Thesis for the Degree of Master of Science

Metabolic regulation of *Ecklonia stolonifera* extract in high-fat diet-induced obese mice

by

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Metabolic regulation of *Ecklonia stolonifera* extract in high-fat diet-induced obese mice
(고지방식이 유도 비만 마우스에서 곰피 추출물의 대사적 조절 연구)

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by
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ABBREVIATIONS

BWG: body weight gain

FER: food efficiency ratio

HOMA-IR: homeostatic index of insulin resistance

AST: aspartate aminotransferase

ALT: alanine aminotransferase

NAFLD: non-alcoholic fatty liver disease

WAT: white adipose tissue

TG: triglyceride

TC: total cholesterol

HDL-C: high-density lipoprotein cholesterol

HTR: HDL-cholesterol/total cholesterol ratio

AI: atherogenic index

qRT-PCR: quantitative reverse transcription polymerase chain reaction

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

SREBP1c: sterol regulatory element-binding protein 1c

ACC: acetyl-CoA carboxylase

FAS: fatty acid synthase

SCD1: stearoyl-CoA desaturase 1

PAP: phosphatidate phosphatase

CPT1a: carnitine palmitoyl transferase

PPARα: peroxisome proliferator-activated receptor alpha

MCP-1: monocyte chemoattractant protein-1

TNF-α: tumor necrosis factor-alpha

IL-6: interleukin-6

TLR2: toll-like receptor 2

TLR4: toll-like receptor 4

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

식이 유도 비만 마우스에서 곰피 추출물의 대사적 조절 연구

조수연

부경대학교 대학원 식품생명과학과

요 약

곰피는 갈조류로 플로로탄닌이라는 폴리페놀성 물질을 함유하고 있으며 이외에도 다양한 생리 활성 물질이 풍부한 것으로 알려져 있다. 곰피는 항산화, 항염증, 항고지혈증 및 항당뇨에 긍정적인 효과를 나타내는 것으로 보고되어 있지만, 곰피의 체지방 조절 및 인슐린 저항성, 비알콜성 지방간, 그리고 염증 반응 등의 비만과 관련된 대사적 조절에 관한 연구는 부족한 실정이다. 본 연구는 식이 유도성 비만 동물 모델을 대상으로 곰피 추출물 (Ethanolic extract of *Ecklonia stolonifera*)이 비만과 관련된 대사적 조절에 미치는 영향과 작용 메커니즘을 규명하고자 하였다.

실험은 수컷 C57BL/6J 마우스를 정상식이군 (ND, normal diet), 고지방식이군 (HFD, high fat-diet, 20% fat), 곰피 추출물군 (ES, HFD + 0.006% *Ecklonia stolonifera* extract)으로 나누어 16주간 진행되었으며, 마우스 체중, 지방조직 중량, 백색지방조직의 지방구 크기, 혈장과 간조직의 지질 함량, 간조직의 지방구 축적, 혈중 adipocytokine 및 aminotransferase 수준, 분변 중 지질 함량, 공복 혈당, 혈장 인슐린, 인슐린 저항성 지표, 그리고 지방조직과 간조직의 생화학적 마커 및 유전자 발현을 분석하였다.

곰피 추출물은 고지방식이만을 섭취한 대조군과 비교하여 마우스의 체중, 식이 효율과 부고환 지방, 신장 주위 지방, 장간막 지방 등 복부 지방 무게를 유의적으로 감소시켰다. 또한, hematoxylin & eosin (H&E) 염색을 통해 곰피추출물이 지방조직의

세포 크기를 감소시켰음을 확인하였다. 곰피 추출물의 보충은 고지방식이에 의해 증가된 공복 혈당, 인슐린 저항성 지표인 HOMA-IR 및 혈장 leptin과 resistin 농도를 유의적으로 감소시켰다. 또한 곰피 추출물은 혈중 지질 농도뿐만 아니라 간 무게, 간조직의 중성지방 및 콜레스테롤 함량, 간조직의 지방구 축적 및 혈장 aminotransferase 수준을 감소시켰다. 분변의 지질 농도는 고지방식이군과 곰피 추출물군에서 비슷한 경향을 보였다. 하지만 곰피 추출물은 지방조직에서 지방산 합성과 관련된 FAS의 효소활성도를 유의하게 감소시켰고, ACC, FAS, SCD1을 포함한 lipogenic gene과 이들의 transcription factor인 SREBP1c mRNA 발현을 감소시켰다. 반면 지방산 산화와 관련된 CPT1a 및 PPARα의 mRNA 발현을 지방조직에서 유의적으로 증가시켰다. 또한, 곰피 추출물은 간조직에서 지방산 β-oxidation 효소 활성도와 관련 유전자인 CPT1a의 mRNA 발현을 유의적으로 증가시켰으며, lipogenic gene인 FAS 및 SCD1의 mRNA 발현을 유의적으로 감소시켰다. 곰피 추출물은 혈장에서 MCP-1 및 TNF-α 농도뿐만 아니라 지방조직과 간조직에서 MCP-1, IL-6, TNF-α, TLR2, TLR4 및 NF-κB를 포함한 염증성 인자의 mRNA 발현을 감소시켰다.

결과적으로, 곰피 추출물은 지방조직 및 간조직에서 지질 대사 조절 효소 활성 및 유전자의 발현을 조절하여 지질 합성을 감소시키고 지방산 산화를 촉진시켜 체지방 감량 및 혈장과 간조직의 지질 축적 감소의 효과를 나타낸다. 또한, 고지방식이로 인한 인슐린 저항성과 염증반응을 억제하였다. 따라서, 곰피는 지방조직과 간조직의 지질 대사 조절, 인슐린 저항성과 염증성 인자의 발현 억제를 통해 비만과 관련된 대사질환을 개선할 수 있는 효과적인 식품 소재로써 활용 가능할 것으로 평가된다.

I. INTRODUCTION

1. Purpose of study

Obesity is thought to be an important problem in adult's health as well as children, and its incidence is steadily increasing worldwide (World Health Organization, 2020). Obesity is a complex disease that can cause a variety of metabolic diseases including type 2 diabetes, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), cancer and cardiovascular disease (Katsiki et al., 2016). Therefore, preventing and treating obesity is essential. Currently, orlistat, naltrexone extended-release (ER)/bupropion ER (Contrave), phentermine/topiramate controlled-release (Qsymia), and liraglutide (Saxenda) are well known as anti-obesity drugs approved by the U.S. Food and Drug Administration (FDA) (Son and Kim, 2020). However, these medications can cause side effects such as insomnia, thirst, headache, constipation, and malformation (Yun, 2010). Since natural products have a low risk of such side effects, many studies have been performed to evaluate the anti-obesity effect of natural products (Asai and Miyazawa, 2001; Wu et al., 2013).

Seaweeds, or marine macroalgae, have been consumed as food mainly in Asia such as China, Japan, and South Korea. In western countries, seaweeds have traditionally been used as animal feed and fertilizers. In the eighteenth and nineteenth centuries, they have been traded in processed form such as medicine, salt and gelling agent. In recent years, seaweeds have been recognized as potential sources of bioactive and pharmaceutical compounds (Gomez-Zavaglia et al., 2019). They are classified as green algae, brown algae and red algae. Today, brown algae (66.5%) is the most consumed, followed by red algae (33%) and green algae (5%) (Lorenzo et al., 2017). Seaweeds are rich in various bioactive substances such as proteins, vitamins, polysaccharides, polyunsaturated fatty acids and polyphenol. Many studies have established the biological and pharmacological properties of

polysaccharides (e.g. fucoxanthin, fucoidan, laminarin, alginate) and polyphenolic compounds (e.g. phlorotannin) found in brown seaweeds (Wan-Loy and Siew-Moi, 2016). In instance, fucoxanthin reduces lipid accumulation during adipocyte differentiation without affecting cell viability in 3T3-L1 cells (Maeda et al., 2006; Seo et al., 2016), and improves inflammation by suppressing activation of NF-kB, MAPK and Akt in LPS-induced BV2 microglial cells (Park et al., 2011). In addition, fucoidan inhibits adipogenesis through the MAPK pathway in 3T3-L1 cells (Kim et al., 2010) and has an antitumor effect *in vitro* and *in vivo* (Oliveira et al., 2020).

Ecklonia stolonifera is a brown alga that is rich in various physiologically active substances including phlorotannins. *Ecklonia stolonifera* has been reported to exhibit antioxidant, anti-inflammatory, hypolipidemic, hepatoprotective, and anti-diabetic activities (Manandhar et al., 2019; Kim et al., 2011; Moon et al., 2011; Murray et al., 2018). However, anti-obesity activity of *Ecklonia stolonifera* and its underlying mechanisms have not been fully elucidated. Therefore, the present study evaluated the effects of *Ecklonia stolonifera* on adiposity, insulin resistance, lipid metabolism and inflammation in diet-induced obese mice and elucidated the underlying mechanism of these effects.

2. Background

2.1. Lipid metabolism and insulin resistance related to obesity

White adipose tissue (WAT) and liver are responsible for lipid synthesis and storage (Laurent et al., 2013). Lipid is an important element of the cell organ that constitutes the cell membrane and functions as energy storage and signal transduction (Pomorski et al., 2001). Lipid metabolism is a process in which lipids such as fatty acid, phospholipid, triglyceride, and cholesterol are synthesized and degraded. WAT stores excess energy in form of triglycerides, and regulates lipid mobilization and distribution in the body, and excessive energy accumulation leads to obesity (Langin, 2011). In contrast, in periods of energy deprivation, triglyceride stored in WAT are broken down into glycerol and free fatty acid (FFA)s through lipolytic pathway, and released FFAs are transported to liver, muscle and other organs and are subsequently utilized by fatty acid oxidation, which drives lipid distribution and controls whole body energy balance (Jung and Choi, 2014). In particular, excessive visceral fat is related to impaired inhibition of FFA release, hypertriglyceridemia and low concentrations of high-density lipoprotein (HDL) cholesterol (Ebbert and Jensen, 2013). Excess released FFAs can accumulate in other tissues including liver, which leads to impaired insulin sensitivity (Jung and Choi, 2014).

NAFLD is characterized by excessive accumulation of fat in the liver and ranges over a wide spectrum from hepatic steatosis to NASH, fibrosis, cirrhosis and hepatocellular carcinoma (Yilmaz et al., 2014; Hassan et al., 2014). Since the accumulation of triglyceride (TG) in the liver results from esterification of glycerol and FFA, aberrant cellular esterification for TG synthesis can causes NAFLD (Fabbrini et al., 2010). Dysregulation of hepatic lipid metabolism that leads to NAFLD can also contribute to the development of atherogenic dyslipidemia, especially increased circulating TG and remnant lipoprotein cholesterol levels, and small dense LDL particles (Deprince et al.,

2020). In patients with NAFLD, cardiovascular disease is reported to be the main cause of death (Byrne and Targher, 2015).

Lipogenic transcription factor, such as sterol regulatory element-binding protein 1c (SREBP1c), is a major regulator of lipid metabolism including lipogenesis and lipolysis (Brown and Goldstein, 1997; Horton and Shimomura, 1999). SREBP1c stimulates upregulation of several lipogenic genes including fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), Stearoyl-CoA desaturase 1 (SCD1) and phosphatidate phosphatase (PAP). ACC catalyzes acetyl-CoA to malonyl-CoA (Brownsey et al., 1997). FAS is known as a major lipogenic enzyme that catalyzes the biosynthesis of long-chain fatty acids from acetyl-CoA precursors (Boizard et al., 1998). SCD1 is a rate-limiting enzyme in the cellular synthesis of monounsaturated fatty acids, which facilitates then the conversion of stearic acid to oleic acid during lipogenesis (Ntambi, 1999). PAP is a rate-limiting enzyme for hepatic triglyceride synthesis. PAP catalyzes the dephosphorylation of phosphatidate and produces diacylglycerol and inorganic phosphate, and it also generates lipid-signaling molecules related to phosphatidate or degrades it (Carman and Han, 2006).

By contrast, lipid accumulation can be reduced in adipose tissue and liver by upregulation of genes involved in β-oxidation including peroxisome proliferator-activated receptor alpha (PPARα) and carnitine palmitoyl transferase 1a (CPT1a). CPT1a promotes the transfer of long-chain fatty acids across the mitochondrial membrane during β-oxidation (Wakil and Abu-Elheiga, 2009; Gao et al., 2011). CPT1a and PPARα upregulate fatty acid β-oxidation in mitochondria and decrease lipid accumulation, and activated PPARα can further enhance the transcription of CPT1a.

Insulin plays important role in promotion of preadipocytes differentiation and increases capacity of TG storage in adipose tissue (Kahn and Flier, 2000). It is reported that increased levels of FFA, glycerol, hormones, pro-inflammatory cytokines and other factors released from adipose tissue are associated with insulin resistance (Kahn et al.,

2006). Insulin resistance suppresses insulin signaling pathway and increases insulin secretion from ß-cell (LeRoith, 2002). In addition, insulin resistance leads to type 2 diabetes and is related to hypertension, hyperlipidemia, atherosclerosis and polycystic ovarian disease (Reaven, 1995).

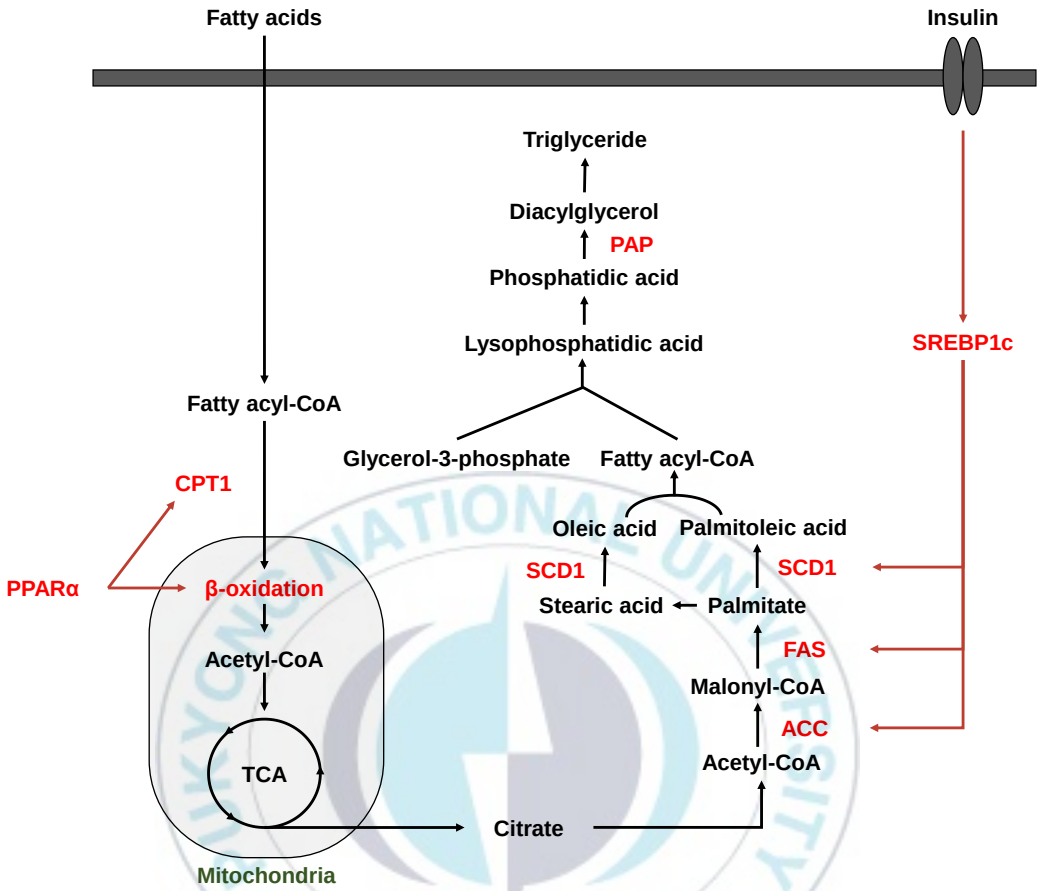


Figure 1. Lipid metabolism pathway.

2.2. Inflammation in obesity

Adipose tissue inflammation is a major cause of obesity-related metabolic syndromes. Adipose tissue is an important endocrine organ, regulating peripheral inflammation as well as lipid metabolism (Chazenbalk et al., 2012). Adipokines secreted from adipose tissue regulate appetite, satiety, immunity, inflammation, adipogenesis and insulin sensitivity (Francisco et al., 2018; Blüher, 2014; Yang et al., 2015). Among various adipokines, MCP-1 increases adipose tissue inflammation in obesity by attracting macrophage infiltration into adipose tissue (Cranford et al., 2015; Xu et al., 2015). TNF-alpha is a cytokine secreted by adipose tissue, and it is related to pro-inflammation and insulin resistance (Lumeng et al., 2007; Khosravi et al., 2013). Leptin, a peptide hormone produced and secreted by adipocyte, regulates energy intake and consumption (Zhang et al., 1994). Leptin induces suppressing appetite and increases energy consumption through the hypothalamus, leading to loss of weight and adipose tissue (Fruhwürth et al., 2018). Obesity induces leptin resistance and hyperleptinemia (Yang and Barouch, 2007). Leptin increases macrophage accumulation and levels of pro-inflammatory cytokines such as TNF-α and MCP-1. Resistin is also related to pro-inflammation and activates NF-κB pathway, an important transcription factor of inflammation.

The occurrence of inflammation also causes serious problems in the liver. Excessive secretion of inflammatory cytokines causes chronic inflammation, which can lead to fatty acid disorders in the liver (Zhao et al., 2015). Obesity is associated with activation of the TNF signaling pathway. In addition, it promotes the generation of cytokines such as IL-6 and TNF, increasing the risk of hepatic inflammation and tumor development, including steatohepatitis (Chen et al., 2017; Park et al., 2010).

II. MATERIALS AND METHODS

1. Preparation of ethanolic extract of *Ecklonia stolonifera*

The powdered leafy thalli of *Ecklonia stolonifera* was extracted with 70% ethanol under reflux and then filtered. The filtrate was concentrated under reduced pressure to obtain 70% EtOH extract (yield 23.5 ± 1.8%). The sample was provided by Prof. Jae Sue Choi of Pukyong National University (Busan, Korea).

2. Animals and experimental design

Male C57BL/6J mice (4 weeks old), purchased from Hana Biotech Inc. (Pyeongtaek, Republic of Korea), were individually housed at a constant temperature (24 ± 2 °C) and humidity conditions (50 ± 5%) with a 12-h light/dark cycle. The mice were fed a pelletized commercial chow diet during 1 week of the acclimation period, and then they were divided into three groups randomly. Thereafter, the mice fed each diet for 16 weeks: normal diet (ND, n=5), high-fat diet (HFD, 20% fat based on the AIN-76 diet plus 1% cholesterol, w/w, n=9), high-fat diet + *Ecklonia stolonifera* extract (ES, 0.006%, w/w, n=9) (Table 1).

During the experimental period, food intake was monitored three times a week, and body weight was measured weekly. Fasting blood glucose levels were measured at 8 and 16 weeks.

All animal experiment were approved by the Animal Ethics Committee of Pukyong National University (Approval No. 2019-25).

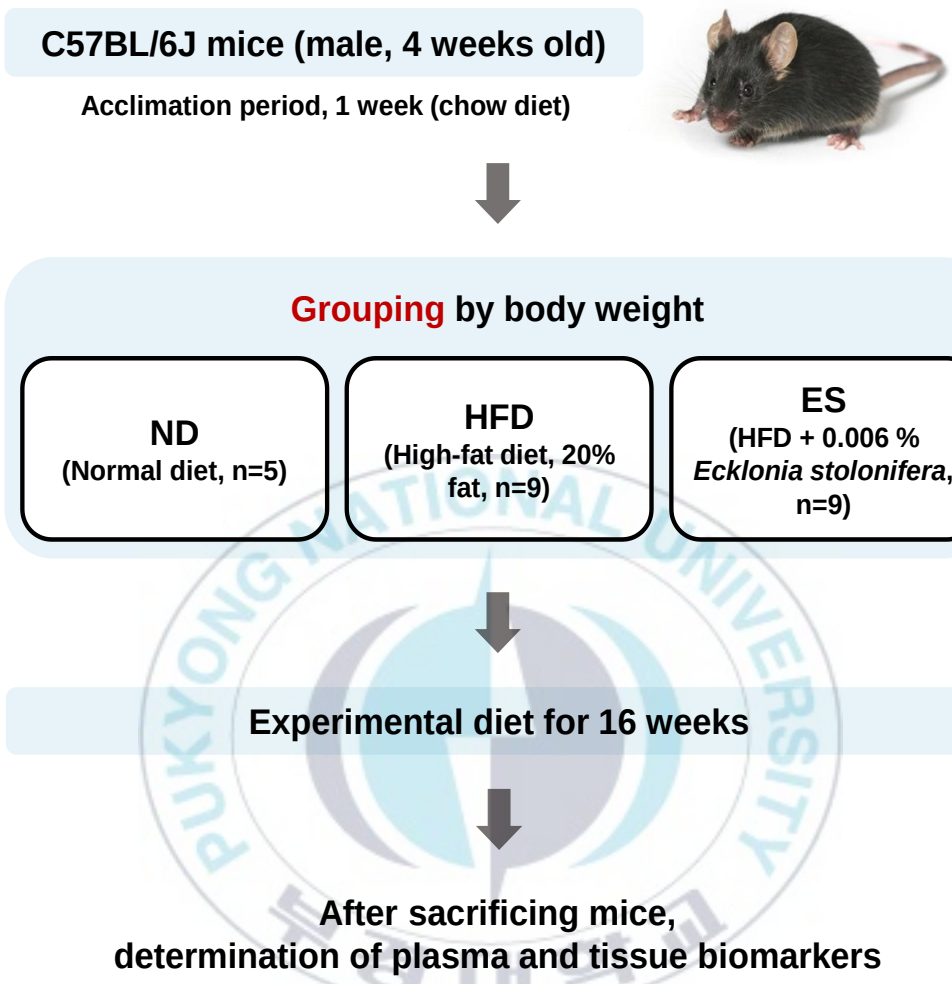


Figure 2. Experimental design. ND, normal diet; HFD, high-fat diet; ES, high-fat diet + 0.006% *Ecklonia stolonifera* extract.

Table 1. Diet ingredient composition (% of diet, w/w)

Ingredient	ND	HFD	ES
Choline Bitartrate	0.2	0.2	0.2
DL-Methionine	0.3	0.3	0.3
Cholesterol	-	1	1
Vitamin mix (AIN-76) [1]	1	1.2	1.2
Mineral mix (AIN-76) [2]	3.5	4.2	4.2
Lard	-	17	17
Sucrose	50	37	37
Corn oil	5	3	3
Cellulose	5	5	5
Corn starch	15	11.1	11.1
Casein	20	20	20
Tert-Butylhydroquinone	0.001	0.004	0.004
Ecklonia stolonifera ethanolic extract	0	0	0.006
Total (g)	100	100	100

ND, normal diet; HFD, high-fat diet (45 kcal% fat, 35 kcal% carbohydrates, 20 kcal% protein); ES; HFD + 0.006% *Ecklonia stolonifera* ethanolic extract. [1] Vitamin mixture AIN-76 (g/Kg): thiamin (81%) 0.6, riboflavin 0.6, pyridoxine HCl 0.7, niacin 3, calcium pantothenate 1.6, folic acid 0.2, biotin 0.02, vitamin B12 (0.1% in mannitol) 1, vitamin A palmitate (500,000 IU/g) 0.8, vitamin E, DL-alpha tocopheryl acetate (500 IU/g) 10, vitamin D3, cholecalciferol (400,000 IU/g in sucrose) 0.25, vitamin K, MSB complex 0.15, sucrose, fine ground 981.08. [2] Mineral mixture AIN-76 (g/Kg): sucrose, fine ground 118.03, calcium phosphate, dibasic 500, sodium chloride 74, potassium citrate, monohydrate 220, potassium sulfate 52, magnesium oxide 24, manganous carbonate 3.5, ferric citrate 6, zinc carbonate 1.6, cupric carbonate 0.3, potassium iodate 0.01, sodium selenite, pentahydrate 0.01, chromium potassium sulfate, dodecahydrate 0.55.

3. Sampling procedure

At the end of the breeding period, the mice were anesthetized with CO_2 after 12-h fasting, and then blood samples were collected from inferior vena cava using a syringe with heparin. The plasma was separated by centrifugation at 3,000 rpm and 4 ℃ for 15 minutes and stored at -80 ℃. The heart, liver, kidney, spleen, and white adipose tissues (WAT; epididymal, perirenal, retroperitoneal, mesentery, subcutaneous WAT) were removed and rinsed with physiological saline solution, and then weighed. After that, liver and epididymal WAT were stored at -80 ℃ for subsequent analyses. For 1 week before the end of the experimental period, the feces were collected and stored at -20 ℃.

4. Biochemical analysis

4.1. Blood glucose analysis

Fasting blood glucose levels were measured in blood collected from the tail vein using blood glucose meter (Accu-Chek Performa, Roche Diagnostics Unc., Indianapolis, IN, USA).

Plasma glucose level was determined with commercial enzymatic kits (Asan Pharm Co., Seoul, Korea).

4.2. Plasma lipids analysis

Plasma triglyceride (TG), total-cholesterol (TC), and HDL-cholesterol (HDL-C) levels were determined by using commercial kits (Asan Pharm Co., Seoul, Korea). The ratio of HDL-cholesterol to total-cholesterol (HTR) and atherogenic index (AI) were calculated using the following formula: HTR (%) = [(HDL-cholesterol)/(total-cholesterol)] × 100; AI = [(total-cholesterol) - (HDL-cholesterol)] / HDL-cholesterol.

4.3. Hepatic lipids analysis

Hepatic lipid extraction was performed by modifying the method of Folch et al. (1957). Hepatic tissue (0.1 g) was homogenized with 2 mL of FM solution ($CHCl_3$: CH_3OH = 2:1, v/v), and then filtered using Whatman filter paper (Whatman Co., Maidstone, UK) and dried with nitrogen gas (60 ℃). The sample was re-dissolved in 1 mL of FM solution, and among them, 200 μL of the sample was dried again with nitrogen gas (60 ℃). Then, the sample was dissolved in 2 mL ethanol. Hepatic TG and TC levels were determined by the same commercial kits used in plasma lipid analysis.

4.4. Fecal lipids analysis

Fecal lipid extraction was performed by modifying the method of Folch et al. (1957). The dried feces ground using a mortar, and then 0.25 g of feces powder dissolved with 5 mL of FM solution for extracting 24 hours at 4 ℃. The fecal lipid extract was centrifuged for 10 minutes at 3,000 rpm and 4 ℃. After that, 3 mL of supernatant was transferred and dried with nitrogen gas (50 ℃). The sample was re-dissolved in 500 μL of FM solution, and among them, 200 μL of the sample was dried again with nitrogen gas (60 ℃). Then, the sample was dissolved in 5 mL ethanol. The concentrations of fecal TG and TC were determined by the same commercial kits used in plasma and hepatic lipid analysis.

4.5. Plasma AST, ALT analysis

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined by using commercial kits (Asan Pharm Co., Seoul, Korea).

4.6. Plasma adipocytokine analysis

Plasma concentrations of Insulin, leptin, resistin, IL-6, MCP-1, and TNF-α were measured using MILLIPLEX map Mouse Adipokine Magnetic Bead Panel kit (MERCK, Darmstadt, Germany). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as follows: [insulin concentration (mU/L) × fasting blood glucose (mg/dl)×0.05551]/22.5.

4.7. Hepatic enzyme activity analysis

4.7.1. Preparation hepatic enzyme source

Hepatic enzyme fractions of mitochondria, cytosol and microsome were prepared by referring to the Hulcher and Oleson (1973). Liver tissue (0.5 g) was homogenized with 3 mL of buffer A (0.1M triethanolamine, 0.02M EDTA-2Na, 0.002M dithiothreitol). After that, it was centrifuged at 3,000 rpm and 4 ℃ for 15 minutes. The supernatant was separated and centrifuged again at 13,000 rpm and 4 ℃ for 15 minutes. The pellet was dissolved with 1 mL of buffer A and centrifuged at 13,000 rpm and 4 ℃ for 20 minutes. After that, the supernatant was removed and the acquired pellet was dissolved with 1 mL of buffer A as a mitochondrial sample. In the previous process, centrifuged at 13,000 rpm and 4 ℃ for 15 minutes, the separated supernatant was centrifuged at 32,500 rpm and 4 ℃ for 1 hour. The supernatant was used for the cytosolic sample. The pellet was dissolved in 1 mL of buffer and centrifuged at 32,500 rpm and 4 ℃ for 1 hour. Then acquired pellet was dissolved with 1 mL of buffer A for using a microsomal sample.

4.7.2. Hepatic enzyme activity

The FAS activity in cytosol was measured by using the method developed by Carl et al. (1975). PAP activity was determined according to the method of Walton et al. (1996). Activities of CPT and beta-oxidation were determined according to the method of Bieber et al. (1972) and Lazarow et al. (1981), respectively. The protein concentration was determined using the Bradford method (1976).

4.8. Epididymal WAT enzyme activities analysis

4.8.1. Preparation of epididymal WAT enzyme source

Epididymal WAT enzyme fraction was prepared by referring to the Hulcher and Oleson (1973). Epididymal WAT (0.5 g) was homogenized with 3 mL of buffer A (0.1M triethanolamine, 0.02M EDTA-2Na, 0.002M DTT), and then it was centrifuged at 3,000 rpm and 4 ℃ for 15 minutes. The supernatant was separated and centrifuged again at 13,000 rpm and 4 ℃ for 15 minutes. After that, the supernatant was centrifuged at 32,500 rpm and 4 ℃ for 1 hour. The acquired supernatant was used for the cytosolic sample.

4.8.2. Enzyme activity in epididymal WAT

The FAS activity in cytosol was measured by the Carl et al. (1975). The concentrations of protein were determined by the Bradford method.

5. Morphological analysis

Liver and epididymal WAT were immediately fixed in 10% formaldehyde after removal. And then, it was embedded in paraffin, and stained with hematoxylin & eosin (H&E). Stained area in the liver and epididymal WAT was analyzed with a microscope (Eclipse E200; Nikon, Tokyo, Japan) at 400× magnification.

6. RNA isolation and quantitative reverse transcription polymerase chain reaction analysis

6.1. RNA extraction and cDNA synthesis

Total RNA was extracted from liver and epididymal WAT with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, USA) using SpeedMill_PLUS (Analytik Jena Co., Jena, Germany). After adding chloroform and leaving it at room temperature for 5 minutes, it centrifuged at 13,000 rpm and 4 ℃ for 15 minutes. The supernatant was transferred and the same amount of isopropanol was added and left at room temperature for 10 minutes, and then centrifuged at 12,000 rpm and 4 ℃ for 10 minutes. After that, the pellet was centrifuged with 75% ethanol at 12,000 rpm and 4 ℃ for 5 minutes for washing. After washing twice, the pellet was dissolved with DEPC-treated water. The RNA concentration, purity, and integrity were determined with a Gen5 3.04 software program (BioTek Inc., Vermont, USA).

cDNA was synthesized from 1μg of RNA using High Capacity cDNA Reverse Transcription Kit (4368814, applied biosystems by Thermo Fisher Scientific, USA). After that sample was stored at -18 ℃.

6.2. Quantitative reverse transcription polymerase chain reaction analysis

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the SYBR Green PCR Master Mix (Applied Biosystems Inc., California, USA) by StepOnePlus™ Real-Time PCR System (Applied Biosystems Inc., California, USA). Primers were synthesized by Genotech Inc. (Daejeon, Korea). Primer sequences were shown in Table 2. The amplification was performed as follows: 10 min at 95 ℃ for 1 cycle, 15 sec at 95 ℃ and 1 min at 60 ℃ for 41 cycles. The cycle threshold (Ct) values were calibrated using the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Relative gene expression was calculated with the $2^{-\triangle\triangle Ct}$ method.

Table 2. Primer sequence used for qRT-PCR

Primer	Sequence (5'-3')
GAPDH-F	TGCAGTGGCAAAGTGGAGAT
GAPDH-R	TTGAATTTGCCGTGAGTGGA
SREBP1c-F	ATCGGCGCGGAAGCTGTCGGGGTAGCGTC
SREBP1c-R	ACTGTCTTGGTTGTTGATGAGCTGGAGCAT
ACC-F	GAATCTCCTGGTGACAATGCTTATT
ACC-R	GGTCTTGCTGAGTTGGGTTAGCT
FAS-F	GCTGCGGAAACTTCAGGAAAT
FAS-R	AGAGACGTGTCACTCCTGGACTT
SCD1-F	TGAGCTCAGTCTCACTCCTT
SCD1-R	AAAAGATTTCTGCAAACCAA
PAP-F	GGGTTCTACTGTGGAGATGA
PAP-R	TGACAGTAGCTGTGATGATGA
CPT1-F	ATCTGGATGGCTATGGTCAAGGTC
CPT1-R	GTGCTGTCATGCGTTGGAAGTC
PPARα-F	CTGAGACCCTCGGGGAAC
PPARα-R	AAACGTCAGTTCACAGGGAAG
MCP-1-F	TCAGCCAGATGCAGTTAACGC
MCP-1-R	TGATCCTCTTGTAGCTCTCCAGC
IL-6-F	GCTACCAAACTGGATATAATCAGG
IL-6-R	CCAGGTAGCTATGGTACTCCAGAA
TNF-α-F	TGGCCCAGACCCTCACACTCAGATC
TNF-α-R	GCCTTGTCCCTTGAAGAGAACCTGG
TLR2-F	GAGCATCCGAATTGCATCAC
TLR2-R	TATGGCCACCAAGATCCAGA
TLR4-F	CCTCTGCCTTCACTACAGAGACTTT
TLR4-R	TGTGGAAGCCTTCCTGGATG
NF-κB-F	CACCTAGCTGCCAAAGAAGG
NF-κB-R	GCAGGCTATTGCTCATCACA

7. Statistical analysis

Results are expressed as mean ± S.E.M. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software program. Student's *t*-test was used to evaluate the differences among groups. Probability value of $p < 0.05$ is considered as statistically significant difference between groups.

III. RESULTS

1. Effects of ES on food intake, body weight, adipose tissue weight, organ weight and morphology in HFD-induced obese mice

1.1. Food intake, body weight, body weight gain and food efficiency ratio

Food intake and body weight were measured for the experimental period. HFD significantly decreased food intake compared to the ND group. There were no significant differences in food intake between HFD and ES groups (Figure 3A). The body weight, body weight gain (BWG), and food efficiency ratio (FER) of the HFD group were markedly increased compared to the ND group (Figure 3B-D). However, ES significantly reduced body weight, BWG ($p < 0.05$) and FER ($p < 0.05$) (Figure 3B-D).

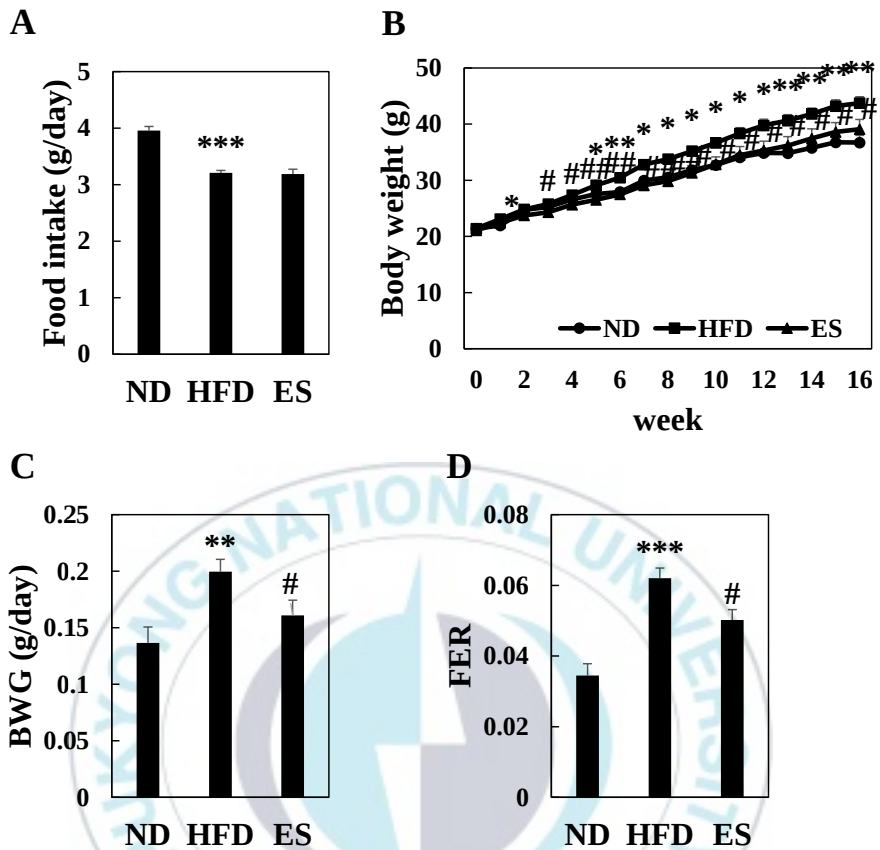


Figure 3. Effects of ES on food intake (A), body weight (B), body weight gain (C) and food efficiency ratio (D) in high-fat diet (HFD)-fed C57BL/6J mice. Data shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ND group versus HFD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; BWG, body weight gain; FER, food efficiency ratio.

1.2. Adipose tissue weight and organ weight

The weights of WAT and organs were calculated to weight per 100 g body weight. The epididymal, perirenal, retroperitoneal, mesentery, subcutaneous and visceral WAT weights were significantly higher in the HFD group compared to the ND group. Epididymal, perirenal, mesentery and visceral WAT weights were markedly decreased in the ES group compared to the HFD group ($p < 0.05$), although there were no significant differences in retroperitoneal and subcutaneous WAT weights between two groups (Figure 4A).

Liver weight was significantly increased in HFD group compared to the ND group and ES significantly reduced liver weight ($p < 0.05$) (Figure 4B). There were no significant differences in heart, kidney and spleen weights between HFD and ND groups (Figure 4C-E).

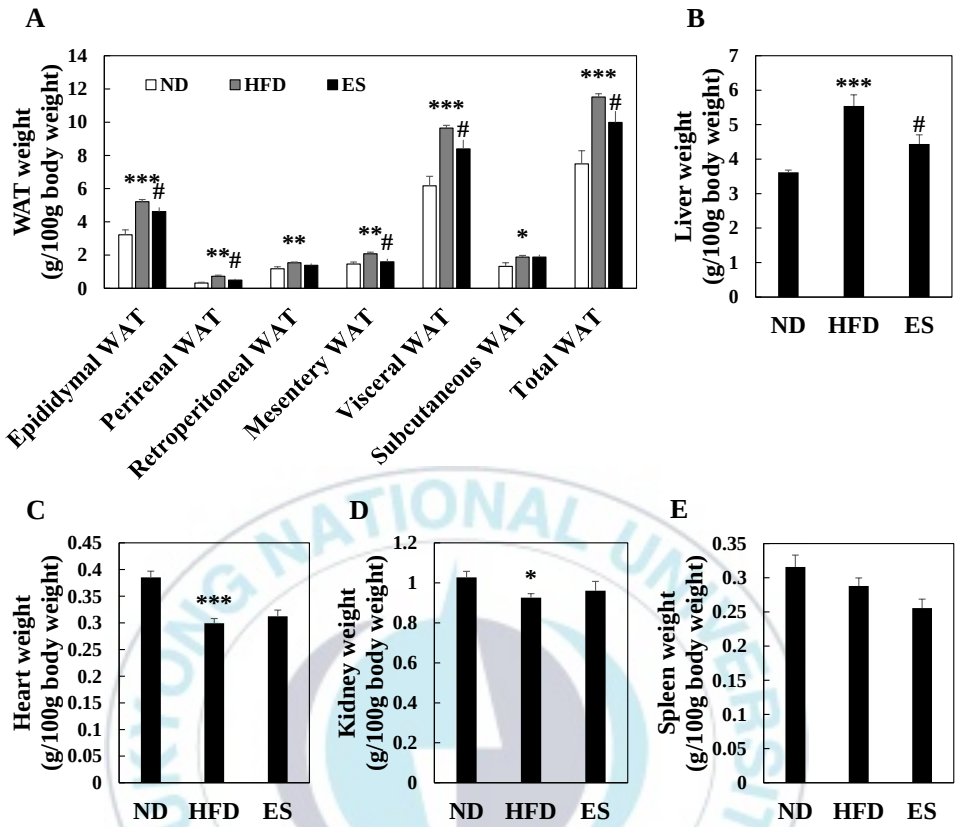


Figure 4. Effects of ES on WAT weight (A), liver weight (B), heart weight (C), kidney weight (D) and spleen weight (E) in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ND group versus HFD group, $^{\#}p < 0.05$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; WAT, white adipose tissue.

1.3. Morphology in epididymal WAT

The epididymal adipocytes were larger in the HFD group than that of the ND group and were smaller in the ES group to a similar level to the ND group (Figure 5).

Figure 5. Effect of ES on hematoxylin and eosin staining of epididymal white adipose tissue in HFD-fed C57BL/6J mice (×400). ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; H&E, hematoxylin and eosin. Representative photomicrographs of epididymal WAT are shown at ×400 magnification.

2. Effects of ES on blood glucose, plasma insulin, HOMA-IR, leptin and resistin levels in HFD-induced obese mice

After 8 weeks of the experimental period, there was no significant difference in fasting blood glucose. However, fasting blood glucose of the HFD group was higher than that of the ND group at 16 weeks. The ES-supplemented mice had a significant decrease in fasting blood glucose ($p < 0.01$) (Figure 6A). The plasma glucose, insulin, HOMA-IR, leptin, and resistin levels were significantly higher in the HFD group compared to the ND group, whereas ES significantly reduced plasma glucose ($p < 0.01$), HOMA-IR ($p < 0.05$), leptin ($p < 0.001$), and resistin ($p < 0.01$) levels compared to the HFD group (Figure 6B-F). The ES also tended to decrease plasma insulin level (diminished by 19%) compared to the HFD group (Figure 6C).

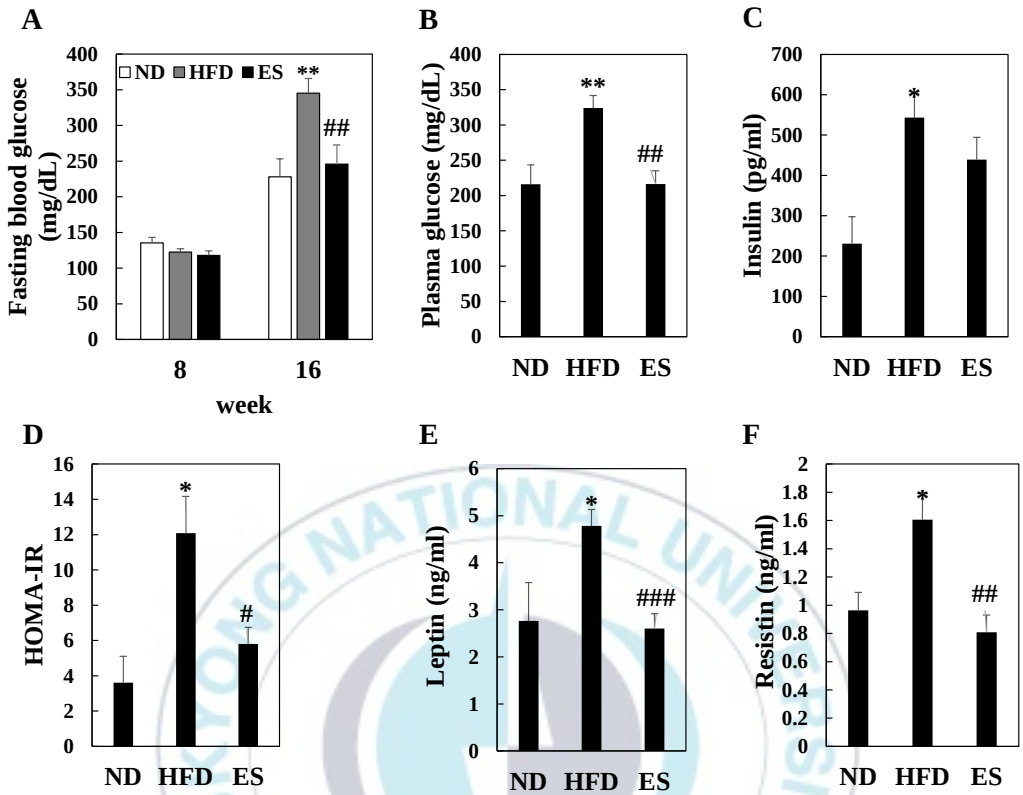


Figure 6. Effects of ES on fasting blood glucose (A), plasma glucose (B), plasma insulin (C), HOMA-IR (D), plasma leptin (E) and resistin (F) in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$, $^{**}p < 0.01$; ND group versus HFD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; HOMA-IR, homeostatic index of insulin resistance, [insulin concentration (mU/L) × fasting blood glucose (mg/dl) × 0.05551]/22.5.

3. Effects of ES on plasma, hepatic, fecal lipids levels and liver morphology in HFD-induced obese mice

3.1. Plasma lipids profile

There was no significant difference in plasma TG level between ND and HFD groups (Figure 7A). Plasma TC and HDL-C levels were significantly higher in the HFD group than in the ND group (Figure 7B), and there were no significant differences in HTR and AI between two groups (Figure 7D-E). ES significantly decreased plasma TG ($p < 0.05$) and TC ($p < 0.05$) levels compared to the HFD group (Figure 7A-B). There were no significant differences in levels of plasma HDL-C, HTR, and AI (Figure 7C-E).

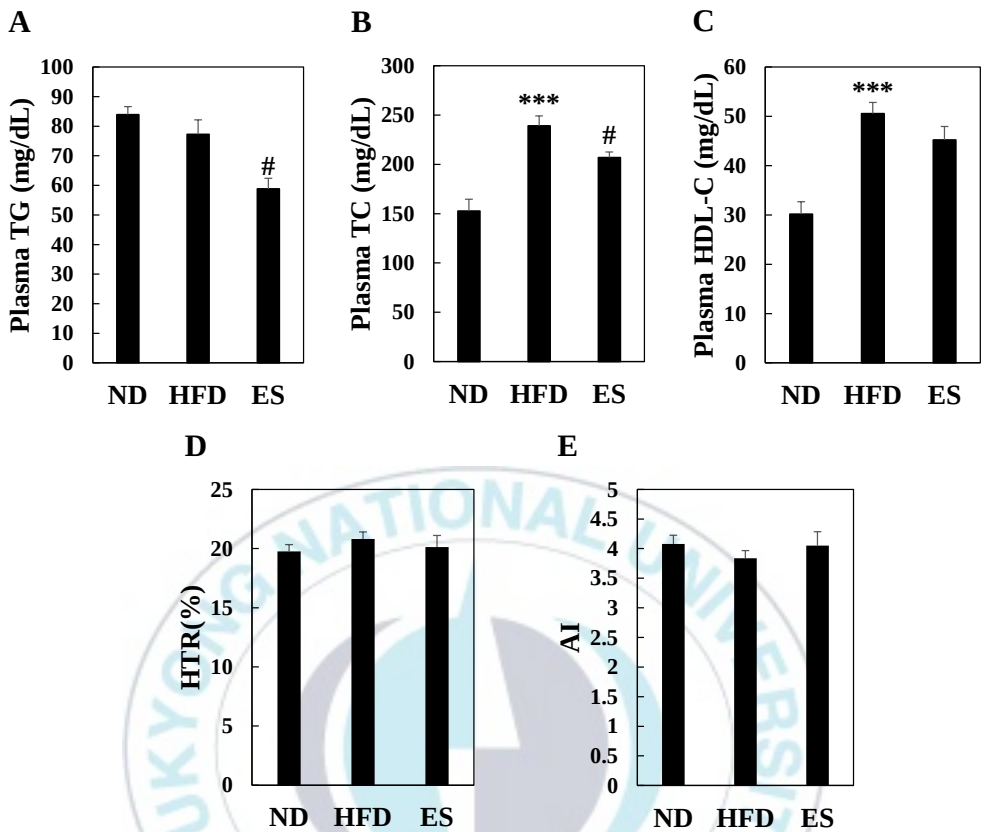


Figure 7. Effects of ES on plasma TG (A), TC (B), HDL-C (C), HTR (D) and AI (E) levels in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{***}p < 0.001$; ND group versus HFD group, $^{\#}p < 0.05$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; HTR, HDL-cholesterol-to-TC ratio; AI, atherogenic index.

3.2. Hepatic lipids profile and liver morphology

The HFD group significantly increased hepatic TG and TC levels compared to the ND group. Significant decreases in the hepatic TG ($p < 0.05$) and TC ($p < 0.05$) levels were observed in the ES group compared to the HFD group (Figure 8A-B).

Hepatic lipids droplet accumulation was markedly increased in the HFD group compared to the ND group, markedly decreased in ES-administrated mice compared to the HFD group (Figure 9).

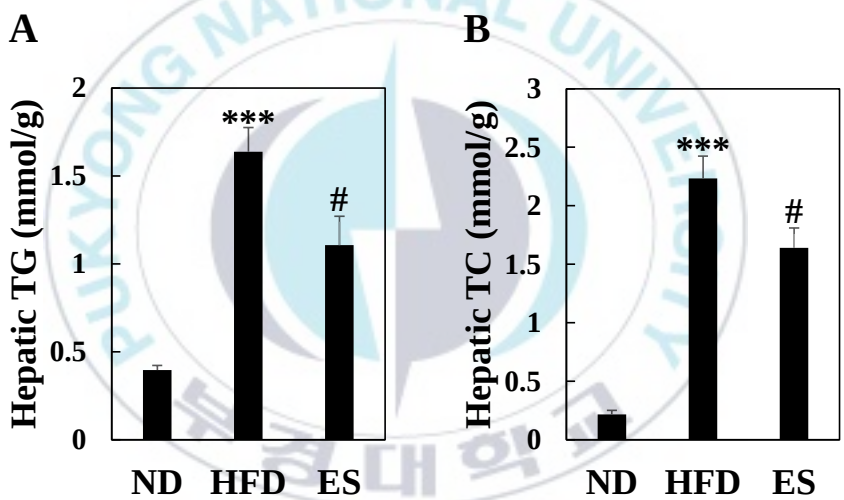
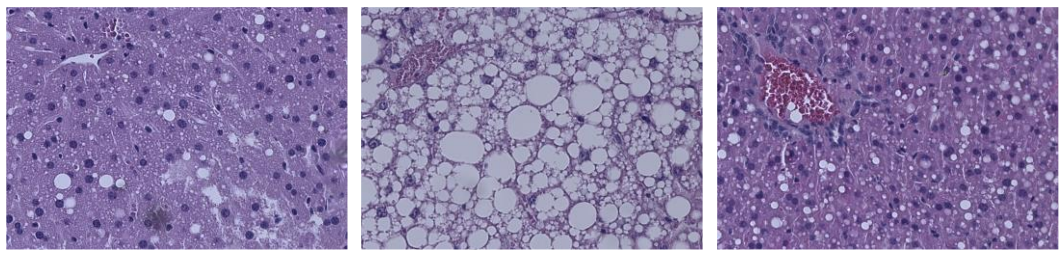


Figure 8. Effects of ES on hepatic TG (A) and TC (B) levels in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{***}p < 0.001$; ND group versus HFD group, $^{\#}p < 0.05$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; TG, triglyceride; TC, total cholesterol.

H&E staining



ND **HFD** **ES**

Figure 9. Effect of ES on hematoxylin and eosin staining of liver tissue in HFD-fed C57BL/6J mice (×400). ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; H&E, hematoxylin and eosin. Representative photomicrographs of liver are shown at ×400 magnification.

3.3. Fecal lipids profile

The HFD group significantly increased TG and TC levels in liver and feces compared to the ND group. Fecal lipids levels in the ES group were similar of those in the HFD group (Figure 10A-B).

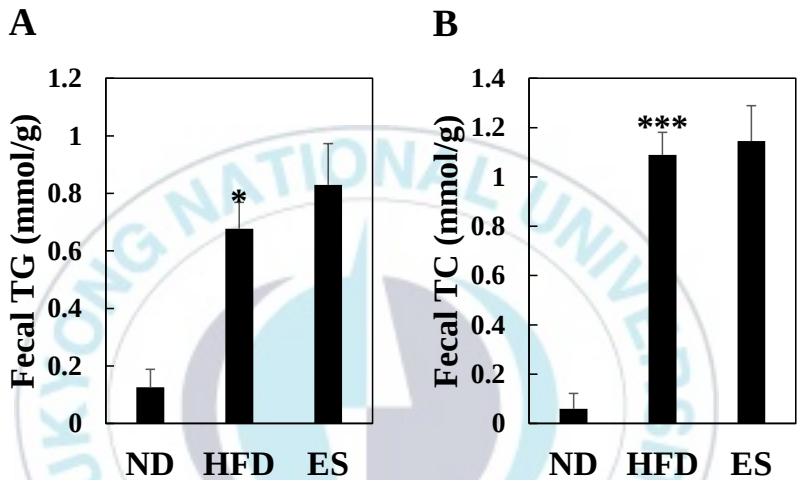


Figure 10. Effects of ES on fecal TG (A) and TC (B) levels in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$, $^{***}p < 0.001$; ND group versus HFD group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; TG, triglyceride; TC, total cholesterol.

4. Effects of ES on aminotransferases levels in HFD-induced obese mice

HFD group exhibited significant increases in plasma AST and ALT levels compared to those of the ND group, and ES group showed significant decreases in plasma AST ($p < 0.05$) and ALT ($p < 0.05$) levels compared to HFD group (Figure 11A-B).

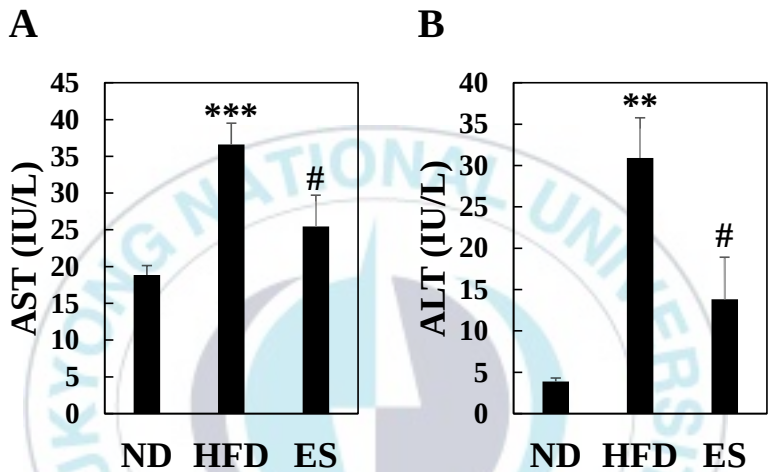


Figure 11. Effects of ES on plasma AST (A) and ALT (B) in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{**}p < 0.01$, $^{***}p < 0.001$; ND group versus HFD group, $^{\#}p < 0.05$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

5. Effects of ES on lipid-regulating enzyme activities and mRNA expression in HFD-induced obese mice

5.1. Effects of ES on lipid-regulating enzyme activities and mRNA expression in epididymal WAT

5.1.1. Epididymal WAT lipid-regulating enzyme activity

FAS activity was significantly increased in epididymal WAT of HFD group compared to the ND group. ES significantly decreased FAS activity in epididymal WAT ($p < 0.05$) compared to the HFD group (Figure 12).

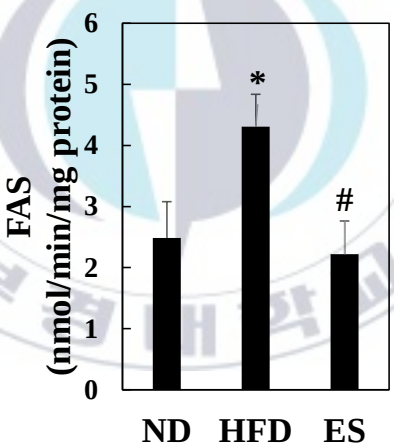


Figure 12. Effect of ES on FAS activity of epididymal WAT in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$; ND group versus HFD group, $^{\#}p < 0.05$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; FAS, fatty acid synthase.

5.1.2. Epididymal WAT lipid-regulating mRNA expression

There was no significant difference in mRNA expression of epididymal WAT SREBP1c, ACC, FAS, SCD1, PAP and CPT1a between ND and HFD groups (Figure 13A-F). SREBP1c ($p < 0.05$), ACC ($p < 0.05$), FAS ($p < 0.05$) and SCD1 ($p < 0.01$) in epididymal WAT were markedly decreased in the ES group compared to the HFD group, although there was no significant difference in PAP mRNA expression between the two groups (Figure 13A-E). The mRNA expression of PPARα was significantly decreased in the HFD group compared to the ND group (Figure 13G). ES significantly increased the CPT1a ($p < 0.05$) and PPARα ($p < 0.05$) mRNA expression in epididymal WAT compared to the HFD group (Figure 13F-G).

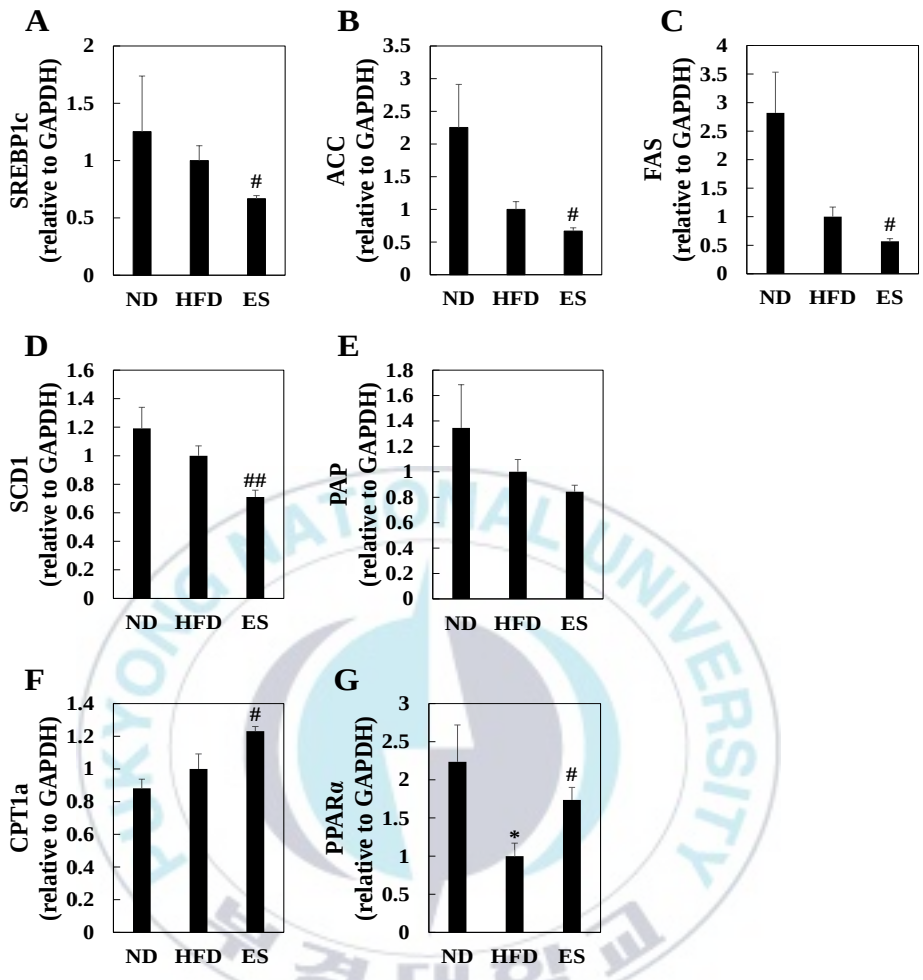


Figure 13. Effects of ES on epididymal mRNA expression of SREBP1c (A), ACC (B), FAS (C), SCD1 (D), PAP (E), CPT1a (F), PPARα (G) in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$; ND group versus HFD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; SREBP1c, sterol regulatory element-binding protein 1c; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD1, stearoyl-CoA desaturase 1; PAP, phosphatidate phosphatase; CPT1a, carnitine palmitoyl transferase 1a; PPARα, peroxisome proliferator-activated receptor alpha.

5.2. Effects of ES on lipid-regulating enzyme activities and mRNA expression in liver

5.2.1. Hepatic enzyme activity

Hepatic PAP activity was significantly increased in HFD group compared to the ND group (Figure 14B). There were no significant differences in hepatic FAS and PAP activities related to lipogenesis between HFD and ES groups (Figure 14A-B). Hepatic CPT1a activity was significantly lower in the HFD group compared to the ND group, however, there was no significant difference between HFD and ES groups (Figure 14C). Activity of hepatic fatty acid β-oxidation ($p < 0.01$) was significantly increased in ES group compared to the HFD group (Figure 14D).

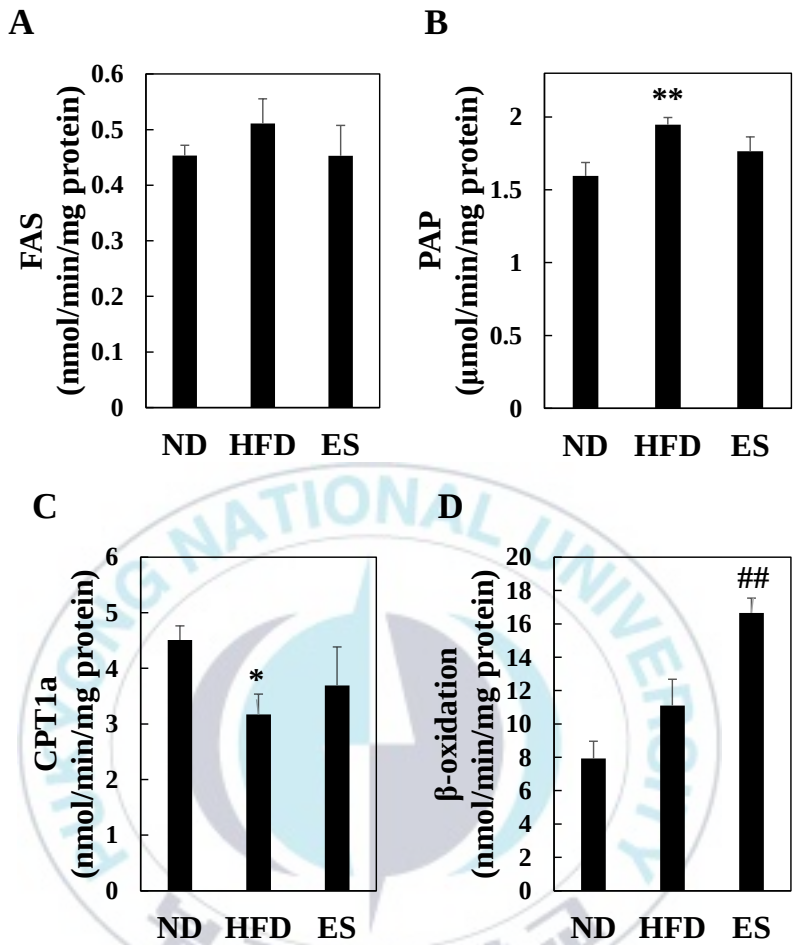


Figure 14. Effects of ES on FAS (A), PAP (B), CPT1a (C) and β-oxidation (D) activities of epididymal WAT in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ND group versus HFD group, $^{\#}p < 0.05$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; FAS, fatty acid synthase; PAP, phosphatidate phosphatase; CPT1a, carnitine palmitoyl transferase 1a.

5.2.2. Hepatic lipid-regulating mRNA expression

The HFD group had significantly higher hepatic SREBP1c, FAS, and PAP mRNA expression than the ND group and had somewhat higher hepatic ACC and SCD1 mRNA expression (Figure 15A-E). ES group had significantly lower hepatic FAS ($p < 0.05$) and SCD1 ($p < 0.05$) mRNA expression than the HFD group (Figure 15C-D), although there were no significant differences in hepatic SREBP1c, ACC, and PAP mRNA expression between two groups (Figure 15A-B, Figure 15E). Hepatic mRNA expression of CPT1a was significantly decreased in HFD group, and ES significantly increased CPT1a mRNA expression ($p < 0.05$) compared to HFD group (Figure 15F). There was no significant difference in hepatic PPARα (Figure 15G).

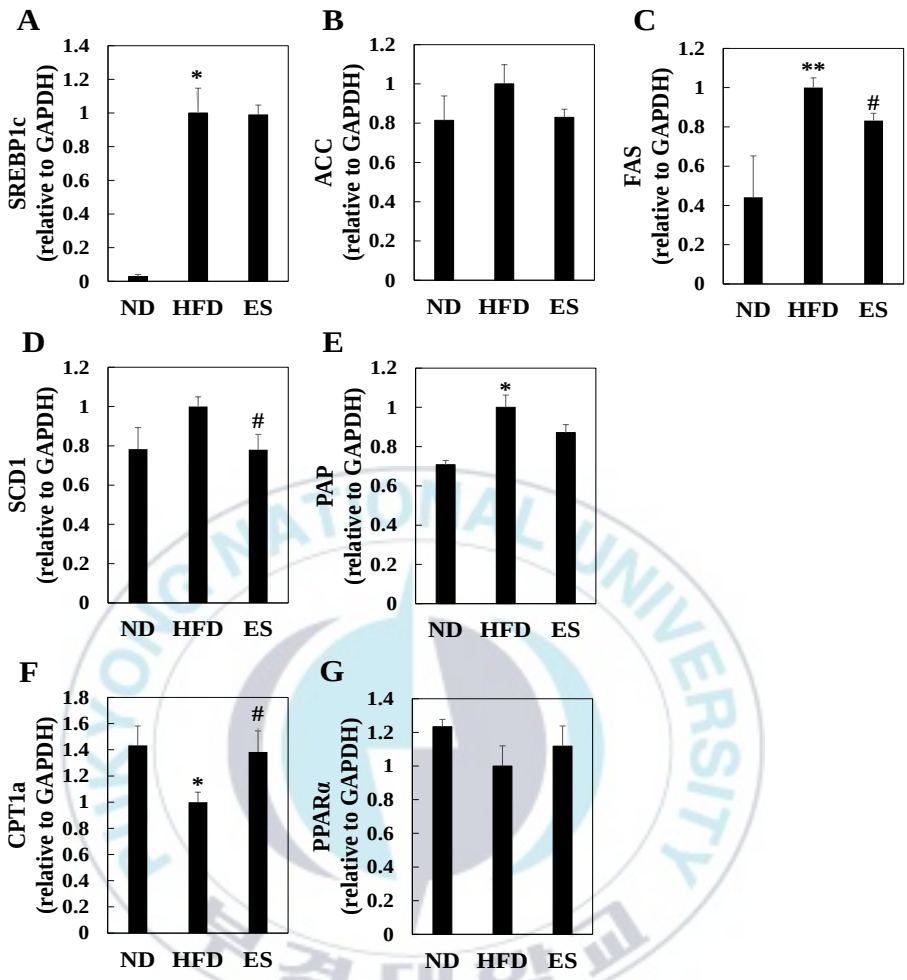


Figure 15. Effects of ES on hepatic mRNA expression of SREBP1c (A), ACC (B), FAS (C), SCD1 (D), PAP (E), CPT1a (F), PPARα (G) in HFD-fed C57BL/6J mice.

Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$, $^{**}p < 0.01$; ND group versus HFD group, $^{\#}p < 0.05$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; SREBP1c, sterol regulatory element-binding protein 1c; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD1, stearoyl-CoA desaturase 1; PAP, phosphatidate phosphatase; CPT1a, carnitine palmitoyl transferase 1a; PPARα, peroxisome proliferator-activated receptor alpha.

6. Effects of ES on plasma cytokine and inflammatory gene expression in HFD-induced obese mice

6.1. Plasma cytokines levels

Plasma IL-6 level was significantly higher in the HFD group compared to the ND group, and ES tended to decrease plasma IL-6 level compared to the HFD group (Figure 16A). HFD group also had somewhat higher plasma MCP-1 and TNF-α levels compared to ND group. ES supplementation markedly decreased plasma MCP-1 ($p < 0.05$) and TNF-α ($p < 0.05$) levels compared to the HFD group (Figure 16B-C).

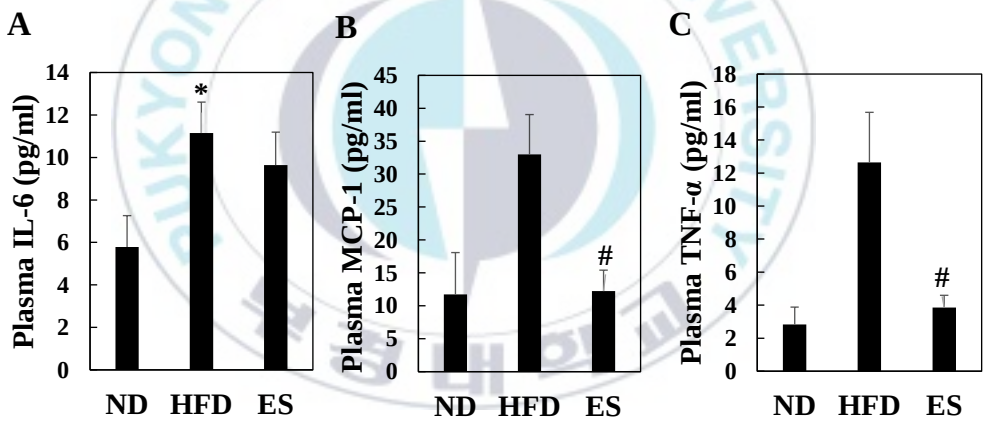


Figure 16. Effects of ES on plasma IL-6 (A), MCP-1 (B), TNF-α (C) levels in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$; ND group versus HFD group, $^{\#}p < 0.05$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-alpha.

6.2. Inflammatory gene expression in epididymal WAT

The mRNA expression of pro-inflammatory genes, MCP-1, TLR2, and TLR4, was significantly higher in epididymal WAT of the HFD group compared to ND group (Figure 17A, Figure 17D-E). IL-6, TNF-α and NF-κB mRNA expression also tended to be higher in epididymal WAT of the HFD group compared to the ND group (Figure 17B-C, Figure 17F). ES administration significantly reduced the mRNA expression of MCP-1 ($p < 0.01$), IL-6 ($p < 0.05$), TLR2 ($p < 0.01$), TLR4 ($p < 0.001$) and NF-κB ($p < 0.01$) in epididymal WAT compared to the HFD group (Figure 17A-B, Figure 17D-F).

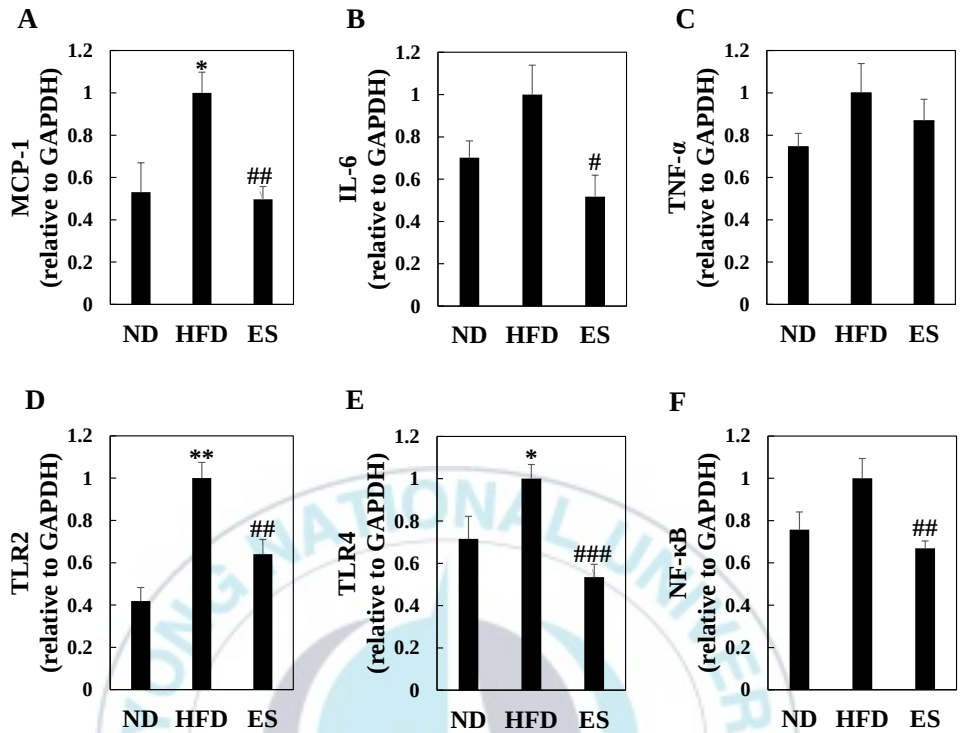


Figure 17. Effects of ES on epididymal WAT MCP-1 (A), IL-6 (B), TNF-α (C), TLR2 (D), TLR4 (E), NF-κB (F) in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$, $^{**}p < 0.01$; ND group versus HFD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; MCP-1, monocyte chemoattractant protein-1; IL-6, interleukin 6; TNF-α, tumor necrosis factor- alpha; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

6.3. Inflammatory gene expression in liver

HFD significantly increased hepatic MCP-1 and TLR2 mRNA expression and tended to increase hepatic IL-6, TNF-α, TLR4 and NF-κB mRNA expression compared to ND group. In liver, ES significantly reduced the mRNA expression of MCP-1 ($p < 0.01$), IL-6 ($p < 0.05$), TNF-α ($p < 0.05$), TLR2 ($p < 0.01$), TLR4 ($p < 0.05$) and NF-κB ($p < 0.05$) compared to HFD group (Figure 18A-F).

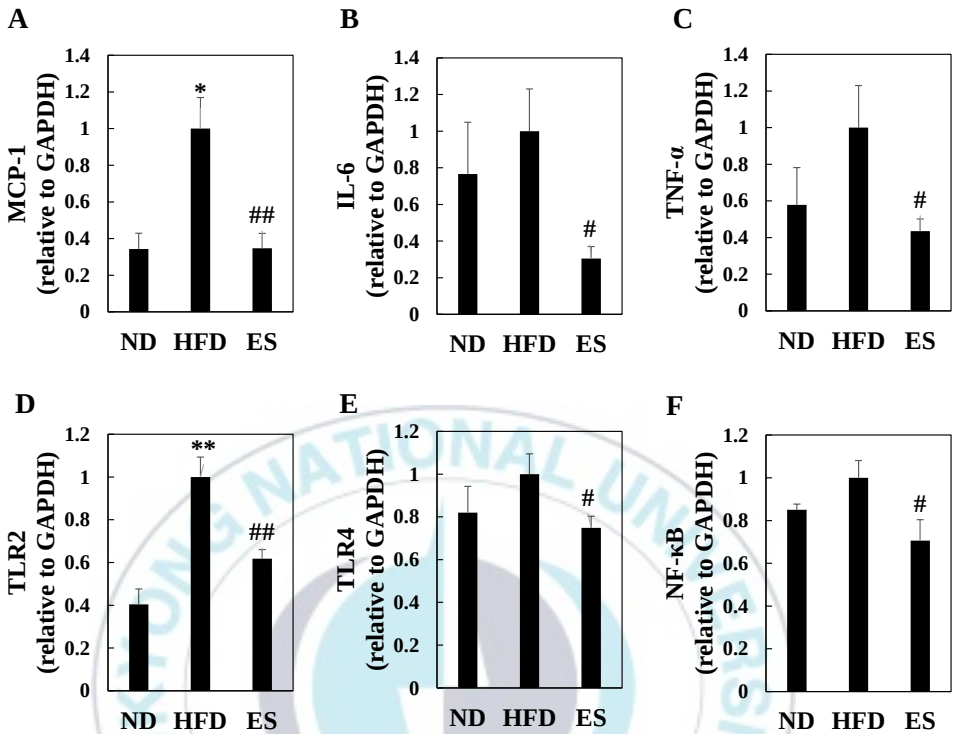


Figure 18. Effects of ES on hepatic MCP-1 (A), IL-6 (B), TNF-α (C), TLR2 (D), TLR4 (E), NF-κB (F) in HFD-fed C57BL/6J mice. Data are shown as the mean ± S.E (ND, n=5; HFD, ES, n=9). Statistical differences among groups were determined using Student's *t*-test.: $^{*}p < 0.05$, $^{**}p < 0.01$; ND group versus HFD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; HFD group versus ES group. ND, normal diet group, HFD, high-fat diet group; ES, high-fat diet + *Ecklonia stolonifera* (0.006%, w/w) group; MCP-1, monocyte chemoattractant protein-1; IL-6, interleukin 6; TNF-α, tumor necrosis factor- alpha; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

IV. DISCUSSION

1. Effect of ES on lipid metabolism and insulin resistance

Obesity is the result of excessive fat accumulation due to an imbalance in energy intake and expenditure. Excessive energy is stored as TG in WAT, which results in hypertrophy (enlarged adipocytes) and hyperplasia (increased numbers of adipocytes) (Choe et al., 2016). Accordingly, in prolonged positive energy balance conditions, the excessive energy is deposited in the form of neutral triglycerides in WAT through the lipogenic pathway, which results in an increase of lipid droplet size and subsequent adipose expansion and obesity. Obesity can increase multiple metabolic risk factors including dyslipidemia (high TG concentration, high TC concentration, low HDL-C concentration and small LDL particles), NAFLD and insulin resistance (Souza et al., 2012; Bays et al., 2013).

This study revealed that ES supplementation significantly reduced body weight, WAT weight, and plasma leptin level in HFD-induced obese mice. ES also markedly reduced plasma and hepatic TG and TC levels and hepatic lipid droplets accumulation. In addition, fasting blood and plasma glucose, HOMA-IR, and plasma resistin levels were significantly decreased in ES-supplemented mice. These results suggest that ES can improve obesity and obesity-related dyslipidemia, NAFLD and insulin resistance in HFD-induced obese mice. Insulin resistance is strongly associated with obesity and leads to hyperinsulinemia, impaired glucose and lipid metabolism (Naowaboot et al., 2016). In this study, ES improves insulin resistance as well as lipid accumulation.

Adipose tissue performs two reciprocal biochemical processes to maintain lipid homeostasis: lipogenesis vs. lipolysis and subsequent fatty acid oxidation (Park et al., 2005). These two processes are regulated by several hormones, lipid metabolites, and nutritional conditions. ES down-regulated SREBP1c, ACC, FAS and SCD1 mRNA expression and

FAS enzyme activity in epididymal WAT compared to the HFD group. ES also up-regulated CPT1a and PPARα mRNA expression in WAT. Our results suggest that ES treatment decreases lipogenesis and increases fatty acid oxidation in epididymal WAT of HFD-induced mice, which can lead to weight loss and alleviating adiposity. Its anti-obesity effect was supported by decreased plasma leptin level observed in ES group. Obesity causes leptin resistance, which loses the ability to inhibit energy intake and increase energy expenditure (Zhang and Scarpace, 2006). Leptin, known as anorexigenic hormone, is involved in food intake and regulation of fatty acid oxidation contacted with brain to prevent lipid accumulation in adipose tissue (Myers et al., 2008; Arch, 2005). In many studies, leptin positively correlated with body weight and fat mass (Choi et al., 2016; Friedman, 1998; Hamann and Matthaei, 1996). In this study, plasma leptin level was increased in obese mice, but ES significantly decreased plasma leptin level, which may be related to the decreased body weight and body fat mass observed in ES group.

Recently, NAFLD related to insulin resistance, obesity, and metabolic syndrome is on an increasing trend (Kim et al., 2018). NAFLD ranges from simple fatty liver (hepatic steatosis) to nonalcoholic steatohepatitis (NASH), a state of steatosis combined with liver cell injury and inflammation, fibrosis, cirrhosis, and even hepatocellular carcinoma (Fabbrini et al., 2010). NAFLD occurs when excessive triglycerides accumulate in the liver, because of an imbalance between lipid deposition and removal (Hassan et al., 2014, Arguello et al., 2015). Furthermore, Hepatic fat overload is increased mitochondrial function including fatty acid oxidation, however, sustained fat overload deposition impairs mitochondrial function (Nassir and Ibdah, 2014). In the present study, ES significantly decreased liver weight, hepatic TG, hepatic TC, and hepatic lipid droplets. We also found that ES group decreased plasma AST and ALT levels compared to those of the HFD group. Levels of AST and ALT are clinically and toxicologically major indicators (Radi et al., 2011). Circulating AST and ALT levels increased with obesity and high levels of AST and ALT are related to NAFLD (Hakkak et al., 2015; Hakkak et al., 2018; Lim et al., 2013). These results suggest that ES

treatment can protect against hepatic steatosis and has a hepatoprotective effect in HFD-fed obese mice. In liver, ES significantly down-regulated FAS and SCD1 mRNA expression and up-regulated CPT1a mRNA expression as well as β-oxidation activity, which may be associated with improved dyslipidemia and NAFLD observed in ES group.

2. Effect of ES on inflammation

High levels of pro-inflammatory cytokines, such as MCP-1, IL-6 and TNF-α, and low anti-inflammatory cytokines levels are observed in obese subject and animals (Maessen et al., 2016; Toffanin et al., 2010; Basu et al., 2011; Engeli et al., 2003; Jorde et al., 2010). Several studies have reported that chronic inflammation is linked to the pathogenesis of obesity-related metabolic dysfunction and that chronic activation of inflammatory pathway has a crucial role in inducing obesity and insulin resistance in various diseases (Ouchi et al., 2011; Sun et al., 2011; Weisberg et al., 2003).

WAT is an endocrine organ. Adipokines/cytokines secreted from adipose tissue regulate energy homeostasis and metabolism depending on the energy requirement of several tissues. Adipokines/cytokines are also known to regulate lipid metabolism and inflammation (Greenberg and Obin, 2006). WAT also modulates lipid metabolism in other organs through secreting various adipokines (or adipocytokines) including leptin, resistin, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which can contribute to the development of various obesity-related metabolic disease (Jung and Choi, 2014).

In addition, leptin, which is mainly expressed in adipose tissue, not only plays an important role in body weight regulation but also regulates the production of pro-inflammatory adipokines/cytokines (Yadav et al., 2012). Obesity activates NF-κB pathway, and then increases several pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 in liver, resulting in increased circulation of MCP-1 (Boden, 2008). Also, hepatic cytokine and chemokine, such as IL-6 and TNF-α caused by obesity, induce hepatic fibrosis and steatosis (Chiang et al., 2011).

The present study found that ES markedly decreased plasma MCP-1 and TNF-α levels. ES also significantly down-regulated mRNA expression of MCP-1, IL-6, TNF-α, TLR2, TLR4 and NF-κB in liver. Along with liver, mRNA expression of inflammatory gene, such

as MCP-1, IL-6, TLR2, TLR4 and NF-κB, was down-regulated in epididymal WAT of ES-supplemented mice. These results indicate that ES inhibits HFD-induced inflammation by down-regulating pro-inflammatory adipokines/cytokines.

V. CONCLUSION

The present study evaluated the anti-obesity efficacy of ES and its underlying mechanisms in HFD-induced obesity mouse model. To determine the anti-obesity effect and underlying mechanism, we investigated the effects of ES on body weight, adiposity, adipokines, lipid metabolism and inflammation related to obesity. ES significantly reduced the body weight, food efficiency ratio and weights of visceral WATs including epididymal WAT, perirenal WAT and mesentery WAT. In addition, significant decreases in fasting blood and plasma glucose, plasma leptin and resistin levels and an improvement in insulin resistance were observed in the ES group. ES significantly reduced plasma TC and TG levels. In addition, ES decreased liver weight, hepatic triglyceride, cholesterol contents, hepatic lipid droplets accumulation and plasma AST and ALT levels. The weight loss and body fat lowering effects were related to the reduction of lipogenesis through down-regulation of SREBP1c, ACC, FAS and SCD1 mRNA expression and promotion of fatty acid oxidation through up-regulated CPT1a, PPARα mRNA expression in epididymal WAT. In liver, ES also significantly down-regulated FAS and SCD1 mRNA expression and up-regulated CPT1a mRNA expression. In addition, ES markedly decreased plasma MCP-1 and TNF-α levels and down-regulated mRNA expression of inflammatory genes including MCP-1, IL-6, TLR2, TLR4 and NF-κB in WAT and liver. Accordingly, the present study demonstrated that ES was effective in improving dyslipidemia, NAFLD, inflammation and insulin resistance in the HFD-fed mice. Hence, ES is expected to become an effective food material that can ameliorate obesity and its comorbid conditions by regulating lipid metabolism in adipose and liver tissue and by improving inflammation and insulin resistance.

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