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Green coffee bean extract improves obesity by decreasing body fat in high-fat diet-induced obese mice

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ABSTRACT

Objectives: To evaluate possible lipid catabolism and body fat regulation effects of 3-caffeoylquinic acid in Green coffee bean extract (GCBE) in high-fat diet (HFD)-induced obese mice.

Methods: Obesity was induced in mice using a HFD for four weeks. Then, mice were fed only HFD or HFD with GCBE at 50, 100, and 200 mg/kg. Fatty acid synthesis mechanism regulation of body fat was investigated through real-time PCR and Western blot assay. Body fat reduction was measured through dual-energy X-ray absorptiometry.

Results: In HFD-induced obese mice, GCBE treatment significantly decreased body weight gain, liver weight and white adipose tissue weights with regulation of adipose tissue lipolysis hormones, like adiponectin and leptin. GCBE treatment decreased mRNA expression levels of adipogenesis and adipocyte metabolism related genes in adipose tissues and the liver, and decreased the corresponding protein expression. Dual energy X-ray absorptiometry measurements were used to compare body fat between mice on high-fat and those treated with GCBE. GCBE treated mice had a lower fat mass compared to HFD alone fed mice and relative body weight and fat mass were markedly decreased.

Conclusions: GCBE has a potential anti-obesity effect with lowering body fat accumulation by regulating adipogenesis and lipid metabolism-related genes and proteins in WAT and liver.

1. Introduction

In recent decades, obesity has become a serious clinical disease that is contributed by a high-fat diet. The World Health Organization defines obesity was abnormal or excessive fat accumulation is present in many diseases. Another definition of obesity is the accumulation of body fat from the imbalance between calorie input and energy expenditure. Incremental evidence suggests obesity is related to epidemiological diseases including diabetes, heart disease, stroke, arthritis, inflammation, and cancers [1]. Therefore, the role of fat in obesity development is important to study to prevent and treat obesity. Adipocytes store energy in triglyceride form and break down lipids into free fatty acids when energy is required [2]. Furthermore, adipocytes play a major role in obesity and related disease through the secretion of wide range of regulatory factors. Remarkably, adipocytes hormonally control metabolism through the secretion of autocrine, paracrine, and endocrine hormones and effect insulin sensitivity, immune function, eating behavior, and most importantly regulate differentiation of preadipocytes into adipocytes [3].

Polyphenols are abundant secondary metabolites in plants and are known to prevent diseases associated with oxidative stress and its related complications. The glycosylated derivate forms of polyphenol, chlorogenic acids (CGA) (ester of caffeic

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acid and quinic acid) are the main polyphenol in coffee [4]. There is an increase in scientific evidence that coffee affects metabolic syndromes such as obesity, type 2 diabetes, atherosclerosis, and insulin-resistance [5–9].

Green coffee is raw coffee beans that have not been roasted. Many different pharmacological studies about green coffee bean extract (GCBE) demonstrates that the CGA in green coffee regulates hypertensive, vasoreactivity, and glucose metabolism [10–12]. There are several prospective studies regarding how 5-caffeoylquinic acid (5-CQA), the major chlorogenic acid in coffee, decreases diabetes risk by decreasing glucose uptake in the small intestine. However, only the short-term effects were analyzed and more research is needed [7,13]. In particular, recent studies propose that attenuation of obesity and lipid accumulation by green coffee bean extract is derived from 5-CQA in diet-induced obesity and insulin resistance [14,15].

In the present study, we investigate a quantitative analysis of the 3-caffeoylquinic acid (3-CQA) in GCBE and examined whether it has an ameliorative effect against high-fat diet (HFD, 60% calories from fat) induced obesity in mice. Furthermore, decreases in lipid accumulation and metabolism related genes, proteins, and body fat composition provide scientific evidence to support GCBE as a supplement to prevent obesity.

2. Materials and methods

2.1. Chemicals and reagents

Isopropanol, TRI reagent and protease inhibitor cocktail were purchased from Sigma–Aldrich (MO, USA). HPLC grade acetonitrile and phosphoric acid were purchased from Merck (Darmstadt, Germany). Polyclonal antibodies against FAS, SREBP-1c, PPARγ, C/EBPα, AMPK, phospho-AMPK, PPARα and β-actin were purchased from Santa Cruz Biotechnology (CA, USA). Horseradish peroxidase-linked anti rabbit IgG and HRP-linked anti mouse IgG were purchased from Bio-Rad (CA, USA). SYBR Green reaction buffer was purchased from Takara (Shiga, Japan).

2.2. Compound analysis of GCBE

GCBE was provided from KPLC group (Montagne, France). Chromatographic analysis of 3-CQA in GCBE was performed using the Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a UV detector. The C18 column (5 μm, 4.6 × 250 mm; Supelco, MO, USA) was maintained at 40 °C for chromatographic separation. The mobile phase was mixture of 0.5% phosphoric acid in distilled water (A) and 0.5% phosphoric acid in acetonitrile (B) and delivered at 1.2 mL/min in a gradient flow as follows: 0 min 8.0%, followed by 20 min 25.0%, 35 min 100.0%, and 45 min 8%. The injected volume was 5.0 μL and optimal detection was achieved at 325 nm. The standard samples for 3-CQA was purchased from Sigma Aldrich (MO, USA) and freshly prepared for analysis.

2.3. Animal studies

Male C57BL/6J mice were obtained from DaeHan BioLink (Chungbuk, South Korea) at four weeks of age. The mice were individually housed in stainless steel cages and were maintained under temperature of (23 ± 3) °C in a humidity-controlled room with a 12–12 h light–dark cycle. All mice were given free access to water and food. After acclimatization for one week, they were fed either the normal-fat diet (NFD, $n = 8$, certified irradiated global 18% protein diet, 2918C, Harlan Laboratories, Indiana, USA) or High-fat diet (HFD, $n = 40$, Rodent diet with 60% Kcal from Fat, #101556; Research Diets, USA) for four weeks to induce obesity. After obesity induction, the mice were divided into five experimental groups ($n = 8$/group) and were matched by body weight.

The following five groups were studied for six weeks: normal-fat diet, HFD, and HFD with oral administration of GCBE at 50 mg/kg of body weight (HFD + GCBE 50); HFD with GCBE at 100 mg/kg of body weight (HFD + GCBE 100); HFD with GCBE at 200 mg/kg of body weight (HFD + GCBE 200). Food intake of the mice was recorded daily and their body weights were measured twice per week. At the end of the experiment, the mice were anesthetized and the liver, kidney, and white adipose tissue (WAT) were excised immediately. Each tissue was then rinsed with phosphate-buffered saline, and stored at −80 °C until analysis. The experimental procedures were approved by Ethics Committee of the Wonkwang University (Iksan, Korea) and the mice were maintained in accordance with their guidelines.

2.4. Plasma biochemical analysis

After six weeks of feeding, 12 h fasted mice were anesthetized using ether and blood was collected. Collected samples were centrifuged at 2 500×*g* for 15 min at 4 °C for biochemical analyses of plasma parameters. The separated plasma was stored at −80 °C until analysis. The levels of serum glucose, total cholesterol (T-CHO), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), Alanine transaminase (ALT), Aspartate transaminase (AST), free fatty acid (FFA), leptin, and adiponectin were measured using commercial kits (Sigma–Aldrich, MO, USA) according to the manufacturer's instructions.

2.5. Histological analysis

Liver and adipose tissue were dissected, fixed in 10% neutral buffered formalin, and embedded in paraffin for histological examination. The formalin-fixed and paraffin-embedded tissue blocks were cut to a thickness of 4 μm and stained with hematoxylin and eosin (H&E). The sections were photographed under 200× magnification.

2.6. Protein extraction and Western blot analysis

Cold phosphate-buffered saline washed adipose and liver tissues were homogenized in RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSE) and 1% protease inhibitor cocktail. The homogenates were centrifuged 8 000×*g* for 15 min at 4 °C and the supernatants were collected. Total protein concentration was calculated by BCA protein assay (Pierce, IL, USA). Proteins were separated on 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% non-fat skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and were incubated overnight at 4 °C with

appropriate antibodies. Following a 16 h incubation at 4 °C, the membranes were washed with TBS-T and then incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies for 1 h. After washing, the immunocomplexes were visualized by chemiluminescence reagents and detected by chemi-luminometer (CLINX Science Instruments Co. Ltd., Shanghai, China).

2.7. Isolation of total RNA and quantitative real-time PCR

Total RNA of adipose and liver tissues was isolated using the RNeasy mini kit (Qiagen, Velno, Netherlands) according to the manufacturer's protocol. The extracted RNA was reverse transcribed to cDNA by using Primescript first strand cDNA synthesis kit (Promega, Fitchburg, USA). Then the RNA expression levels of adipogenesis, beta-oxidation, and lipolysis related genes were analyzed by a real-time PCR LightCycler 96 system (Roche, Basel, Switzerland) using a SYBR Green Master PCR Kit (Takara, Shiga, Japan) according to the manufacturer's protocols. Primer sequences were as follows (forward and reverse, respectively): β-actin, TGT CCA CCT TCC AGC AGA TGT and AGC TCA GTA ACA GTC CGC CTA GA; Peroxisome proliferator-activated receptor α (PPARα), CAG CGA GTA GCG CAT AGT CA and GGA TGT CAC ACA CAA TTC; PPARγ; TCA CAA GAG GTG ACC CAA TG and CCA TCC TTC ACA AGC ATG AA; Adiponectin, GCA CTG GCA AGT TCT ACT GCA A and GTA GGT GAA GAG AAC GGC CTT GT; Adipose triglyceride lipase (ATGL), AAC ACC AGC ATC CAG TTC AA and GGT TCA GTA GGC CAT TCC TC; Hormone-sensitive lipase (HSL), ACC GAG ACA GGC CTC AGT GTG and GAA TCG GCC ACC GGT AAA GAG; Sterol regulatory element binding protein-1c (SREBP-1c), GGC ACT AAG TGC CCT CAA CCT and GCCACA TAG ATC TCT GCC AGT GT; SREBP-2, GGC ACT AAG TGC CCT CAA CCT and CAC CAT TTA CCA GCC ACA GG; Carnitine palmitoyl transferase-1 (CPT-1), AAA GAT CAA TVG GAC CCT AGA and CAG CGA GTA GCC CAT AGT CA; and CCAAT/enhancer-binding protein α (C/EBPα), GTG TGC ACG TCT ATG CTA AAC and GCC GTT AGT GAA GAG TCT CAG. The real-time PCR cycling conditions were as follows: 2 min at 95 °C; 45 cycles of 20 s at 95 °C, 20 s at 60 °C, 40 s at 72 °C, and 30 s at 72 °C; and a final extension for 5 min at 72 °C followed by a melting curve analysis. The mRNA levels of the target genes were normalized to the expression level of β-actin and the results were presented as the fold changes relative to the HFD group.

2.8. Body fat composition analysis

Dual energy X-ray Absorptiometry (DXA) measurements were used to compare body fat between C57BL6J mice on HFD and treated with GCBE. DXA measurements were performed after six weeks of administration using a total-body scanner (InAlyzer dual X-ray absorptiometry, Medikors, Gyeonggi, Korea). DXA measures one time with low energy and one time with high energy to separate the images into bones and tissues in gram units by separating them into fat and lean before analysis. Radiography of body fat was displayed by three modes according to low density fat (blue color), medium density fat (yellow color) and high density fat (red color).

2.9. Statistical analysis

Statistical evaluations of the data were expressed as the mean ± SEM. The statistical significance of the differences between the mean values for the treatment groups was analyzed by Student's *t*-tests and one-way analysis of variance (ANOVA) using the software, Origin 7 (Microcal Software, USA). Values of $P < 0.05$ and $P < 0.01$ were considered statistically significant.

3. Results

3.1. CQA composition in GCBE

To determine the composition of 3-CQA in GCBE, we compared the chromatographic profiles of a standard on HPLC analysis (Figure 1). Total CQA content in the GCBE was 50% and the 3-CQA composition in the GCBE was approximately 27.5% and was confirmed by comparing the chromatogram of the standard. The optimized green coffee bean was used in all subsequent experiments.

3.2. Effects of GCBE on food intake and body weight gain

We elucidated the effects of GCBE supplementation on HFD induced obese mice. Food intake and body weight gain were compared with those of mice fed normal diet, HFD, and HFD with GCBE (Figure 2). The initial body weights were not different among the groups. The body weight gain in HFD groups was higher than in the normal diet group. Whereas, the body weight of HFD with GCBE at 100 and 200 mg/kg were observed to significantly decrease body weight gain compared to the HFD group ($P < 0.05$ and $P < 0.01$, respectively).

3.3. Effects of GCBE on organ and white adipose tissue weights

The effects of GCBE on the relative weight of liver, perirenal, retroperitoneal, and epididymal WAT were compared to
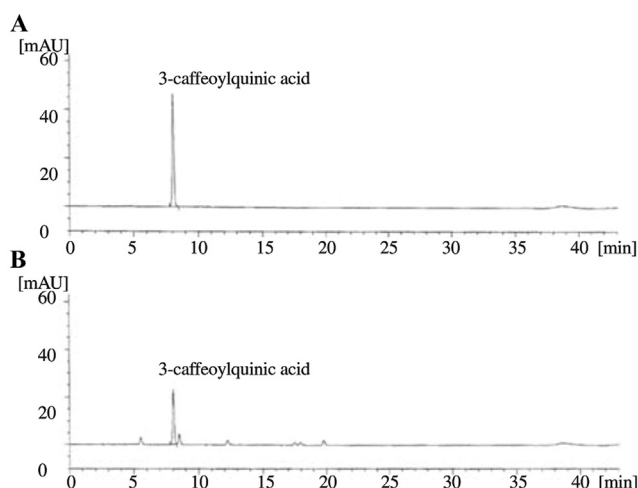

Figure 1. Representative HPLC chromatograms of 3-CQA (A) in GCBE (B) measured at a wavelength of 325 nm.
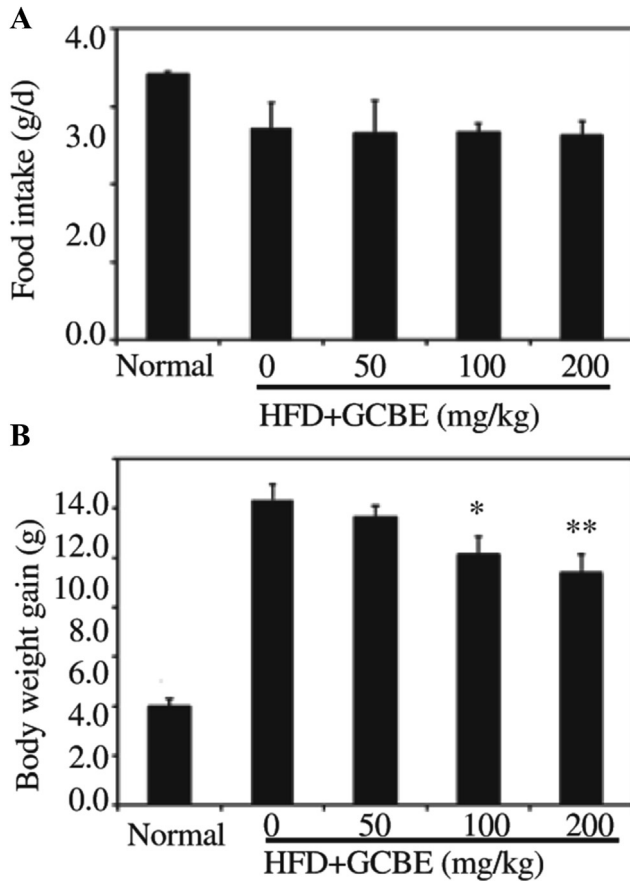
Figure 2. Effects of GCBE on body weight gain and food intake rate in HFD-induced obese mice for six weeks. (A) Food intake rate. (B) Body weight gain.
*$P < 0.05$ and **$P < 0.01$ compared to the HFD group ($n = 8$ per group).

Figure 3. Effects of GCBE on organ weight in mice fed a HFD for six weeks.
*$P < 0.05$ and **$P < 0.01$ compared to the HFD group ($n = 8$ per group).

the HFD group (Figure 3). The organ weights from mice in the HFD group were higher than in the normal diet group. However, the HFD with GCBE group showed a decreased liver, perirenal, retroperitoneal, epididymal, and total WAT weights. However, the relative organ weights in the HFD + GCBE 200 group exhibited a significant reduction compared to the HFD group ($P < 0.05$).

3.4. Effects of GCBE on plasma biochemical parameters

Table 1 shows the effects of GCBE on the plasma biochemical parameters. In the plasma biochemical analysis glucose, T-CHO, TG, LDL-C, HDL-C, ALT, AST, and leptin levels in the normal diet group were significantly lower than in the HFD group (all, $P < 0.01$). Analysis of lipid levels in the HFD + GCBE 200 group had significantly reduced plasma T-CHO, TG, and LDL-C levels (all, $P < 0.01$), whereas HDL-C levels in HFD with GCBE groups increased when compared to the HFD group. ALT, AST, and FFA were significantly reduced in the HFD with GCBE 100 and 200 groups when compared to the normal diet group (all, $P < 0.01$). The concentration of leptin in the HFD with GCBE 100 and 200 groups displayed a statistically significant reduction compared to the HFD group ($P < 0.05$ and $P < 0.01$, respectively). However, the adiponectin levels in mice fed GCBE showed significant increases ($P < 0.05$).
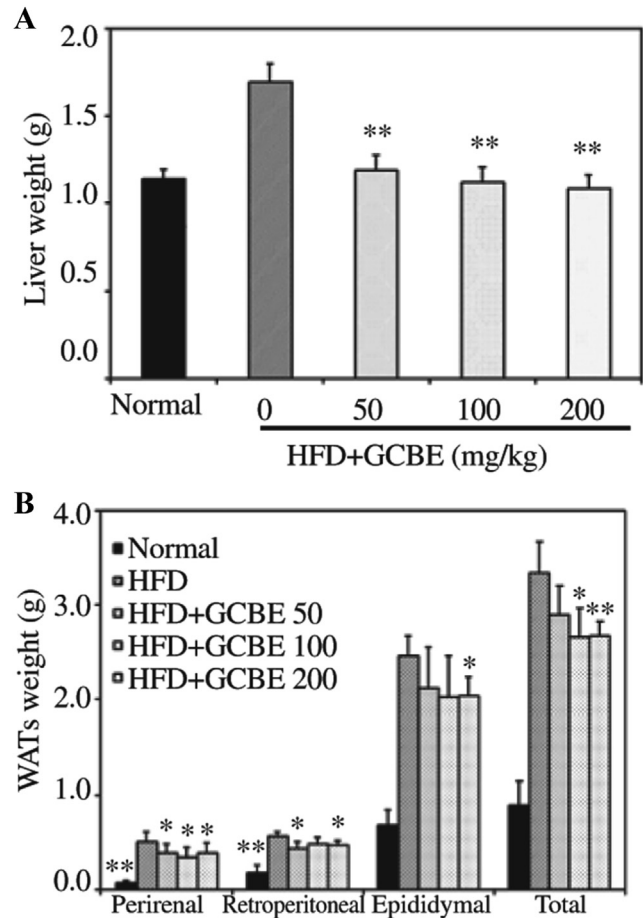
3.5. Effects of GCBE on liver and epididymal adipose tissue morphology

Figure 4 shows microphotographic observations of hepatic tissues and epididymal adipose tissue sections by hematoxin and eosin (H&E) staining. Experimental mice in the figure are as follows: Normal diet, HFD, HFD with 50 mg/kg, HFD with 100 mg/kg, and HFD with 200 mg/kg groups. In the case of the liver, large macrovascular adipocyte were found in the HFD group when compared to the normal diet group. However, the HFD with GCBE 50, 100, and 200 mg/kg groups showed markedly reduced hepatic steatosis compared to the HFD induced group (Figure 4A).

Lipid accumulation and cellular morphology in epididymal WAT was examined by H&E staining. The adipocyte size in the HFD group showed an increase compared to the normal diet group. In the HFD with GCBE groups, we observed a marked reduction of adipocyte hypertrophy compared to mice fed the HFD alone (Figure 4B).

3.6. Effects of GCBE on mRNA expression of genes in adipose tissues

We evaluated the effects of GCBE on the expression of adipogenesis and adipocyte metabolism related genes by

Table 1
Effects of GCBE on plasma biochemical parameters in HFD-induced obese mice.

Parameters	Normal	HFD	HFD + GCBE 50	HFD + GCBE 100	HFD + GCBE 200
Glucose (mg/dL)	121.00 ± 3.17[**]	181.25 ± 7.93	166.75 ± 7.70	142.30 ± 4.35[**]	169.00 ± 8.02
T-CHO (mg/dL)	77.87 ± 4.03[**]	179.75 ± 11.40	152.87 ± 8.40	145.75 ± 5.85[*]	141.00 ± 5.44[**]
TG (mg/dL)	83.62 ± 6.09[**]	103.12 ± 3.38	77.62 ± 4.09[**]	82.20 ± 3.35[*]	79.50 ± 2.80[**]
LDL-c (mg/dL)	3.87 ± 0.44[**]	36.90 ± 4.60	23.12 ± 3.28[*]	18.12 ± 2.68[**]	17.75 ± 2.66[**]
HDL-c (mg/dL)	84.37 ± 3.93[**]	140.50 ± 8.59	157.87 ± 6.31	145.87 ± 3.99	143.12 ± 3.77
ALT (U/L)	40.62 ± 1.99[**]	151.0 ± 13.34	62.42 ± 8.04[**]	65.33 ± 7.79[**]	55.28 ± 8.46[**]
AST (U/L)	155.25 ± 29.07[**]	252.60 ± 17.10	202.37 ± 11.00[*]	196.37 ± 13.96[**]	186.50 ± 10.37[**]
FFA (mEq/L)	1.42 ± 0.03	1.42 ± 0.03	1.10 ± 0.04[**]	0.98 ± 0.03[**]	1.04 ± 0.03[**]
Leptin (mg/mL)	1.60 ± 0.27[**]	53.94 ± 4.36	45.18 ± 6.04	42.77 ± 2.36[*]	29.40 ± 5.39[**]
Adiponectin (mg/mL)	12.07 ± 0.41[**]	9.46 ± 0.30	10.53 ± 0.51	11.30 ± 0.28[*]	11.89 ± 1.01[*]

[*]$P < 0.05$ and [**]$P < 0.01$, compared to the HFD group ($n = 8$ per group).
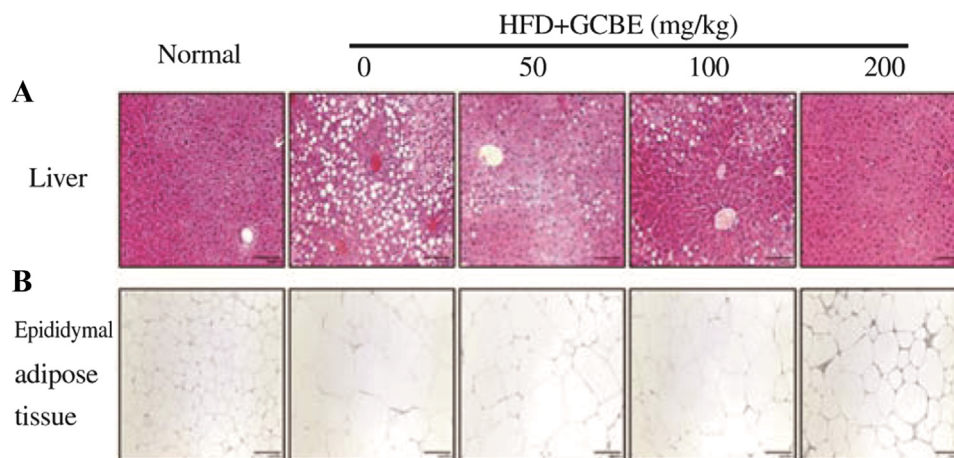
Figure 4. Histological observations of liver sections and epididymal adipose tissue.
(A) Liver and (B) epididymal adipose tissue were stained with hematoxylin and eosin, and were viewed under a microscope (×200). Representative sections are from three mice from each treatment group.

analyzing the mRNA expression levels in the epididymal adipose tissues using quantitative real-time PCR. As shown in Figure 5, peroxisome PPARα, CPT-1, Adiponectin, ATGL, and HSL mRNA expression levels in the HFD group were decreased compared to the normal diet group. However, in the HFD with GCBE groups, the mRNA levels of PPARα, Adiponectin, ATGL, and HSL were significantly upregulated in a dose-dependent manner. In HFD plus GCBE 200 group, significantly increased mRNA levels of ATGL and HSL, whereas CPT-1 mRNA expression levels increased compared with levels in adipose tissues of HFD group. The expression levels of adipogenesis related genes displayed whether GCBE affects adipose tissues of HFD group. C/EBPα, SREBP-1c and PPARγ were significantly downregulated in the HFD with GCBE 200 group compared with those in HFD group ($P < 0.01$ and $P < 0.05$, respectively).
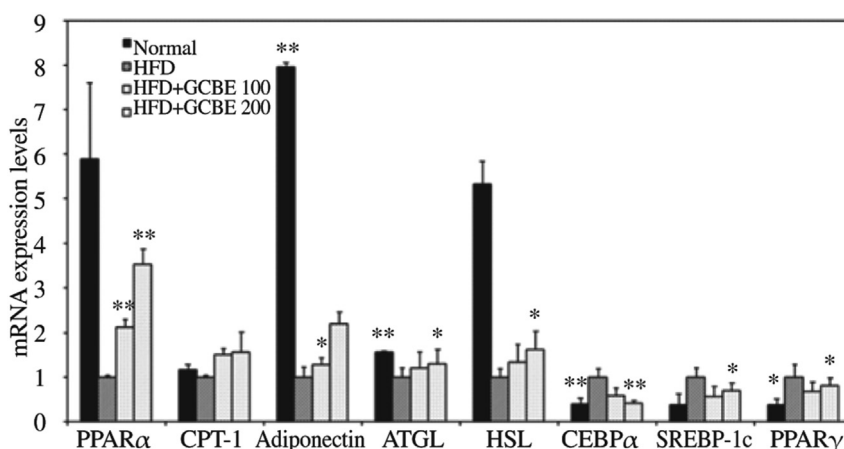
Figure 5. Effects of GCBE on genes expression levels in epididymal adipose tissues.
The mRNA expression levels of genes were evaluated by quantitative real-time PCR. *$P < 0.05$ and **$P < 0.01$ compared to the HFD group ($n = 8$ per group).

3.7. Effects of GCBE on mRNA expression of genes and proteins in the livers

To determine the mode of action of GCBE on HFD induced obesity, we examined the mRNA and Protein expressions in the liver. The adipogenesis and adipocyte metabolism related gene expression levels in HFD with GCBE groups, we found that PPARα, CPT-1, Adiponectin, ATGL, HSL, C/EBPα, SREBP-1c, and PPARγ were affected by GCBE in mice (Figure 6).

In the liver tissue, compared to the HFD group, mRNA expression levels of the normal group displayed reductions in C/EBPα, SREBP-1c, SREBP-2, and PPARγ; and statistically significant increases in PPARα and CPT-1 (Figure 6A) (both, $P < 0.05$). In the HFD with GCBE groups, PPARα mRNA levels were significantly upregulated ($P < 0.05$). The mRNA expression levels of CPT-1 also showed a significant increase in a dose-dependent manner ($P < 0.05$). We found that in the HFD with GCBE groups, the adipogenesis related genes C/EBPα, SREBP-2, and PPARγ expression levels also significantly decreased when compared to the mice fed HFD (Figure 6A). The

transcription factor SREBP-1c, a major lipogenic related protein, was also significantly reduced in HFD with GCBE groups (Figure 6A). Figure 6B and C show protein expression levels differences in GCBE supplemented groups. The target protein of SREBP-1c, FAS was statistically significantly reduced in the GCBE 200 treated group compared with the HFD group. Consistently, the C/EBPα protein expression level in the HFD with GCBE groups was significantly decreased in a dose-dependent manner. Additionally, GCBE treatments decreased protein expression of PPARγ levels.

We also investigated the phosphorylation of AMPK and PPARα protein expression. In the HFD group, pAMPK and PPARα protein levels in the liver were markedly decreased compared to the normal group. In contrast, GCBE 100 and 200 treatments significantly increased pAMPK expression compared to the HFD group ($P < 0.01$ and $P < 0.05$, respectively), similar to the normal group. Interestingly, we observed that the PPARα protein was markedly and dose dependently increased in the mice fed HFD with GCBE groups, 5.6 times more than in the HFD alone group (Figure 6C).
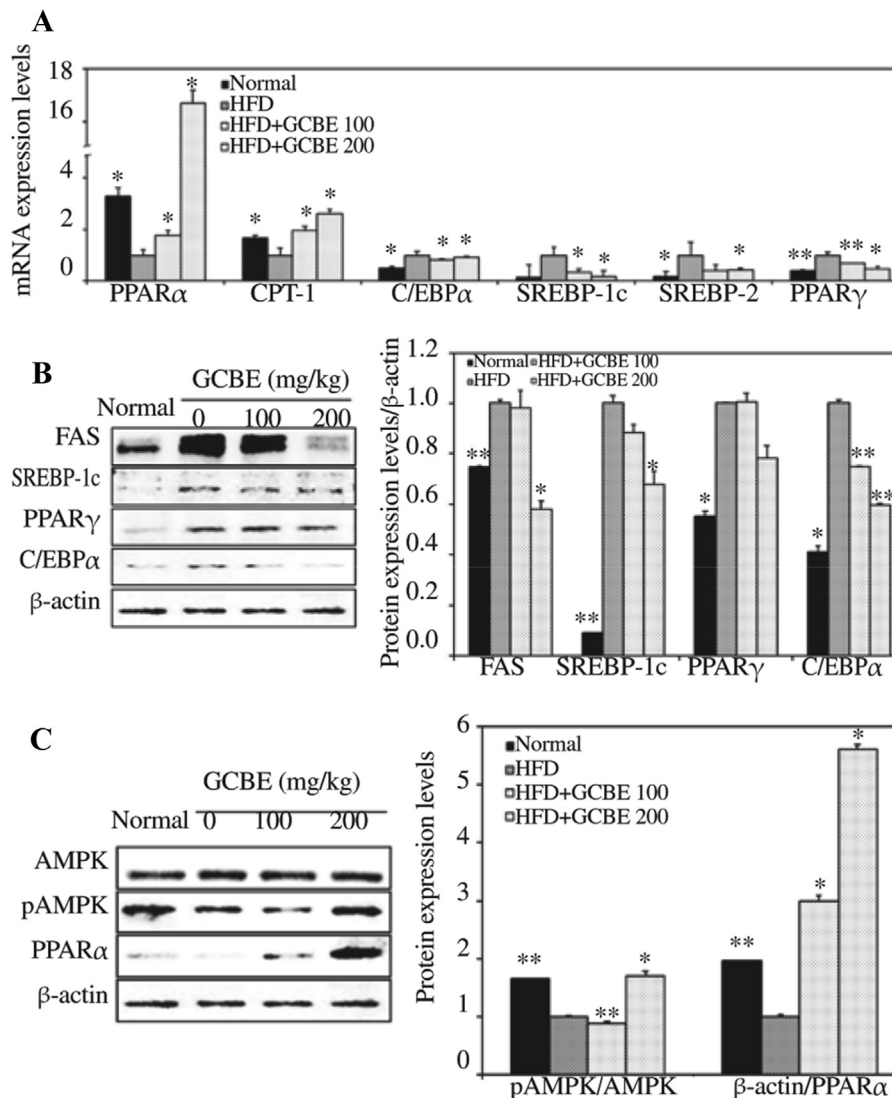

Figure 6. Effects of GCBE on gene expression levels and proteins in liver tissues.
(A) The mRNA expression levels of adipogenesis related genes were evaluated by quantitative real-time PCR. (B) and (C) The protein expression levels were detected by Western blot analysis. *$P < 0.05$ and **$P < 0.01$, compared to the HFD group ($n = 8$ per group).
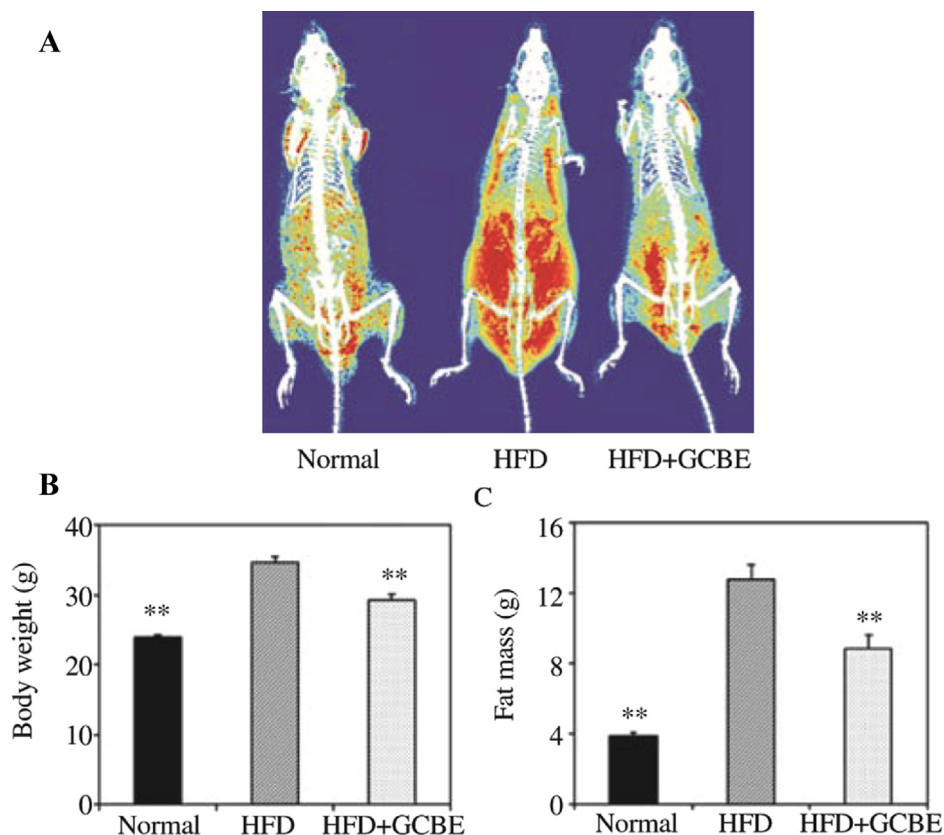
Figure 7. Distribution of fat mass and body weight measurements by DXA on mice fed a normal fat diet, high fat diet, and high fat diet with GCBE 100 mg/kg.
(A) The radiography of body fat is displayed by three modes according to low density fat (blue), medium density fat (yellow), and high density fat (red). (B) The body weight, and (C) fat mass were measured by DXA. $*P < 0.05$ and $**P < 0.01$ compared to the HFD group ($n = 8$ per group).

3.8. Distribution of fat mass and body weight measurements by DXA

To determine the effects of GCBE on HFD induced body composition, fat mass and body weight were determined by DXA. The DXA scan of the mice fed normal diet, HFD and HFD with GCBE 200 mg/kg is presented as radiography of body fat (Figure 7A). The HFD induced obese mice presented more large red color area than blue and yellow, meaning they had more high density of fat than low and medium density fats. The GCBE supplement for six weeks significantly reduced the body weight in the HFD induced obese mice. Furthermore, the relative distribution of fat mass noticeably decreased (Figure 7B and C) ($P < 0.01$).

4. Discussion

The chlorogenic acid was glycosylated derivate form of polyphenol, caffeic and quinic acid combined form. Coffee has high concentrations of chlorogenic acid as most common form is 5-CQA [1]. Previous studies have indicated that chlorogenic acid has many potent biological properties [16–18]. Additionally, recent reports of chlorogenic acid provide regulatory effects of glucose and lipid concentrations in diabetes and obesity [19–22].

Song et al. [15] have reported that 5-CQA was the most abundant and active compound in decaffeinated GCBE and has weight lowering and insulin resistance effects in mice fed the HFD at a dose of 300 mg/kg GCBE. Although previous reports show the effects of GCBE on the development of obesity, the effects of GCBE in HFD induced obese mice measuring total body fat mass has not been investigated. Thus, our study presents that feeding 3-CQA rich GCBE ameliorates and suppresses dietary-induced obesity and decreases body fat mass, directly. Our data show that GCBE has anti-obesity effects and confirms previous studies. Further, we show that GCBE effects the regulation of adipogenesis and lipid metabolism, directly effecting the distribution of fat mass.

Increased fat intake leads to obesity with body weight gain and glucose intolerant in mice [23]. We confirmed that six weeks of GCBE (Total chlorogenic acid was 50%, which was including 27.5% of 3-CQA) supplementation in a HFD did not affect the food intake in HFD-induced obese mice. Additionally, body weight gain in GCBE fed mice significantly decreased compared to mice fed a HFD alone. This phenomenon supports previous reports that GCBE has anti-obesity effects [15].

Our results also show that GCBE supplementation significantly lowers plasma T-CHO, TG, FFA, and LDL-C levels while increasing plasma HDL-C levels in the mice fed HFD. These results suggest that GCBE could reduce HFD induced hyperglycemia and hyperlipidemia.

Leptin and adiponectin regulate lipolysis through opposing activities in adipocytes [24]. Mature adipocytes produce adiponectin, which increases insulin sensitivity, glucose uptake, fatty acid oxidation, and anti-inflammatory effects in hormone-stimulated lipolysis [25]. To prevent lipid accumulation in adipose tissue, adipocytes secrete leptin to regulate food intake and fatty acid oxidation contacted with brain [26]. The plasma concentration of adipose tissues and endocrine

hormones revealed that GCBE treatment reduced leptin concentrations and inversely increased adiponectin, in a dose dependent manner.

In addition, we confirmed that mice fed GCBE showed significant decreases in liver and WAT weight in HFD-induced obese mice. Moreover, in liver and adipocytes histological analysis, GCBE treatment showed smaller adipocyte sizes and number in the mice fed a HFD. These results indicate that GCBE could decrease adipocytes, which demonstrates lipid accumulation and un-oxidized fatty acid induces lipotoxicity and dysregulation, which then decreases stimulation of lipolysis.

Another important finding in our study is that GCBE may improve adipogenesis and lipid metabolism in HFD-induced obese mice. Adipogenesis is the development of adipocyte differentiation with the related gene transcription factors, C/EBPs and PPARs [27]. In the mouse models, adipose tissue regulation was related with PPARγ and C/EBPα with HFD feeding. GCBE treatment reduced C/EBPα, SREBP-1c, and PPARγ mRNA expression levels in expression [28,29]. PPARγ expression is activated in adipocytes genes [30]. The lipogenic transcription factor, SREBP-1c, is a key regulator for fatty acid and cholesterol, as well as, LDL receptor that activates lipogenesis [31].

To examine the underlying mechanisms, we induced obesity with hyperglycemia and hyperlipidemia epididymal adipose tissue. Additionally, we investigated lipid catabolism activity in adipose tissues. Adipocytes have excess energy and store fatty acids in the form of TG in lipid droplets. Consequently, energy regulation of the catabolic pathway, including fatty acid oxidation and glycolysis, is a strong candidate for obesity.

We also studied whether GCBE has anti-lipolytic effects in adipocytes. PPARα is a nuclear receptor, which increases the expression of gene CPT-1 to induce mitochondrial fatty acid to dispose through β-oxidation [32]. GCBE treatment increases the mRNA expression of PPARα and CPT-1, indicating GCBE promotes energy dissipation in adipocyte tissues. The adiponectin plasma concentration regulates lipid profiles. Gene expression levels of adiponectin were also up-regulated by GCBE treatment. HFD-induced obese mice presented a decrease of adiponectin gene expression in adipose tissues [33]. Moreover, Qiao *et al.* [25] proposed adiponectin inhibits lipolysis and regulates lipid metabolism suppressing catabolites of TG by inhibiting ATGL and HSL in mouse adipocytes. Our study showed that lipolysis activity of GCBE was induced by adiponectin, HSL, and ATGL mRNA expression in adipocyte tissues. Based on these mRNA expression results in adipose tissue, GCBE leads to reduced adipogenesis and lipid accumulation in the adipose tissues of HFD-induced obese mice.

Of the adipogenesis-related gene and protein expression levels in the liver, we found that the dose of GCBE treatment significantly up-regulated the lipid oxidation related genes, like PPARα and CPT-1, and down-regulated the transcription factors that regulate adipocyte differentiation, like C/EBPα, SREBPs, and PPARγ. There is a possibility GCBE treatment affects the expression levels of adipogenesis-related genes and proteins. It is likely that the anti-obesity effects of GCBE treatment are caused by the suppression of gene expression and proteins involved in fatty liver induced obesity.

In obesity development, AMPK is a key mediator that switches adipocytes from anabolizing to catabolizing lipids. In the liver, AMPK altered fatty acid oxidation and glycogenesis, while switching off the synthesis of fatty acids, gluconeogenesis and ATP consumption with PPARα [34,35]. In this study,

phosphorylation of AMPK and PPARα increased in GCBE treated groups in a dose dependent manner. From these results, GCBE induced AMPK activation causes anabolic stages involving fatty acid, glucose oxidation and inhibition of adipocytes proliferation in obese mice.

Several epidemiologic studies have reported that body fat, especially abdominal and visceral fat, in adiposity related obesity is associated with increased risk of metabolic syndrome [36]. Excess adiposity was measured using an imaging technique, to investigate the fat tissue and fat distribution. DXA quantifies the past photon peaks through tissue-organs *ex vivo* with high accuracy to understand the mechanisms involved in metabolic regulation by measurement of fat distribution and composition [37,38]. Simultaneously, we measured DXA fat distribution, body weight, and total fat mass. Notably, our results show that the mice supplemented with a HFD have a higher body weight, fat mass, and distribution of high density fat compared to mice fed a normal diet. When we compared GCBE and HFD treatment groups, the GCBE group displayed a significant reduction of fat distribution and concentration, with less body weight and fat mass.

In conclusion, this study shows GCBE treatment markedly ameliorated body fat accumulation in HFD induced obesity and reduced body weight gain, fat mass, adipocyte sizes, WAT weight, and plasma lipid levels. We confirm that the active compound 3-CQA from GCBE, reduces body fat accumulation by the regulation of adipogenesis and lipogenesis in obesity. Thus, these findings demonstrate that GCBE represents a potential activity that prevents the development of obesity, hyperlipidemia and its complications in diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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