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A THESIS FOR THE DEGREE OF
MASTER OF SCIENCE IN FOOD AND NUTRITION

Effect of Citrus Unshiu Peel Extract
Fermented by *Aspergillus niger* on Obesity
and Hyperglycemia in High - Fat Diet Fed
C57BL/6J Mice

고지방 식이를 섭취한 C57BL/6J 마우스에서
발효진피추출물이 비만과 혈당 개선에 미치는 영향

FEBRUARY, 2017

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Abstract

Effect of Citrus Unshiu Peel Extract Fermented by *Aspergillus niger* on Obesity and Hyperglycemia in High - Fat Diet Fed C57BL/6J Mice

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Citrus unshiu peel is a Korean citrus fruit that has abundant bioactive compounds including flavonoids like naringin, hesperidin, and narirutin. In this study, we examined the anti-obesity and anti-hyperglycemia effects of citrus unshiu peel extract fermented by mycotoxin non-producing *Aspergillus niger* FMB S46494 using high-fat diet induced obese mice. The total flavonoid contents of citrus unshiu peel extract (CPE) and fermented citrus unshiu peel extract (FCPE) were 55.6 mg/g NE (naringin equivalents) and 173.5 mg/g NE respectively. The contents of flavonoid were determined by HPLC. Narirutin and hesperidin were the major flavonoids in citrus peel extract. Naringenin level reached 14.1 mg/g after fermentation. Four-week-old male C57BL/6J mice were divided into five groups

(n = 8) and fed the respective experimental diets for 13 weeks ; LFD (low - fat diet), HFD (high - fat diet), CPE (HFD + 1% (w/w) CPE), FCPE 0.3% (HFD + 0.3% (w/w) FCPE) and FCPE 1% (HFD + 1% (w/w) FCPE). FCPE 0.3% group and FCPE 1% group had significantly lower body weight gains (14.1% and 13.2% less respectively, $p < 0.05$) and epididymal fat weight (28.8% and 23.5% less respectively, $p < 0.05$). The fasting blood glucose levels were significantly lower in the FCPE groups in comparison to the HFD group ($p < 0.05$). In intraperitoneal glucose tolerance test, AUC level was significantly lower in the FCPE 0.3% group compared to the HFD group ($p < 0.05$). In morphology of liver, lipid droplet accumulation was less pronounced in the FCPE 0.3% group and FCPE 1% group. Adipocyte size of epididymal fat was lower in the FCPE groups. Furthermore, the levels of total cholesterol and LDL cholesterol in plasma were significantly lower in the FCPE groups ($p < 0.05$). Expression of genes related to hepatic glucose homeostasis (*Gk*, *G6pase*, *Pepck*), hepatic glucose transporter (*Glut2*), hepatic lipogenesis and fatty acid oxidation (*Srebp1c*, *Acc*, *Fas*, *Cpt1*) as well as hepatic cholesterol homeostasis (*Srebp2*, *Hmgr*, *Pcsk9*, *Acat2*, *Cyp7a1*, *Ldlr*) were determined by real - time PCR. The mRNA levels of *Gk*, *Glut2* and *CPT1* were significantly higher in the FCPE groups compared with the HFD group ($p < 0.05$). In addition, FCPE groups had significantly lower *Srebp1c*, *Srebp2*, *Fas*, *Acc*, *Hmgr* and *Pcsk9* mRNA expression ($p < 0.05$). In conclusion, FCPE supplementation may function to improve hepatic lipid and cholesterol metabolism as well as hepatic glucose metabolism in high - fat fed mice. These results suggest that FCPE, which contains naringenin, is a fermented food material that has promising effect in amelioration of obesity and obesity - induced hyperglycemia.

Key words: Citrus unshiu peel, fermentation, *Aspergillus niger*, flavonoid, anti - obesity, anti - hyperglycemia

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List of abbreviation

A. niger : *Aspergillus niger*

PDA : Potato dextrose agar

CPE : Citrus unshiu peel extract

FCPE : Fermented citrus unshiu peel extract

LFD : Low fat diet

HFD : High fat diet

IPGTT : Intraperitoneal Glucose Tolerance Test

FER : Food efficiency ratio

SREBPs: Sterol regulatory element binding proteins

GLUT - 2 : Glucose transporter protein type 2

GK : Glucokinase

PEPCK : Phosphoenolpyruvate carboxykinase

G6pase : Glucose - 6 - phosphatase

HMGR: 3 - hydroxy - 3 - methyl - glutaryl coenzyme A (HMG - CoA) reductase

LDLR : Low density lipoprotein (LDL) receptor

CPT1 : Carnitine palmitoyl transferase 1

FAS : Fatty acid synthase

ACC : Acetyl - coA carboxylase

ACAT2 : Acyl - coenzyme A (CoA):cholesterol acyltransferases 2

PCSK9 : Proprotein convertase subtilisin/kexin type 9

CYP7A1 : Cholesterol 7 alpha - hydroxylase

1. Introduction

Obesity is a serious problem with a high public - health impact that may cause type 2 diabetes and metabolic syndrome (MetS) [1]. The MetS is associated with a large mortality worldwide, which is a cluster of insulin - resistance, diabetes, dyslipidemia, visceral obesity and hypertension [2, 3]. Recently, there have been growing interests in bioactive substance from natural food materials which can ameliorate the obesity and metabolic syndrome without side effects [4].

Citrus fruits have abundant flavonoid compounds, which are classified as the six groups; flavanones, flavanans, isoflavones, flavonols, anthocyanidins and flavanols according to their structural characteristics [5]. Flavonoids have been widely known to possess biological activities with strong anti - oxidant properties. Citrus flavonoids have anti - obesity, anti - inflammatory and anti - diabetic effects in vitro and in vivo [6, 7, 8]. Citrus unshiu (*Citrus unshiu* Marcov., Family: Rutaceae) is a Korean citrus fruit that accounts for 30% of total fruits produced in Korea [9]. Traditionally, citrus peels have been used in Asia countries as a folk medicine [10]. It was reported that citrus peels have higher concentrations of flavonoid than the flesh part [11]. It has been demonstrated that citrus unshiu peels have a positive effect on inflammation [12]. Mice fed citrus unshiu peel showed a significant decrease in body weight gain and epididymal white adipose tissue weight. In addition, significant decrease in blood glucose level in mice fed citrus unshiu peel was partially mediated through the inhibition of gene expression of hepatic gluconeogenic enzyme and the induction of

insulin/glucagon secretion. The amelioration of hepatic steatosis of citrus unshiu peel was correlated to the inhibition of gene expression related to the lipogenesis and fatty acid oxidation in the liver [9].

Major flavonoids in citrus unshiu peels exist as flavanone glycosides (narirutin, naringin and hesperidin) [13, 14]. However, glycoside is absorbed slowly because glycoside is broken down to aglycoside form by microflora of the large intestine and then absorbed from the intestine [15]. Therefore, glycoside forms require hydrolysis to aglycone forms for the improvement of bioavailability [16]. Bioconversion of Jeju Hallabong tangor peels with cytolase may enrich aglycoside flavanones, naringenin and hesperetin, and it enhanced the anti-inflammatory effects of citrus peels in RAW 264.7 cells compared to flavanone glycosides [17]. Aglycone form of flavonoid in the fermented citrus peel extract inhibited oxidative damage in pancreatic beta cell [13]. Previous study suggested that naringenin, which is an aglycoside form of narirutin and naringin, suppress diet-induced weight gain and prevent dyslipidemia by inhibiting gene expression of *Srebp1c* and activating hepatic fatty acid oxidation. In addition, naringenin treatment improved insulin sensitivity and glucose tolerance [18]. Naringenin supplementation (0.003%, 0.006% and 0.012% of diet) upregulated fatty acid oxidation target genes in the hepatic tissue, resulting in lowered plasma level of triglyceride and total cholesterol and triglyceride contents in liver and adipose tissue [19].

Fungal fermentation is one of the oldest biotechnologies and an inexpensive technique that enhances nutrient content of foods or other desirable attributes [20]. *Aspergillus niger* is widely used in the fermentation process due to its ability to produce various

glycosidases. In addition, *A. niger* was used to produce enzyme that hydrolyses naringin to naringenin [21, 22]. Citrus peel extract biotransformed with *A. niger* had more physiological effect than non-fermented citrus peel extract through the fermentation [23]. Therefore, we assumed that fermentation would improve several physiological effects of citrus unshiu peel extract.

Although previous studies revealed that the effect of citrus unshiu peel extract on hyperglycemia and dyslipidemia in *db/db* mice, research on the protection of obesity and obesity-induced hyperglycemia using citrus unshiu peel extract and fermented citrus *unshiu* peel extract remains insufficient. Thus, this study investigated whether citrus unshiu peel extracts after fermentation by mycotoxin non-producing *Aspergillus niger* FMB S46494 ameliorated the obesity and its related hyperglycemia in high-fat diet fed C57BL/6J mice.

2. Materials and method

2.1. Preparation of extracts

Dried citrus unshiu peels were purchased from Barun Yakcho (Yongin, Korea). Samples were prepared by adding 6 L of 70% ethanol to 600 g of dried citrus peel. Extraction was performed at 70°C for 4 h, and then the solution was cooled and filtered with Whatman paper (No. 41). The filtered solution was concentrated with a speed vacuum (Scanspeed 40, LaBoGene Aps, Denmark), freeze-dried to a powder at -80°C with a freeze dryer (FD8508, Ilshin BioBase, Korea) and stored at -20°C until use.

2.2. Fungal strains and cultures

A. niger FMB S46494 strain was provided by the Food Microbiology Laboratory at the Food and Nutrition Department at Seoul National University. *Aspergillus niger* FMB S46494 do not produce mycotoxin [24]. *A. niger* was grown on potato dextrose agar (PDA) (Becton, Dickinson and Company, MD, USA) at 30°C under aerobic conditions for 7 days. The spores from the subcultured PDA plate were scrapped and suspended in the 0.9% NaCl solution with 0.005% Tween 80 to make spore suspension solution.

2.3. Fermentation of citrus unshiu peel extracts

The dried citrus unshiu powder (1 g) was mixed with 30 ml of distilled water and sterilized in an autoclave at 121°C for 15 min. *A. niger* FMB S46494 spores were inoculated at 10^6 spores/ml into the citrus peel suspension and fermented in a shaking incubator at 30°C under 150 rpm for 7 days. The fermented extract was freeze-dried at -80°C with a freeze dryer (FD8508, Ilshin BioBase, Korea), then extracted with 70% ethanol overnight at room temperature. The extract was filtered with Whatman paper (No. 41).

2.4. Measurement of total flavonoid contents

Total flavonoid contents were analyzed according to the method [25]. The sample (15 μ l) was mixed with 150 μ l of diethylene glycol and 15 μ l of 1 N NaOH. The mixture was shaken thoroughly, and incubated for 1 h at room temperature. The absorbance of the solution showing orange - yellowish in color, was measured at 420 nm. The result was determined using a standard calibration curve obtained from various concentrations of naringin (25, 50, 100, 200 and 400 mg/l). Total flavonoid content of the sample was expressed as naringin equivalents in mg/g NE of dried powder.

2.5. Analysis of flavonoid composition by HPLC

The freeze-dried powder of citrus peel extract (CPE) and fermented citrus peel extract (FCPE) were dissolved in dimethyl sulfoxide (DMSO) - methanol for experiment. The extracted solution was filtered through a 0.2 μm syringe filter (PALL Life Sciences, USA) before high-performance liquid chromatography (HPLC) analysis.

The system used for HPLC analysis was the 1090 Series - II Model HPLC System (Hewlett Packard, USA). The flavonoid compounds were monitored at 280 nm using an Eclipse XDB - C18 column (150 mm long, 3mm inside diameter, 5 μm particle size; Agilent Technologies, USA). The column temperature was 35°C, and the flow rate was 0.5 mL/min. The UV detection wavelength was 280 nm. The mobile phase consisted of two solvents: 0.1% acetic acid (A) and 100% acetonitrile (B). The solvent gradient in volume ratios was as follows: 0 - 15 min 10 - 20% B, 15 - 25 min 20 - 25% B, 25 - 30 min 25 - 30% B, 30 - 35 min 30% B.

2.6. Animals and diets

Four - week - old male C57BL/6J mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea). The mice were maintained at $23 \pm 3^\circ\text{C}$ with relative humidity of $50 \pm 10\%$ and a 12 h/12 h light/dark cycle. Mice were randomly divided into five groups ($n = 8$) after two weeks of acclimation period ; LFD (low fat diet containing 10.2% kcal fat, 69.8% kcal carbohydrate, 20.1% kcal protein, TD.06416, Harlan Teklad), HFD (high fat diet containing 60.3% kcal fat, 21.3% kcal carbohydrate and 18.4% kcal protein, TD.06414, Harlan Teklad), CPE (HFD + 1% (w/w) CPE powder), FCPE 0.3% (HFD + 0.3% (w/w) FCPE powder), and FCPE 1% (HFD + 1% (w/w) FCPE powder). The caloric density of the low fat diet and high fat diet was 3.7 kcal/g and 5.1 kcal/g respectively. The mice were allowed free access to water and diet.

Mice were fed *ad libitum* for 13 weeks. Food intake and body weight were measured every other day and once a week, respectively. At the end of experimental period, mice were fasted for 12 h and anaesthetized with Zoletil (Virbac Lab., Carros, France)/Rompun (Bayer, Leverkusen, Germany), and blood samples were collected by cardiac puncture through heparin - coated tube. The Zoletilel/Rompun solution was diluted with saline and injected into thigh muscle. The livers and adipose tissues were removed, weighted and frozen at -70°C until analyzed. All studies and protocol of the animals were approved by Institutional Animal Care and Use Committee (IACUC) (Seoul National University, Korea).

2.7. Intraperitoneal glucose tolerance test (IPGTT)

At the 12th week of treatment, fasting blood glucose levels were measured via tail vein with Accu-check Active (Roche Diagnostics, Indianapolis, IN, USA) after an overnight fasting. Additional blood glucose levels were measured at 15, 30, 60, and 120 min after glucose solution (2 g/kg body weight) administration by intraperitoneal injection.

2.8. Analysis of plasma lipid levels

The plasma levels of triglyceride (TG), total-cholesterol (TC) and high density lipoprotein cholesterol (HDL-C) were analyzed by enzymatic methods using commercial assay kits (Asan Pharmaceutical Co., Seoul, Korea). The levels of low density lipoprotein cholesterol (LDL-C) were calculated by applying the formula ; $LDL-C = TC - (TG/5) - HDL-C$.

2.9. Histological analysis of liver and epididymal fat

Morphological analysis of liver and epididymal fat tissue were conducted to determine hepatic lipid droplet accumulation and adipocyte size of the epididymal fat. Liver and epididymal fat were fixed overnight in 10% neutral formalin (Wako Pure Chemical Industries, Japan) and embedded in paraffin. The 4- μ m sections were prepared, stained with haematoxylin-eosin and viewed under an

optical microscope (Olympus, Japan) with a magnifying power of $\times 200$. Cell length (μm) of 12 adipocytes per group was measured with the open - source image analysis program ImageJ.

2.10. RNA extraction and quantitative real - time reverse transcriptase polymerase chain reaction (RT - PCR).

Total RNA from the liver was isolated using TaKaRa MiniBEST Universal RNA Extraction kit (Takara Bio Inc, Japan) according to the manufacturer's instructions. The purity and concentration of RNA were evaluated using a Nano - Spectrophotometer (NANO - 200, BIOAND, Korea). Total RNA (500 ng) was reverse - transcribed to cDNA using the PrimeScript RT Master Mix (Takara Bio Inc, Japan). mRNA expression was analyzed by real - time quantitative PCR with a SYBR premix Ex Taq (Takara Bio Inc, Japan) using StepOne Real - time PCR system (Applied Biosystems, CA, USA). The mRNA levels were normalized with GAPDH and expressed as values of relative expression compared to that of the HFD group. The initial denaturation was conducted at 95°C for 30 s, then followed by 40 cycles of 5 s at 95°C and 34 s at 60°C. The primer sequences are shown in Table 1.

Table 1. Primer sequences for hepatic genes used in real - time PCR.

gene	sequence (5' → 3')
<i>Gapdh</i>	(forward) AGG TCG GTG TGA ACG GAT TTG
	(reverse) TGT AGA CCA TGT AGT TGA GGT CA
<i>Glut2</i>	(forward) GGC TAA TTT CAG GAC TGG TT
	(reverse) TTT CTT TGC CCT GAC TTC CT
<i>Gk</i>	(forward) CAG GAC AGT GGA GCG TGA AGA C
	(reverse) TTA CAG GGA AGG AGA AGG TGA AGC
<i>G6pd</i>	(forward) AAC GCC TTC TAT GTC CTC TTT C
	(reverse) GTG GTT GCT GTA GTA GTC GGT GTC C
<i>Pepck</i>	(forward) TGC CTC TCT CCA CAC CAT TGC
	(reverse) TGC CTT CCA CGA ACT TCC TCA C
<i>Hmgr</i>	(forward) TTC ACG CTC ATA GTC GCT GGA TAG
	(reverse) TGG TTC AAT TCT CTT GGA CAC ATC TTC
<i>Srebp1c</i>	(forward) CGG AAG CTG TCG GGG TAG
	(reverse) GTT GTT GAT GAG CTG GAG CA
<i>Srebp2</i>	(forward) GCT GGT TTG ACT GGA TGG TT
	(reverse) ACC TTT GGC GAG GTC TAG GT
<i>Ldlr</i>	(forward) ACC CCT CAA GAC AGA TGG TC
	(reverse) CAG CCC AGC TTT GCT CTT AT
<i>Cyp7a1</i>	(forward) GTG GTA GTG AGC TGT TGC ATA TGG
	(reverse) CAC AGC CCA GGT ATG GAA TCA
<i>Pcsk9</i>	(forward) TTG GAT TGA GGC CAT AGG AG
	(reverse) GAC GGC ATA GAC ACC CTC AC
<i>Acat2</i>	(forward) GCA GGG AAG TTT GCC AGT GAG A
	(reverse) GAA CAC GGT CTT GAG CTT TGG C
<i>Fas</i>	(forward) CTG AGA TCC CAG CAC TTC TTG A
	(reverse) GCC TCC GAA GCC AAA TGA G
<i>Acc</i>	(forward) GAA TCT CCT GGT GAC AAT GCT TAT T
	(reverse) GGT CTT GCT GAG TTG GGT TAG CT
<i>Cpt1</i>	(forward) ATC TGG ATG GCT ATG GTC AAG GTC
	(reverse) GTG CTG TCA TGC GTT GGA AGT C

2.11. Statistical analysis

The results were expressed as mean \pm standard error of means (S.E.M.). Statistical differences were evaluated by one - way analysis of variance (ANOVA) followed by the Duncan's multiple range tests using the SPSS statistical package (Chicago, IL, USA). Significant results were considered at $p < 0.05$.

3. Results

3.1. Total flavonoid contents

The total flavonoid contents in the dried powder of the CPE and FCPE were 55.6 mg NE/g and 173.5 mg NE/g of dry material respectively. The final percentage yield from the lyophilized powder of the CPE and FCPE were 32.4% and 12.7% respectively.

3.2. Flavonoid composition

The flavonoid components of CPE and FCPE were analyzed through the HPLC analysis. Table 2 shows the flavonoid composition of CPE and FCPE. The major flavonoids in CPE were narirutin (17.5 mg/g) and hesperidin (8.8 mg/g), and those in FCPE were narirutin (35 mg/g), hesperidin (21.9 mg/g). In addition, naringenin at the level of 14.13 mg/g, which is aglycone - form of narirutin, was found in the fermented sample. However, hesperetin, which is aglycone - form of hesperidin, was not found.

Table 2. Composition of the flavonoid from CPE and FCPE

Sample	Flavonoid contents (mg/g dry weight)		
	Narirutin	Hesperidin	Naringenin
CPE	17.5	8.8	ND*
FCPE	35.0	21.9	14.1

*ND : Not detected

3.3. Food intake, body weight, epididymal fat weight and liver weight

Table 3 and Fig. 1 show the body weight changes and the amounts of food intake. Final weight and body weight gain in the both FCPE 0.3% and FCPE 1% group were significantly lower compared to the HFD group and CPE group. But there was no significant difference in the body weight between the CPE group and HFD group. The average daily food intake in the HFD group was the lowest among the experimental groups. Thus, mice fed CPE and FCPE supplementation showed significantly lower food efficiency ratio (FER) than the HFD group. The mice fed high fat diet with CPE and FCPE supplementation had significantly lower amount of the epididymal fat pad compared to the mice fed only high fat diet (Fig. 2A). However, the liver weights and liver - to - body weight percentage of the CPE and FCPE supplementation groups were not significantly altered (Fig. 2B and Fig. 2C).

Table 3. Food intake, body weight and food efficiency ratio.

	LFD	HFD	CPE	FCPE 0.3%	FCPE 1%
Initial BW* (g)	21.9 ± 1.5	21.4 ± 0.9	21.5 ± 1.4	21.5 ± 0.9	21.4 ± 1.2
Final BW(g)	31.6 ± 3.1 ^c	47.0 ± 2.1 ^a	46.9 ± 1.4 ^a	43.5 ± 3.9 ^b	43.6 ± 2.3 ^b
BW gain (g)	9.8 ± 2.1 ^c	25.6 ± 2.1 ^a	25.4 ± 2.1 ^a	22.0 ± 3.1 ^b	22.3 ± 2.1 ^b
Food intake (g/day)	2.6 ± 0.3 ^a	2.3 ± 0.2 ^c	2.6 ± 0.2 ^b	2.5 ± 0.2 ^b	2.5 ± 0.2 ^b
FER**	4.1 ± 0.9 ^d	12.0 ± 1.0 ^a	10.9 ± 0.9 ^b	9.6 ± 1.3 ^c	9.9 ± 0.9 ^{bc}

*BW ; Body weight

**FER, food efficiency ratio = total body weight gain (g)/ total food intake (g) × 100.

The data are mean ± S.E.M. (n=8 for each group).

^{abcd}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$.

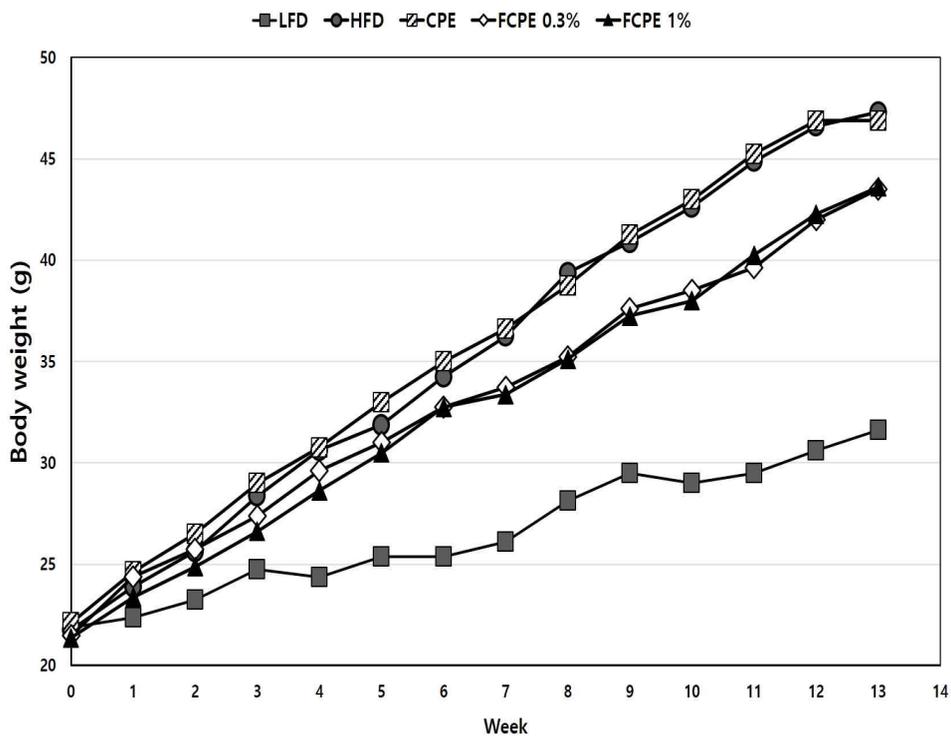


Fig. 1. Body weight curves of mice.

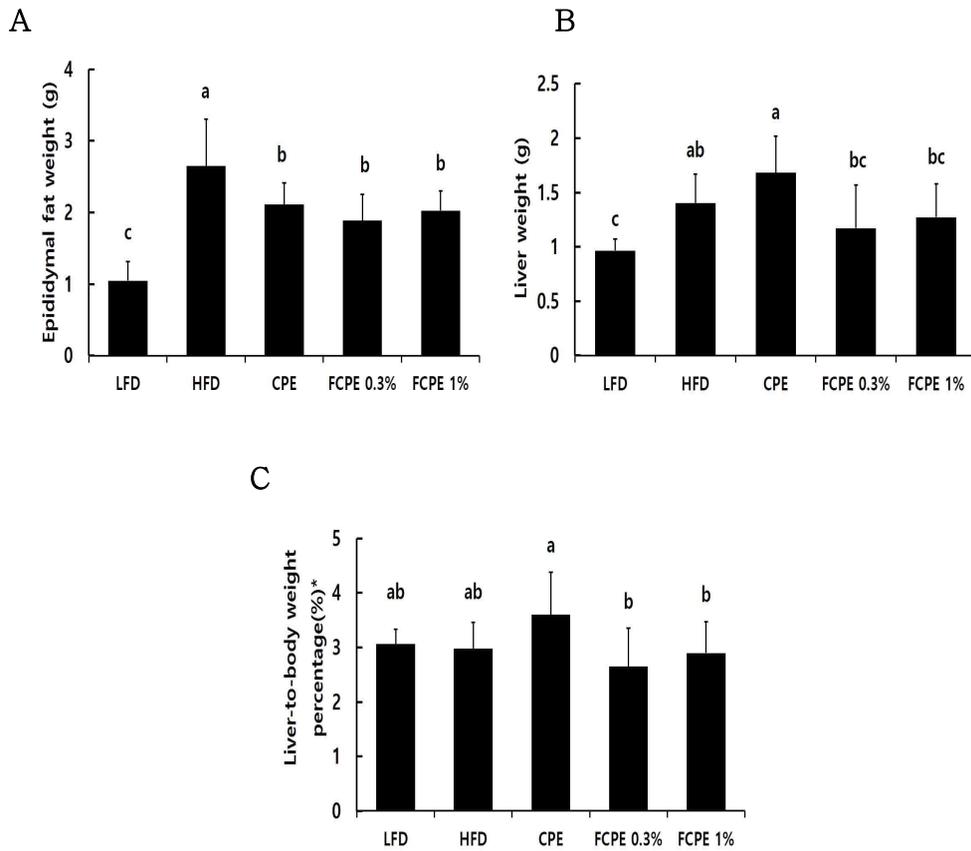


Fig. 2. Epididymal fat weight and liver weight.

A, epididymal fat weight ; B, liver weight ; C, liver - to - body weight percentage (%).

*Liver - to - body weight percentage(%) = liver weight (g)/ body weight (g) × 100. The data are mean ± S.E.M.(n=8 for each group).

^{abc}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$.

3.4. Fasting blood glucose level and glucose tolerance test

Fig 3 shows the fasting blood glucose levels after 12 weeks of experimental feeding. The fasting blood glucose levels in the FCPE 0.3% and FCPE 1% groups were significantly lower than these in the HFD group and CPE group.

IPGTT was further performed at 12th week (Fig.4 and Table 4). Compared to the HFD group, FCPE 0.3% group exhibited significantly lower blood glucose levels at 60 and 120 min after the injection of glucose and FCPE 1% group showed significantly lower blood glucose level at 120 min. However, the blood glucose levels following the administration of glucose solution did not differ between the CPE and HFD group. Based on the result of IPGTT, the AUCs were calculated by the excel program. The FCPE 0.3% group showed significantly lower AUC level than the HFD group (Fig. 4B).

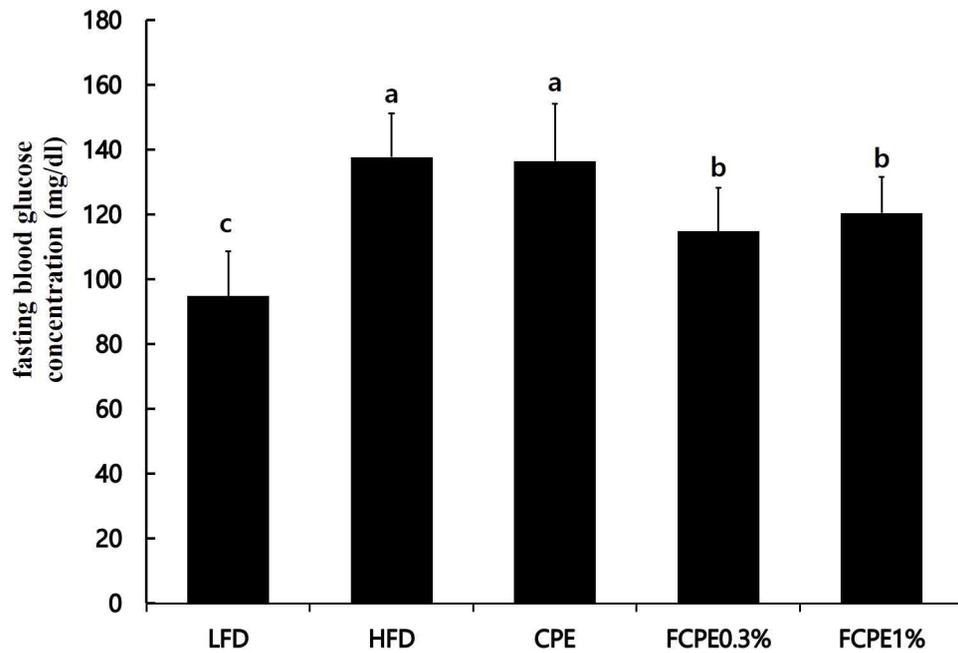


Fig. 3. Fasting blood glucose levels at 12th week.

The data are mean \pm S.E.M. (n=8 for each group). ^{abc}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$.

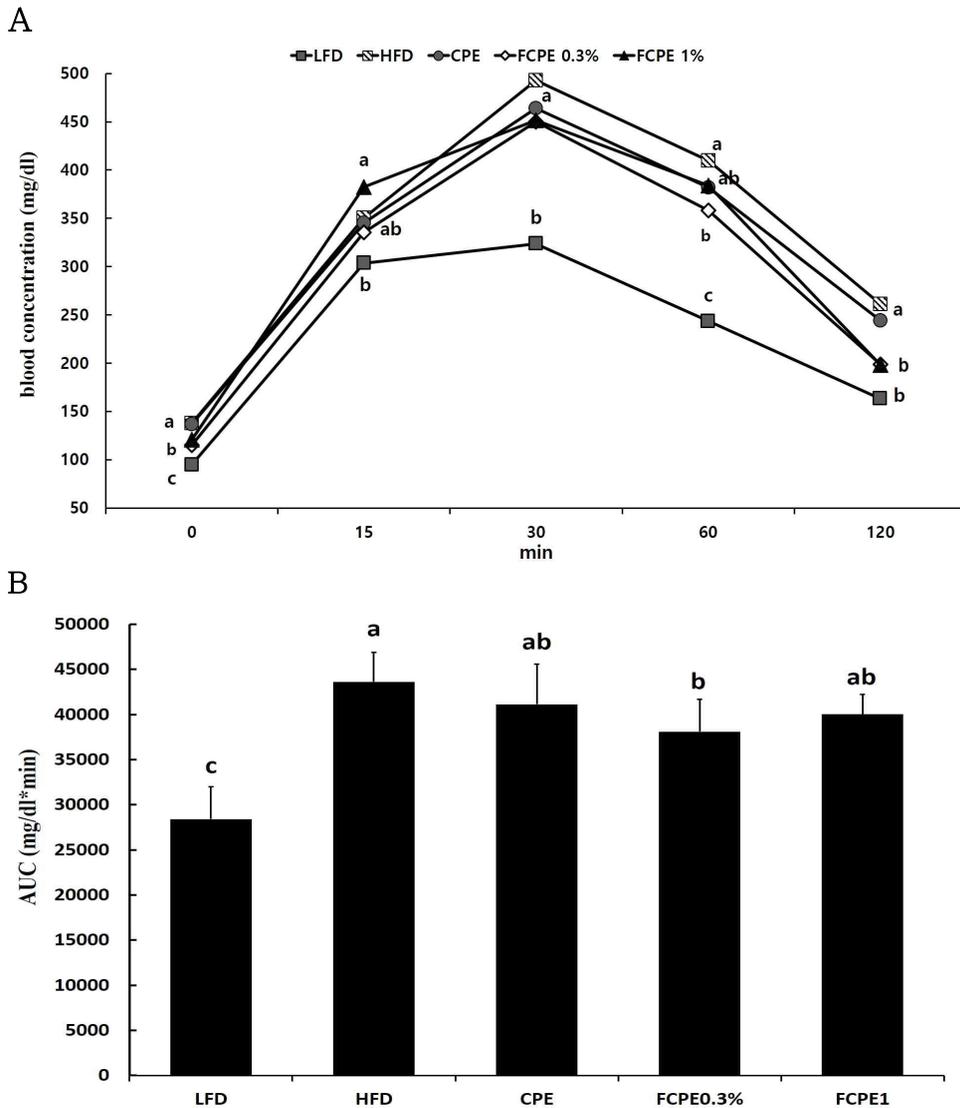


Fig. 4. The result of IPGTT at 12th week.

A, Serum glucose levels at 0, 15, 30, 60 and 120 min after the glucose solution injection (mg/dl) ; B, Calculated AUCs (area under the curve)

The data are mean \pm S.E.M. (n=8 for each group). ^{abc}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$.

Table 4. The result of IPGTT at 12th week.

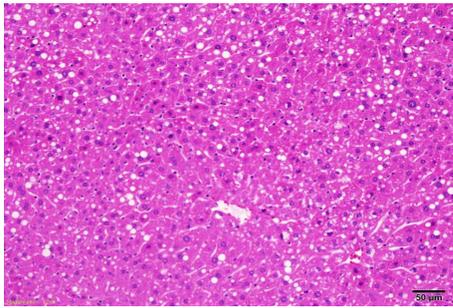
blood glucose (mg/dl)	LFD	HFD	CPE	FCPE 0.3%	FCPE 1%
0 min	94.9 ± 13.7 ^c	134.0 ± 13.4 ^a	136.5 ± 17.7 ^a	114.9 ± 13.3 ^b	120.5 ± 11.1 ^b
15 min	303.6 ± 37.3 ^b	350.5 ± 33.7 ^{ab}	345.4 ± 55.6 ^{ab}	335.5 ± 56.6 ^{ab}	382.5 ± 33.2 ^a
30 min	323.8 ± 44.9 ^b	492.6 ± 57.1 ^a	464.0 ± 52.0 ^a	450.3 ± 41.0 ^a	451.8 ± 57.8 ^a
60 min	243.4 ± 40.0 ^c	400.3 ± 48.1 ^a	381.8 ± 65.4 ^{ab}	358.3 ± 43.9 ^b	383.8 ± 33.1 ^{ab}
120 min	163.1 ± 22.7 ^b	260.9 ± 33.0 ^a	244.3 ± 49.9 ^a	198.9 ± 28.9 ^b	198.1 ± 26.3 ^b

The data are mean ± S.E.M. (n=8 for each group).

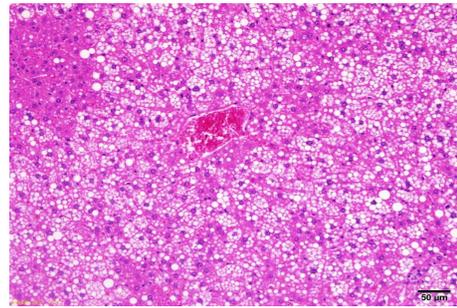
^{abc}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$.

3.5. Histological analysis of liver

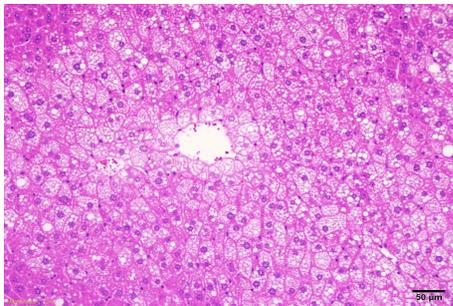
In contrast to the liver weights, the analysis of hepatic morphology demonstrated that the HFD and CPE group showed the highest lipid accumulation in the liver, while minimal hepatic lipid accumulation was found in the FCPE 0.3% group and FCPE 1% group (Fjg. 5).



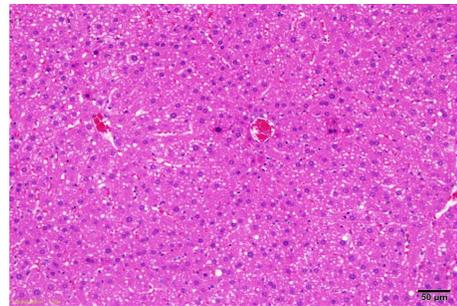
LFD



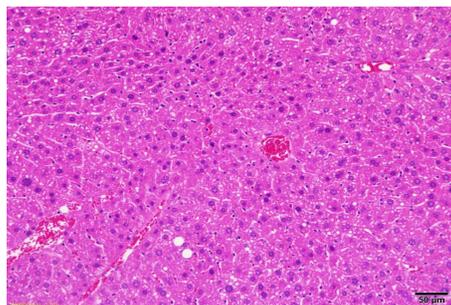
HFD



CPE



FCPE 0.3%



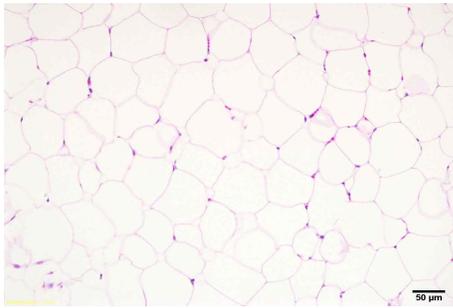
FCPE 1%

Fig. 5. The morphology of hepatic tissue.

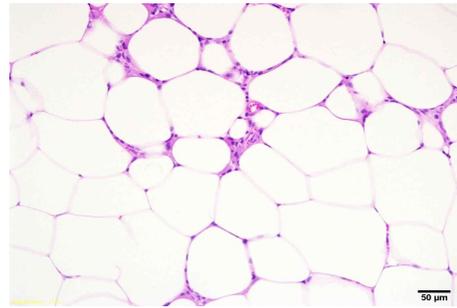
Hepatic lipid droplet accumulation in liver sections stained with H&E. Magnification ($\times 200$).

3.6. Histological analysis of epididymal fat

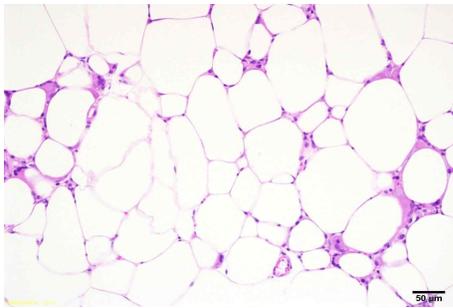
The adipocyte sizes were significantly smaller in the CPE and FCPE supplementation groups than in the HFD group (Fig. 6 and Fig. 7). Fig. 6 shows lots of crown-like structures (CLSs) in the HFD group and CPE group. Apparently numerous macrophages were infiltrated into adipocytes to form crown-like structures. On the other hand, macrophage infiltration was inhibited in the FCPE 0.3% and 1% groups.



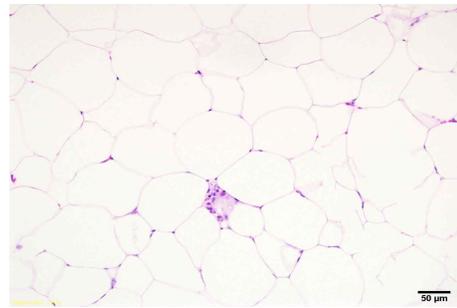
LFD



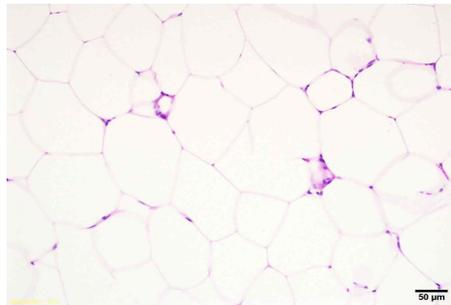
HFD



CPE



FCPE 0.3%



FCPE 1%

Fig. 6. The morphology of epididymal adipose tissue.

Sections of epididymal fat were stained with H&E. Magnification ($\times 200$).

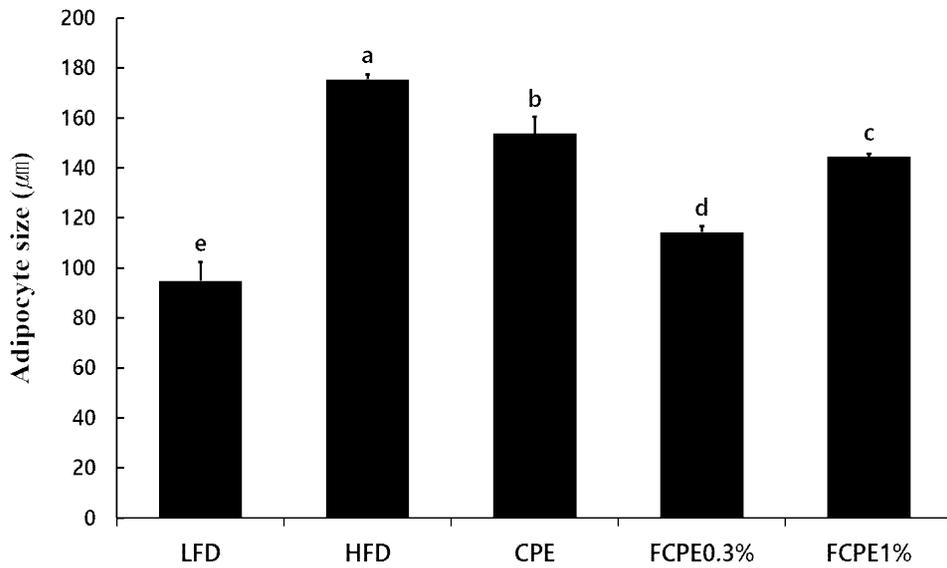


Fig. 7. Adipocyte size in the epididymal adipose tissue.

The data are mean \pm S.E.M. ^{abcd}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$.

3.7. Plasma lipid levels

The plasma TG levels and HDL - C levels were not significantly different among the experimental groups (Fig. 8A and 8C). However, the levels of TC were lower in the 0.3% FCPE and 1% FCPE group. The HFD group and CPE group showed the significantly high levels of TC (Fig. 8B).

Furthermore, significant suppressions were observed in the LDL - C levels of the FCPE 0.3% and FCPE 1% groups compared to the HFD group. In contrast, the CPE group showed significantly higher level of LDL - C compared with the HFD group (Fig. 8D).

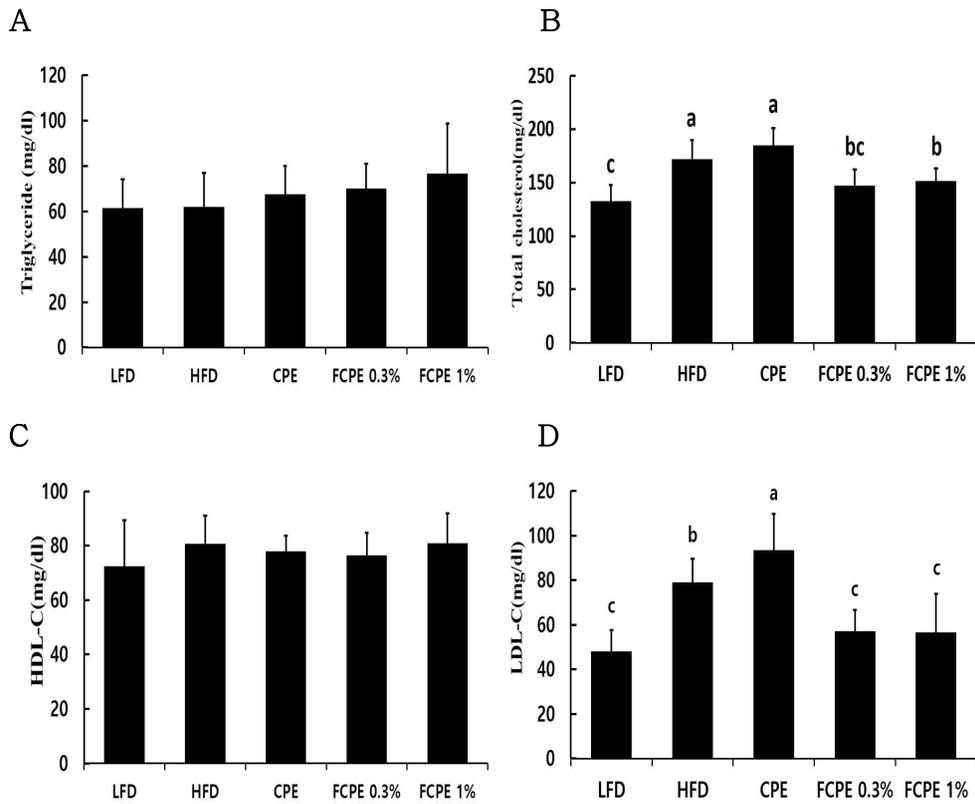


Fig. 8. Plasma lipid profile.

A, Triglyceride concentrations in plasma; B, Total cholesterol concentrations in plasma; C, HDL - C concentrations in plasma; D, LDL - C concentrations in plasma.

The data are mean \pm S.E.M. (n=8 for each group). ^{abc}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$

3.8. Expression of genes involved in hepatic glucose homeostasis and glucose transporter

We performed real-time PCR analysis to investigate the mechanism through which FCPE attenuates blood glucose levels (Fig. 9). FCPE supplementation groups showed significantly higher mRNA levels of *Gk* (glucokinase) than the control group. Moreover, FCPE 1% group had significantly higher expression of *Glut2* (glucose transporter protein type 2) than the control group. Although CPE group had no effect on mRNA levels of *Gk* and *Glut2*, *G6pase* (glucose-6-phosphatase) mRNA level, gluconeogenic enzyme, was significantly lower than the control and FCPE 1% group. There was no difference in the mRNA levels of *Pepck* among the groups.

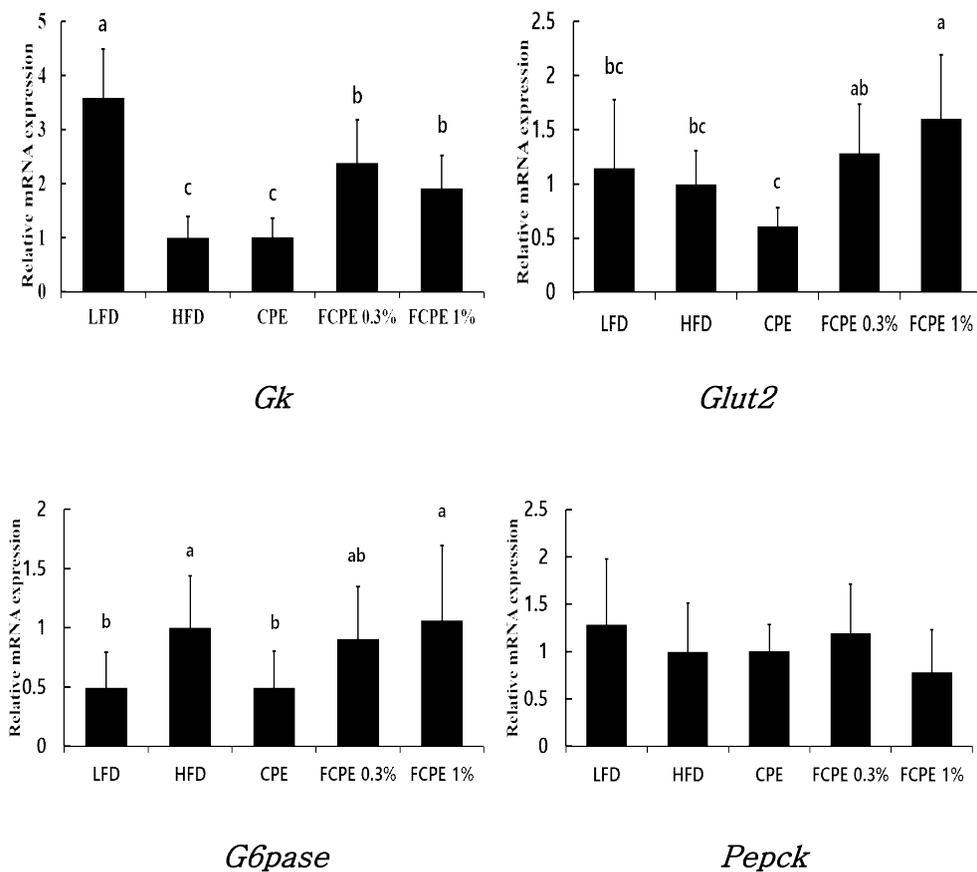


Fig. 9. The mRNA (*Gk*, *Glut2*, *G6pase*, *Pepck*) levels of genes related to hepatic glucose homeostasis and glucose transporter.

The data are mean \pm S.E.M. (n=8 for each group). ^{abc}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$.

3.9. Expression of genes involved in hepatic lipogenesis and fatty acid oxidation

To examine whether FCPE influenced hepatic lipogenesis and fatty acid oxidation, the mRNA levels of *Srebp1c* (sterol regulatory element binding protein 1c), *Acc* (acetyl-CoA carboxylase), *Fas* (fatty acid synthase) and *Cpt1* (carnitin palmitoyl transferase 1) were measured (Fig. 10). FCPE supplementation groups showed significantly lower levels of *Srebp1c* than HFD group. The mRNA levels of *Fas* were significantly lower in the FCPE 0.3% and slightly reduced in the CPE and FCPE 1% group, whereas *Acc* mRNA levels were significantly lower in the FCPE 1% group and slightly lower in CPE and FCPE 0.3% group. Moreover, *Cpt1* mRNA level was significantly higher in the FCPE 1% group than the HFD group.

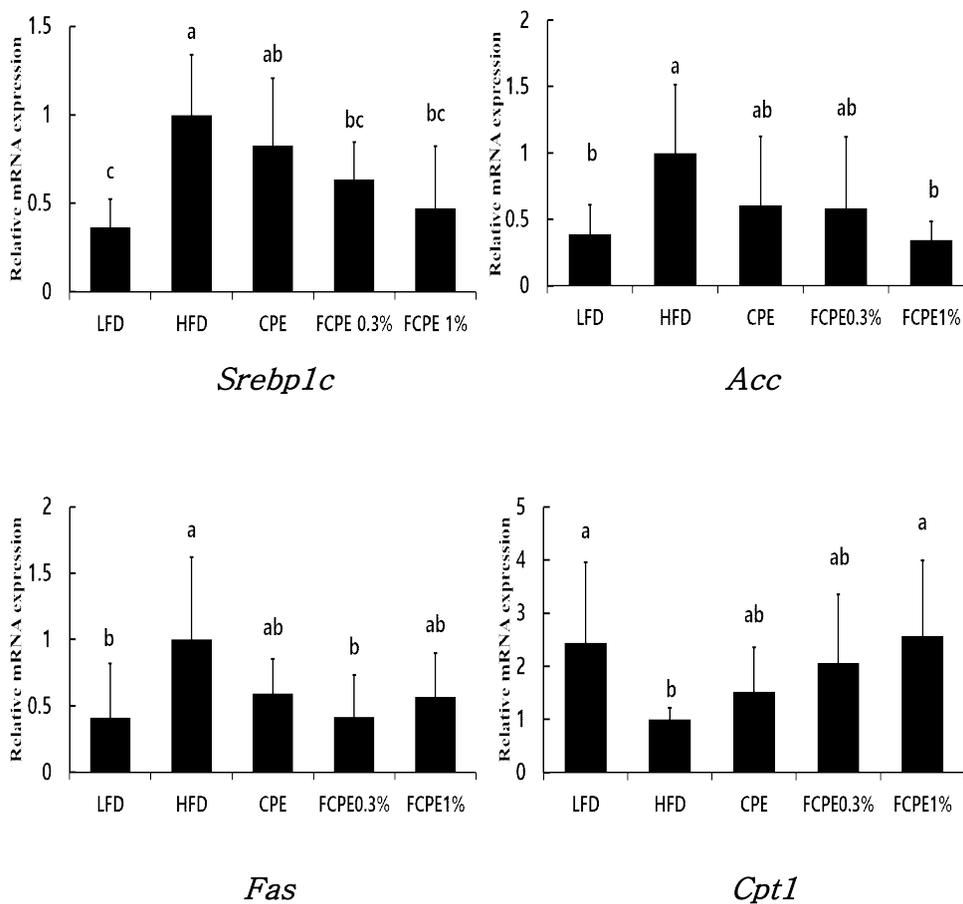


Fig. 10. The mRNA (*Srebp1c*, *Acc*, *Fas*, *Cpt1*) levels of genes related to hepatic lipogenesis and fatty acid oxidation.

The data are mean \pm S.E.M. (n=8 for each group). ^{abc}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$.

3.10. Expression of genes involved in hepatic cholesterol homeostasis

To determine whether the effect of FCPE on low levels of TC and LDL - C in the plasma was associated with changes in genes related to cholesterol homeostasis in the liver, we measured the mRNA levels of *Srebp2* (sterol regulatory element binding protein 2), *Hmgr* (HMG - CoA reductase), *Pcsk9* (proprotein convertase subtilisin/kexin type 9), *Ldlr* (LDL receptor), *Cyp7a1* (cholesterol 7 alpha - hydroxylase) and *Acat2* (acyl - CoA cholesterol acyltransferase 2) (Fig. 11 and Fig. 12). The mRNA levels of *Srebp2* were significantly lower in the FCPE groups. The mRNA levels of *Hmgr* were also significantly lower in the FCPE 0.3% and FCPE 1% groups. In addition, the expression levels of *Pcsk9*, which could lead to the degradation of LDL receptor [26], were also significantly lower in the both FCPE groups. However, no significant differences were observed in mRNA levels of *Ldlr*, *Cyp7a1* and *Acat2* among the groups.

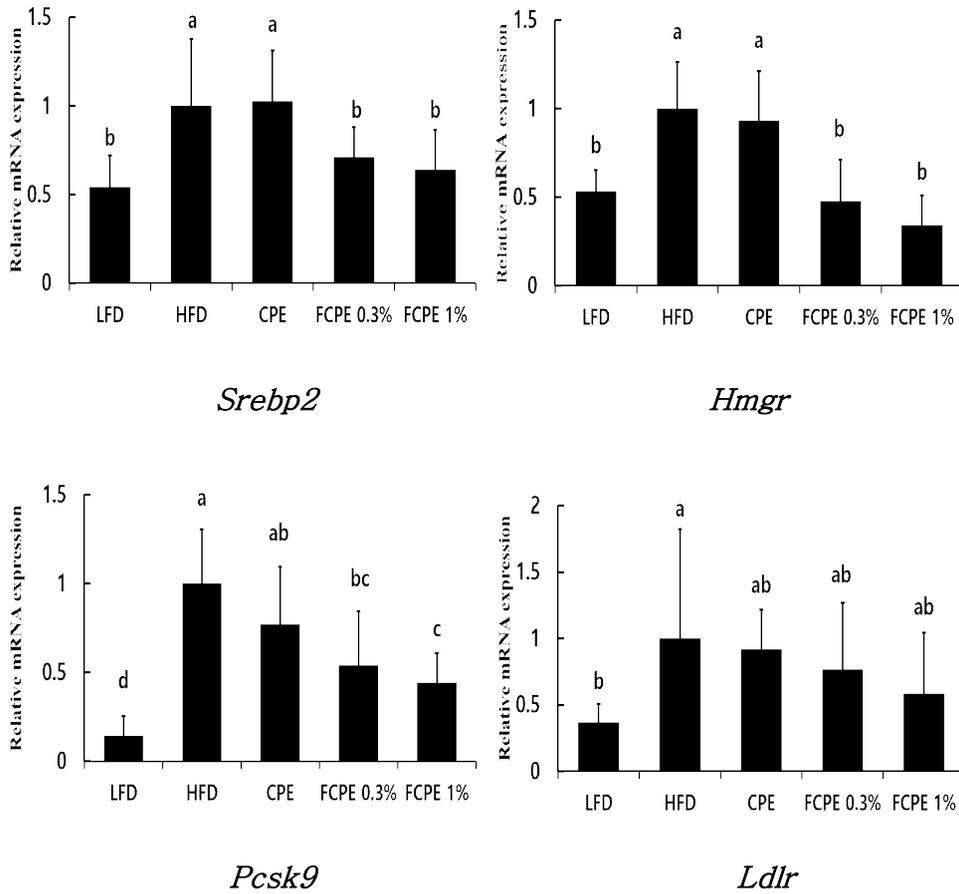


Fig. 11. The mRNA (*Srebp2*, *Hmgr*, *Pcsk9*, *Ldlr*) levels of genes related to hepatic cholesterol homeostasis.

The data are mean \pm S.E.M. (n=8 for each group). ^{abcd}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$.

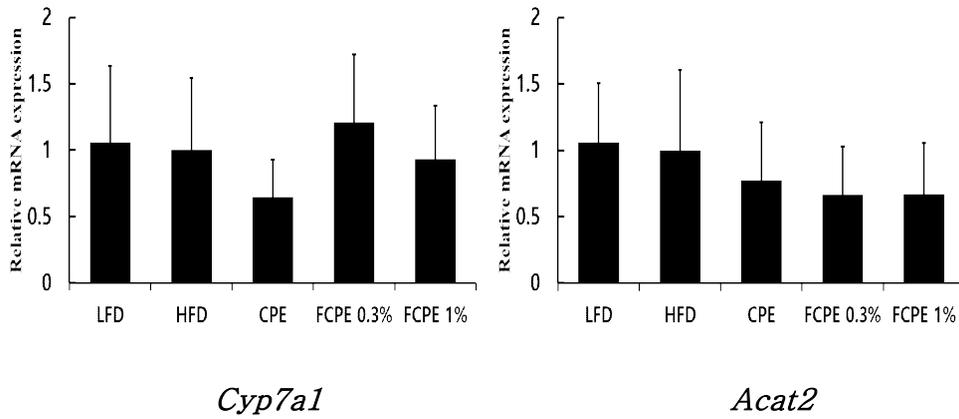


Fig. 12. The mRNA (*Cyp7a1*, *Acat2*) levels of genes related to hepatic cholesterol homeostasis.

The data are mean \pm S.E.M. (n=8 for each group). ^{abc}Means in the same row not sharing a common letter are significantly different groups at $p < 0.05$.

4. Discussion

This study investigated the anti-obesity and anti-hyperglycemia effect of fermented citrus unshiu peel extract in the high-fat diet fed mice. The present study showed that the FCPE supplementation groups had significantly lower body weight gains and epididymal fat weight compared to the HFD group. In addition, lipid droplet accumulation was less pronounced in the FCPE groups. Adipocyte size of epididymal fat was significantly lower in the FCPE groups. The levels of TC and LDL-C in plasma were significantly lower in the FCPE groups. Furthermore, the fasting blood glucose levels were significantly lower in the FCPE groups in comparison to the HFD group.

The major flavonoids in the CPE were narirutin and hesperidin in agreement with the other studies reporting that the major composition of the flavonoids in citrus unshiu peel extract were narirutin and hesperidin [27]. The narirutin content was higher than that of hesperidin content in the CPE, whereas other studies showed that hesperidin content was the highest among the flavonoids in the citrus unshiu peel extract [14, 27]. However, the glycoside forms, narirutin and hesperidin, require hydrolysis to convert them to their active aglycone forms, naringenin and hesperitin, for a more effective bioactivity [16]. Thus, citrus unshiu peel extract was fermented by mycotoxin non-producing *Aspergillus niger* FMB S46494 to convert glycoside form to the aglycone form. As a result, 14.1 mg/g of naringenin was found in the FCPE. *A. niger* FMB S46494 has

been shown to have a strong deglycosylating activity that efficiently transforms protopanaxadiol (PPD) type ginsenosides in the Korean ginseng berry to compound K, which is the deglycosylated forms, through fermentation [28].

In the current study, high-fat diet successfully induced increase of body weight, epididymal fat weight and adipocyte size as well as elevated the plasma TC and LDL-C and deposition of hepatic lipid droplets in C57BL/6J mice. With FCPE supplementation, body weight and epididymal fat weight was lowered. Epididymal fat weight also lowered in the CPE group. The food intakes were rather increased in the CPE and FCPE groups. Thus, the FERs of CPE and FCPE were lower than that of the HFD. Therefore, mice fed CPE or FCPE supplementation showed lower efficiency of converting the feed nutrients into their own energy than mice fed only high-fat diet. These results are supported by a previous study which indicated that dietary citrus ichangensis peel extract in high-fat induced mice also exhibited weight loss due to the flavanones such as naringin and hesperidin [29].

Moreover, accumulation of lipid droplets in the liver was less pronounced in the FCPE supplementation groups. These results were accompanied by the down-regulation of *Srebp1c*, *Fas* and *Acc*. *Srebp1c*, which is a transcription factor, has a fundamental role in regulating *de novo* fatty acids synthesis by inducing the expression of the genes including *Fas* and *Acc* in the liver [30]. *Fas* and *Acc* are enzymes for *de novo* fatty acid synthesis. Moreover, the upregulated expressions of *Cpt1*, which is a critical enzyme for fatty acid oxidation, could lead to prevention

of lipid accumulation in the liver. Thus improvement in hepatic lipogenesis and fatty acid oxidation by FCPE supplementation contributes to attenuation of hepatic lipid accumulation. These findings correspond well with a previous report that naringenin supplementation prevented hepatic lipid accumulation by suppressing the expression of *Srebp1c* mRNA and stimulating the expression *Cpt1* mRNA in the liver [18]. However, hepatic lipid accumulation was identified through the morphological analysis not through the measurement of TG and TC levels in the liver. Therefore, further measurement of the hepatic TG and TC contents to verify the effect of FCPE on hepatic steatosis will be necessary in the future study.

In addition, the levels of plasma TC and LDL - C were suppressed in the FCPE groups. This result was caused by suppression of the *Srebp2*, *Hmgr* and *Pcsk9* mRNAs. *Srebp2*, which favors *de novo* cholesterol synthesis, induces the expression of HMG - CoA reductase, *Pcsk9* and other enzymes of cholesterol biosynthesis pathway [31]. *Hmgr* is the rate - limiting enzyme in the cholesterol biosynthesis pathway. Therefore, regulation of hepatic *Hmgr* is necessary to maintain cholesterol homeostasis in the circulation and liver [32]. Moreover, *Pcsk9*, which could lead to the degradation of LDL receptor [23], is known to prevent LDL uptake. Therefore, poor expression of *Pcsk9* is correlated with reduced circulating LDL - C levels and cardiovascular risk [33]. Interestingly, these findings do not correspond with the results that citrus unshiu peel extract, which contains narirutin and hesperidin, and citrus depressa Hayata, which contain nobiletin and tangeretin, do not affect the plasma

total cholesterol [9, 34]. In contrast, naringenin, the aglycoside form of naringin and narirutin, supplementation reduced plasma and hepatic cholesterol concentrations by suppressing *Hmgr* in high - cholesterol diet fed rats [35, 36]. These findings suggest that naringenin in the FCPE may partially contribute to a significant reduction of plasma cholesterol levels.

Furthermore, high - fat diet led to the elevation of fasting blood glucose levels and impaired glucose tolerance in this study. The high - fat diet could be the major cause of impaired glucose tolerance and condition of early type 2 diabetes in C57BL/6J mice [37]. In contrast to the HFD group, the FCPE supplementation groups showed a lower fasting blood glucose level and an attenuated impaired glucose tolerance than the HFD group. These positive effects seem to be associated with the enhanced *Glut2* and *Gk* mRNA expression. *Glut2*, primarily found in cell membranes of liver, is a major glucose transporter. Increased expression of *Glut2* in the liver could decrease blood glucose and insulin levels [38]. *Gk* is an enzyme that phosphorylates glucose to glucose - 6 - phosphate. Increased intracellular glucose - 6 - phosphate by *Gk* stimulates glycolysis and glycogen synthesis [39]. In addition, several studies have suggested that *Glut2* and *Gk* have central roles in the regulation of glucose metabolism [40, 41]. Previous studies have also shown that a normal diet with 2% citrus unshiu peel extract ameliorates hyperglycemia in *db/db* mice [9] and naringenin supplementation improved glucose tolerance in high - fat fed mice [18].

Surprisingly, the CPE did not exhibit beneficial effects on plasma lipids and blood glucose levels. This result is inconsistent with previous studies in which citrus unshiu peel extract ameliorated serum TG, hyperglycemia and hepatic lipid accumulation [9. 42]. The content of flavonoids, duration of study, differences in species and experimental food and significantly higher food intake than control group could have different effects on these results.

Although other studies of citrus peel extract have demonstrated that flavonoids mainly have an effect on physiological effects, other components in CPE and FCPE might have partially contributed to the presently observed anti-obesity and anti-hyperglycemia effect.

In conclusion, naringin in citrus unshiu peel extract was converted into naringenin through fermentation with *A. niger*. FCPE supplementation resulted in lower the body weight gain, food efficiency ratio and epididymal fat weight. In addition, hepatic lipid accumulation and plasma TC and LDL-C levels were lowered by inhibiting the mRNA expression for fatty acid synthesis and cholesterol synthesis and by activating the mRNA expression for fatty acid oxidation. Furthermore, FCPE lowered fasting blood glucose levels by up-regulating the GK and GLUT-2 mRNA expression. This result suggests that FCPE, which contains aglycoside flavanone, is a more potent material that could ameliorate obesity and obesity-induced hyperglycemia than that of CPE.

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국문초록

진피는 한국에서 생산되는 감귤의 껍질로써 플라보노이드를 포함한 다양한 생리활성 물질을 함유하고 있다고 알려져 있다. 그 중 진피에는 헤스페리딘, 나리루틴과 같은 플라보노이드를 풍부하게 함유하고 있으며, 이 성분들은 비만, 간지방증, 당뇨 질환의 예방 및 개선효과 등 다양한 생리적인 효과를 보인다고 보고되고 있다. 본 연구에서는 진피를 곰팡이 독소를 생산하지 않는 *Aspergillus niger* FMB S46494로 발효하여 배당체 형태의 나리루틴을 비배당체 형태의 나린제닌으로 전환하였다. 그 후, 고지방 식이에 진피 추출물과 발효한 진피 추출물을 첨가하였을 때, 비만과 고혈당에 미치는 영향을 확인하고자 하였다. HPLC 분석을 통해 나리루틴과 헤스페리딘이 진피 추출물 속의 주요 플라보노이드 성분인 것을 확인 하였으며, 발효 진피 추출물 속에는 나리루틴이 발효를 통해 나린제닌으로 일정량 전환되어 14.1 mg/g이 검출되었다. 동물 실험을 위해 4주령의 C57BL/6J 마우스를 각 여덟 마리씩 다섯 군으로 나누어 다섯 가지 실험 식이를 각각 13주간 제공하였다. 실험 식이는 총 식이 칼로리의 10%를 지방으로 공급하는 저지방 식이 (LFD), 총 식이 칼로리의 60%를 지방으로 공급하는 고지방 식이 (HFD), 그리고 고지방 식이에 진피 추출물을 1% 첨가한 식이 (CPE)와 발효 진피 추출물을 각각 0.3%와 1%을 첨가한 식이 (FCPE 0.3% 또는 FCPE 1%)로 구성되었다. FCPE 0.3%군과 FCPE 1%군에서는 HFD군에 비해 체중 증가량 ($p < 0.05$)과 부고환 지방무게 ($p < 0.05$)가 유의적으로 낮았다. 또한 공복혈당 수치도 유의적으로 낮게 나왔으며, 포도당 내성 검사를 통해 FCPE 0.3%군, FCPE 1%군은 포도당 투여 각각 60분, 120분 후부터 유의성 있는 혈당 감소를 보였다 ($p < 0.05$). 혈청 지질 검사를 통해 FCPE군 모두 총 콜레스테롤 수치와 LDL 콜레스테롤 수치가 HFD군에 비해 유의적으로 낮은 것 ($p < 0.05$)을 확인하였다. 간에서 해당과정과 당신생 과정에 참여하는 효소 (*Gk*, *G6pase*, *Pepck*), 간 내 포도당 운반체 (*Glut2*), 간에서의 지방산 합성과 산화 (*Srebp1c*, *Acc*, *Fas*,

CPT1), 간에서의 콜레스테롤 항상성 (*Srebp2*, *Hmgr*, *Pcsk9*, *Cyp7a1*, *Acat2*, *Ldlr*) 관련 유전자의 mRNA 발현량을 Real-time PCR 로 측정하였다. 간에서 *Gk*, *Glut2* 와 *CPT1*이 FCPE군들에서 높은 발현량을 보였다. 또한 *Srebp1C*, *Srebp2*, *Acc*, *Fas*, *Hmgr* 및 *Pcsk9*은 FCPE군들에서 모두 낮은 발현량을 보였다. 이를 통해 발효 진피 추출물이 혈액으로부터 간으로의 당의 흡수와 간에서의 지방산 산화를 촉진시키며, 반대로 간에서 지방산 및 콜레스테롤 생성을 억제할 수 있다는 것을 알 수 있다. 결론적으로 본 연구에서는 발효를 통해 배당체 형태의 플라보노이드인 나리루틴이 비배당체 형태인 나린제닌으로 전환되었으며, 나린제닌이 함유된 발효 진피 추출물이 비만과 고혈당을 개선할 수 있음을 시사한다.

주요어: 진피, 발효, *Aspergillus niger*, 플라보노이드, 항비만, 고혈당개선

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