μg/mL) and/or insulin (5 μg/mL). Cytotoxicity was assessed based on the MTT assay (A). *Significantly different from vehicle ($p<0.05$); #significantly different from insulin alone ($p<0.05$)

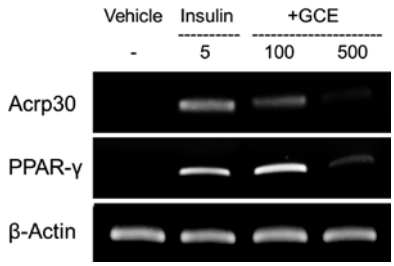

Fig. 4. Effects of GCE on insulin-induced mRNA expression of Acrp30 and PPAR-γ. 3T3-L1 cells were treated with GCE (100 or 500 μg/mL) and insulin (5 μg/mL), and mRNAs were analyzed using RT-PCR with optimal primers.

(27,28). GCE decreased amounts of epididymal, perirenal, and mesenteric adipose tissues, indicating that GCE has a beneficial effect on the amount of abdominal fats that can induce the metabolic syndrome (27).

The fat-lowering activity of GCE-A was much weaker than the effect of GCE. The contents of CA and caffeic acid, and the derivatives (82.3%) in GCE were higher than in GCE-A (60.2%), suggestive of a superior efficacy for GCE since caffeic acid and caffeine are also good lipolytic ingredients (14,15,18). However, controversial results exist for the body weight-lowering effects of CA and caffeine. Both were ineffective in C57BL/6 and ddy mice and in Wistar rats (29-31), in contrast to a high efficacy in ICR mice (18). Therefore, the overall anti-obesity effects of GCE from *C. canephora* Robusta should be due to a mixed function of CA, caffeic acid, and the derivatives including quinic acids with diverse chemical structures. No significant animal model differences were found in the present and previous studies for the extracts including CA, caffeine, and the derivatives (18,29).

Anti-adipogenic effects of GCE Treatment of 3T3-L1 preadipocytes with the hormone sensitizer insulin increased TG synthesis in cells (Fig. 2). This hormone-induced adipogenesis was significantly ($p<0.05$) inhibited by treatment with GCE in a concentration-dependent manner, compared with the control treated with insulin lone (Fig. 2, 3A). TG synthesis was suppressed to a normal level by 500 μg/mL of GCE, a concentration without cytotoxicity in an MTT assay (Fig. 3B).

Expression of mRNA from adipogenesis-specific Acrp30 and PPAR-γ genes in RT-PCR analysis was markedly increased by the insulin inducer (Fig. 4). GCE at 100 μg/mL remarkably suppressed Acro30 expression, but was ineffective on PPAR-γ. However, a higher concentration (500 μg/mL) of GCE almost fully inhibited expressions of both Acrp30 and PPAR-γ mRNA.

Since body fats are synthesized in mature adipocytes through processes of differentiation and maturation of preadipocytes, many investigations have focused on adipogenic signal transduction for development of anti-obesity materials

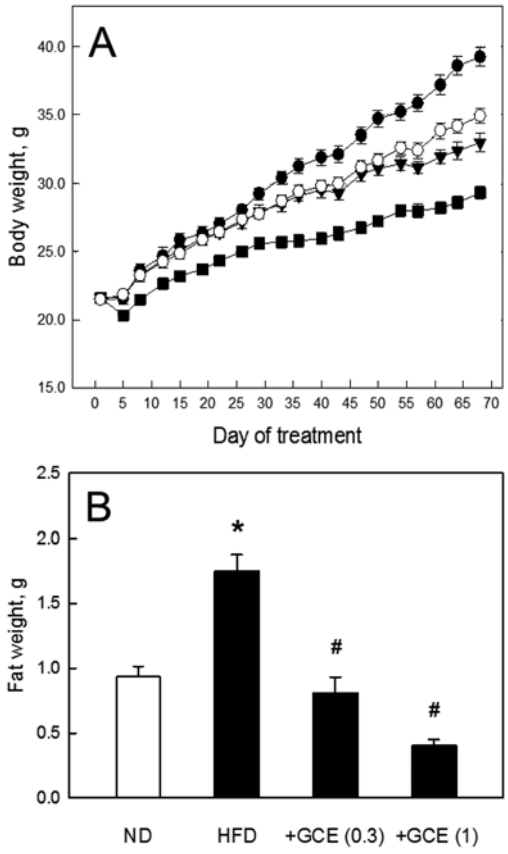

Fig. 5. Effects of GCE on the body weight gain (A) and epididymal fat weights (B) of C57BL/6 mice fed a high-fat diet (HFD). ○, normal diet (ND); ●, HFD alone; ▼, HFD+0.3% GCE; ■, HFD+1.0% GCE. *Significantly different from ND (p<0.05); #significantly different from HFD alone (p<0.05)

(11). Preadipocytes differentiate and mature in response to hormones, including insulin, via expression of the adipogenic gene PPAR-γ, CCAAT/enhancer-binding proteins (C/EBP), and sterol regulatory element-binding proteins (SREBP), to produce TG (32,33). In this study, 3T3-L1 preadipocytes stimulated using insulin (5 μg/mL), dexamethasone (0.25 μM), and IBMX (0.5 mM) produced more TG (200% increase) than non-stimulated cells. GCE (100-500 μg/mL) suppressed maturation and adipogenesis of 3T3-L1 cells at concentrations without cytotoxicity. Analyses of transcriptional factors have demonstrated that the anti-adipogenic effect of GCE is due to inhibition of Acrp30 and PPAR-γ gene expression (23,24). A previous study demonstrated that silk and silkworm pupa peptides inhibited expressions of Acrp30, PPAR-γ, and leptin following TG accumulation (11).

Anti-obesity effects of GCE HD12451-HFD-fed C57BL/6 mice displayed body weight gains higher than for animals fed a D12450B normal diet (Fig. 5A). However, GCE (0.3%) supplementation attenuated the body weight

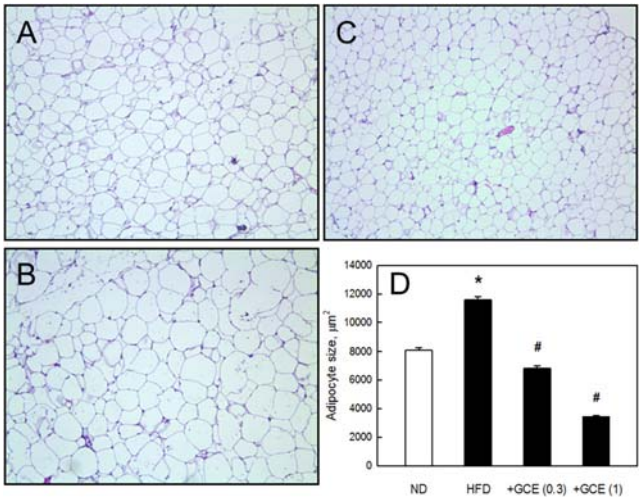

Fig. 6. Effects of GCE on the size of epididymal adipocytes of C57BL/6 mice fed a high-fat diet (HFD). A, normal diet (ND); B, HFD alone; C, HFD+1% GCE. *Significantly different from ND (p<0.05); #significantly different from HFD alone (p<0.05)

increase, leading to a level similar to normal diet-fed mice. Notably, 1% GCE exhibited a higher body weight-suppressive efficacy, resulting in a 25.6% decrease, compared with HFD-fed mice after a 10-week feeding period.

The amount of epididymal adipose tissue was increased 2x in HFD-fed mice, compared with the normal diet-fed group (Fig. 5B). This HFD-induced increase in fat tissue was fully suppressed by supplementation of the diet with 0.3% GCE. A higher concentration (1%) of GCE further decreased the fat weight to values lower than for normal diet-fed animals. Similar fat-lowering effects of GCE were achieved for perirenal and mesenteric adipose tissues (data not shown).

The size of epididymal adipocytes increased by 50% in HFD-fed mice, compared with cells in normal diet-fed animals (Fig. 6). An increase in the cell size was inhibited by supplementation with both 0.3 and 1% GCE, resulting in suppression to a level lower than for normal diet-fed animals at a 1% supplementation level. These powerful reducing effects for adipocyte size were also attained in perirenal and mesenteric adipose tissues (data not shown). The livers of HFD-fed mice contained many lipid droplets (Fig. 7). However, steatosis was attenuated by supplementation with GCE at both the 0.3 and 1% levels. GCE fully resolved the fatty liver.

Concentrations of TC, LDL, and HDL in the blood of HFD-fed mice were significantly (p<0.05) higher than in normal diet-fed animals (Table 1). GCE supplementation reduced blood lipid levels, including TG, TC, and HDL, in a concentration-dependent manner. HFD also greatly increased the blood leptin level, which was fully reversed by GCE treatments at 0.3-1% levels.

Reduction of fat weights was due to reduced amounts of

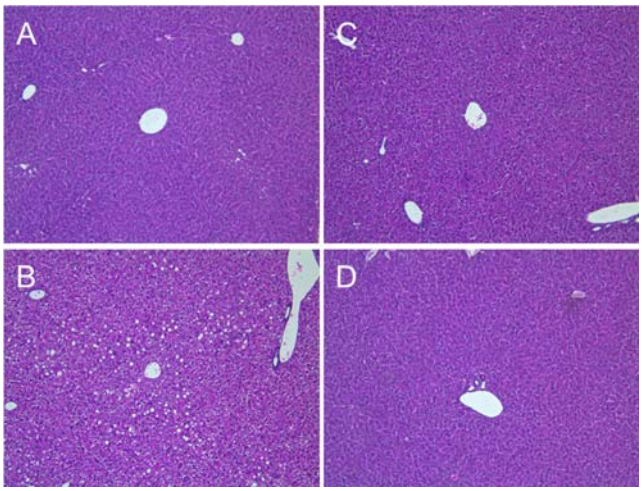

Fig. 7. Representative microscopic images showing lipid accumulation in the livers of C57BL/6 mice fed a high-fat diet (HFD) containing GCE. A, normal diet; B, HFD alone; C, HFD+0.3% GCE; D, HFD+1% GCE

fat tissue and decreases in the size of adipocytes, confirmed based on image analysis of adipocytes. The adipocyte size that was increased by the HFD was drastically reduced to a size smaller than for normal diet-fed animals with GCE supplementation (1%), indicative of the suppressive effects of GCE on maturation and activation of adipocytes for TG synthesis *in vitro*.

Long term use of the HFD increases blood lipid levels and the increase reflects changes in levels of the regulatory hormone leptin (11). In addition, the HFD caused hepatic steatosis that was confirmed by microscopic examination. GCE reversed increases in blood parameters of lipid metabolism and related hormones, and lipid deposition in the liver. These beneficial effects of coffee extracts and ingredients on blood and tissue lipids have been previously reported (18,19,29,30). The lipid and/or glucose-controlling effects of CA and caffeic acid have been attributed to down-regulation of blood leptin activity and insulin secretion (18). However, in this study, the additional molecular signaling factors Acrp30 and PPAR-γ were shown to also be involved in anti-adipogenic effects, although the PPAR-γ effect of GCE was less than the effect on Acrp30.

Supplementation with green *C. canephora* Robusta bean extracts can attenuate body weight gain in HFD-fed animals by decreasing accumulation of body fat and the size of adipocytes. GCE can be used for treatment of dietary obesity.

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Disclosure The authors declare no conflict of interest.

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Table 1. Effects of GCE on the blood concentrations of lipids, glucose, and leptin of mice fed a high fat diet after treatment for 10 weeks

Treatment[1)] (%)	TG (mg/dL)	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	Glucose (mg/dL)	Leptin (ng/mL)
ND	89.6±8.74	134.6±6.37	15.7±1.98	112.1±5.29	276.8±22.37	12.1±4.21
HFD alone	90.9±9.22	160.9±5.88*[2)]	25.3±2.94*	131.8±4.44*	239.8±27.15	77.0±20.92*
+GCE (0.3)	75.1±7.33	148.5±5.47	21.2±1.65	123.0±4.58	248.4±12.11	11.9±3.97[#]
+GCE (1)	63.6±7.47[#]	137.2±5.78[#]	19.7±1.30	114.8±3.51[#]	216.3±3.79	4.0±2.62[#]

[1)]GCE, green coffee bean extract; ND, normal diet; HFD, high-fat diet; TG, triglycerides; TC, total cholesterols; LDL, low-density lipoproteins; HDL, high-density lipoproteins
[2)]*Significantly different from normal diet, $p<0.05$; [#]significantly different from HFD alone, $p<0.05$

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