Thesis for the Degree of Master of Pharmacy

Anti-obesity effects of ethanol extract of *Laminaria japonica* Areshoung in high-fat diet-induced obese rats

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by

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Contents

Table Contents

Figure Contents

List of Abbreviations

LE: *Laminaria japonica* Areshoung ethanol extract

ACC-1: acetyl-CoA carboxylase-1

ACC-2: acetyl-CoA carboxylase-2

ACO: acyl CoA oxidase

BUN: blood urea nitrogen.

CPT-1: carnitinepalmitoyl-transferase 1

FAS: fatty acid synthase

FFA: free fatty acid

GOT: glutamic oxaloacetic transaminase;

GPT: glutamic pyruvic transaminase;

GPAT: glycerol-3-phosphate acyltransferase

LDL-C: low-density lipoprotein-cholesterol

PPARα: peroxisome proliferator-activated receptor α

SCD-1: stearoyl-CoA desaturase

SREBP-1c: sterol regulatory element-binding protein-1c

TNF-α: tumor necrosis factor-α

TC: total-cholesterol

HDL-C: high-density lipoprotein cholesterol

UCP2: uncoupling protein 2

UCP3: uncoupling protein 3

Abstract

Anti-obesity effects of ethanol extract of *Laminaria japonica* Areshoung in high-fat diet-induced obese rats induced obese rats

By Woong Sun Jang

Master of Pharmacy in Fundamental Pharmaceutical Science

Graduate School of Kyung Hee University

Directed by Prof. Se Young Choung

Laminaria japonica Areshoung, a widely consumed marine vegetable, has seen traditional use in Korean maternal health. The present study investigated the anti-obesity effects of *Laminaria japonica* Areshoung ethanol extract (LE) and its molecular mechanism in high-fat diet-induced obese rats. Six-week-old Sprague–Dawley male rats were separately fed a normal diet (ND) or a high-calorie high-fat diet (HD) for 6 weeks, then they were treated with LE (100, 200, or 400 mg/kg) or tea catechin (100 mg/kg) for another 6 weeks.

LE administration significantly decreased body weight gain, fat-pad weights, and serum and hepatic lipid levels in HD-induced obese rats. Histological analysis revealed that LE-treated group showed significantly decreased number of lipid droplets, and size of adipocytes compared to the HD group.

To elucidate the mechanism of action of LE, the levels of genes and proteins involved in obesity were measured in liver and skeletal muscle. LE treatment resulted in increased expression of fatty acid oxidation and thermogenesis-related genes (PPARα, ACO, CPT1, UCP2, and UCP3) in obese rats. Conversely, the expression of fat intake-related gene (ACC2) and lipogenesis-related genes (SREBP-1c, ACC1, FAS, SCD-1, GPAT, AGPAT and DGAT) were reduced by LE treatment.

Additionally, LE treatment increased phosphorylation of AMP-activated protein kinase (AMPK), and its direct down-stream protein, acetyl coenzyme A carboxylase (ACC), which are one of the rate-limiting enzymes in fatty acid synthesis pathway.

These finding demonstrate that LE treatment have a protective effect against a high fat diet-induced obesity in rats through regulation of expression of gene and protein involved in lipolysis and lipogenesis.

Key words

Laminaria japonica Areshoung; anti-obesity; lipogenesis; lipolysis; high-fat diet; rat; H&E staining; fatty acid oxidation; AMPK; ACC

Ⅰ. Introduction

Obesity is a chronic metabolic disorder that results from the imbalance between energy intake and energy expenditure. It is characterized by enlarged fat mass and elevated lipid concentration in blood [1-2].

On a global scale, obesity has reached epidemic proportions and is a major contributor to the global burden of chronic disease and disability. Currently, more than one billion adults worldwide are overweight and at least 300 million of them are clinically obese [3]. Importantly, obesity is often associated with a variety of chronic diseases such as hyperlipidemia, diabetes mellitus, hypertension coronary artery disease and certain cancers [4-7]. Therefore, prevention and treatment of obesity are important for a healthy life [8].

Although a number of pharmacological approaches to the treatment of obesity have been recently investigated, only a few drugs have been approved for clinical usage. Current therapies for obesity treatment include the reduction of nutrient absorption and the administration of drugs that affect lipid mobilization and utilization (e.g. orlistat and sibutramine) [9]. However, owing to the adverse side effects associated with many anti-obesity drugs, more recent trials have focused on screening natural sources that have been reported to reduce body weight and that generally have minimal side effects [10]. This may be an excellent alternative strategy for developing effective and safe anti-obesity drugs in the future [11-13].

A variety of natural products, including crude extracts and isolated compounds from plants, have been widely used traditionally to treat obesity [14-16]. A wealth of information indicates numerous bioactive components from nature are potentially useful in obesity treatments. A good example of this is polyphenolic compounds showing strong anti-obesity activity and include apigenin, genistein, and the catechins [16-18].

Laminaria japonica Areshoung is widely consumed as a marine vegetable, and has been used to promote maternal health in Korea. Recently, it has been reported that *Laminaria japonica* Areshoung possesses various biological functions including anti-inflammatory [19], anti- tumor [20], anti- atherosclerosis [21] and anti-diabetic activity [22]; however, little is known about the effects of *Laminaria japonica* Areshoung on obesity in in vivo experiments.

Therefore, the aim of this study is to investigate the molecular mechanisms underlying its

anti-obesity action.

In the present study, we investigated the anti-obesity effects of ethanol extract of *Laminaria japonica* Areshoung (LE) in high fat diet (HD)-induced obese rats. Body weight gain, food intake, fat-pad weights, and serum and hepatic lipid levels were measured. Green tea catechin, which has shown anti-obesity, anti-diabetic, and cardioprotective effects in animal and human studies, was used as a positive control. To gain insight into the molecular mechanisms underlying the effects described above, we investigated the expression of genes and proteins related to lipid metabolism in LE-treated obese rats. Our results demonstrate the great potential of LE for the treatment of obesity.

Ⅱ. Materials

2-1. Regents

High fat diet was purchased from Research Diets Inc (New Brunswick, NJ, USA). Total-cholesterol (TC), triglyceride, high-density lipoprotein cholesterol (HDL-C), glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and blood urea nitrogen (BUN) kit were purchased from Asan Pharmaceutical (Seoul, Korea). Free fatty acid (non-esterified fatty acid, NEFA) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Maxime^{TM} RT-PCR PreMix kit was purchased from Intron (Seoul, Korea). Adiponectin, leptin, glucose, tumor necrosis factor-α (TNF-α), and insulin kit were purchased from Shibayagi (Gunma, Japan).

2-2. Instruments

Spectrophotometer (Jasco: V-530)
ELISA reader (Bio-Tek instruments: powerwave XS microplate spectrophotometer)
Centrifuge (Vision scientific Co, Ltd.: VS-5500N)
Water bath (Sangwoo)
Spectrophotometer (Nanodrop ND-1000)
Microtome
Reseach microscope (Olympus: BX51)
Microscope digital camera (Olympus: DP71)
RT-PCR machine (TaKaRa Laboratories)

Ⅲ. Methods

3-1. Experimental material preparation

3-1-1. Preparation of L. japonica

L. japonica was collected from Gijang aquaculture farm, Busan, South Korea, in May 2010, and a voucher specimen was deposited in the laboratory (H. R. Kim). Samples were rinsed in tap water to remove salt and dried in an air dryer at 60 ℃ for 40 h. A dried sample was ground with a hammer mill, and the powder was stored at -20 ℃ until used.

3-1-2. Extraction and isolation

Dried powder (2.5 kg) of *Laminaria japonica* Areshoung was extracted three times with 96 % (v/v) ethanol (EtOH) for 3 h at 70 ℃. The combined extracts were filtered and concentrated under reduced pressure to obtain the EtOH extracts (446.6 g) (Fig. 1).

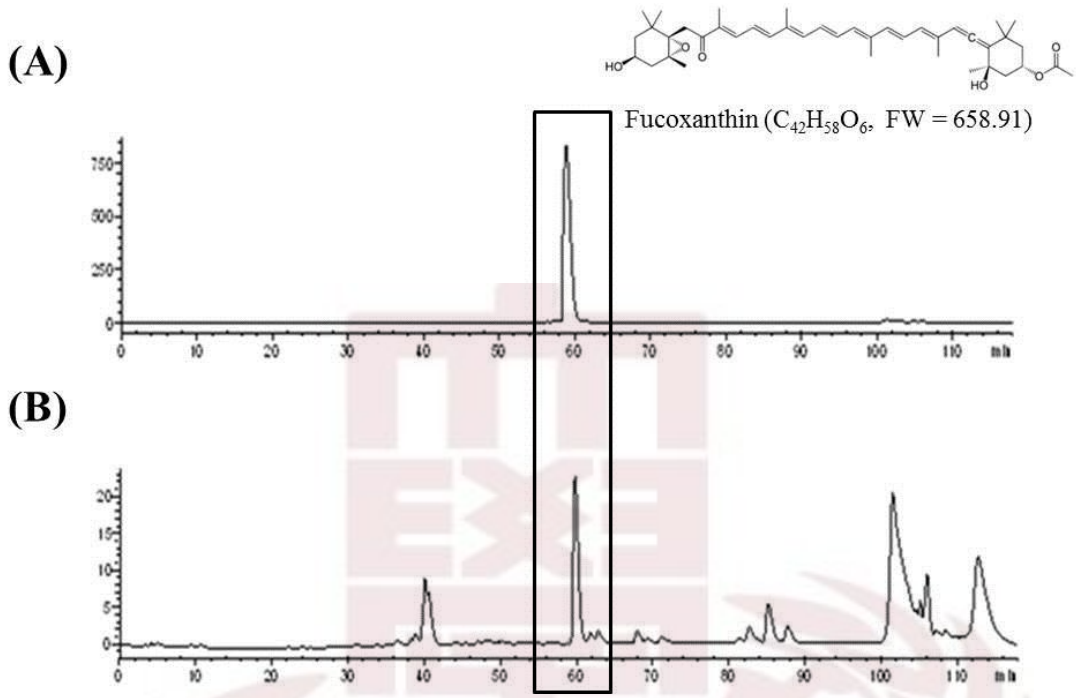

Figure 1. HPLC chromatograms of fucoxanthin (A) and ethanol extract of *L. japonica* (B).

3-2. Animals and experimental diets

Sixty 6-wk-old male Sprague–Dawley rats (Koatech, Pyeongtaek, Korea) were individually housed in standard cages and placed in a room where the temperature was kept at 21 ± 2.0 ℃, the relative humidity at 50 ± 5 %, and the light on a 12-h light/dark cycle. All the rats consumed a commercial diet for 1wk. After that, the animals were separately fed a normal diet (ND) or high fat diet (HD) (Research Diets Inc., New Brunswick, USA) for 6 weeks. Then the animals were subdivided into six groups (n=6)—ND group, HD group, HD + LE (100, 200, or 400 mg/kg) groups, and HD + Tea (tea catechin, 100 mg/kg) group—and treated with LE or tea catechin for another 6 weeks. Tea catechin was kindly provided by Amore Pacific Corp. (Seoul, Korea). Diet compositions are shown in Table 1. Food intake was recorded daily, and body weights were monitored every 2 days during the feeding period. The epididymal, abdominal, visceral, and brown fat-pads and livers were removed, rinsed with phosphate-buffered saline, and then weighed. The liver and fat pad samples were stored at -70 ℃ until analysis. This study was approved by the Institutional Animal Care and Use Committee of Kyung Hee University, Seoul.

Table 1. Composition of normal diet and high-fat diet

	Group	
Ingredient	ND	HD
Casein	80.0	80.0
Cornstarch	60.0	60.0
Sucrose	200.0	122.6
Corn oil	45.0	0
Lard	0	219.2
AIN-76 Mineral Mix	0	0.0
AIN-76 Vitamin Mix	4.0	4.0
DL-Methionine	1.2	1.2
Choline bitartrate	0	0
Energy		
kcal/100 g of diet	390.2	487.0
Calories from fat (%)	11.5	45.0

HD, high-fat diet; ND, normal diet

3-3. Biochemical analysis

At the end of treatment, biochemical analysis were performed after 12-h fasting. Blood was drawn from the inferior vena cava into a heparin-coated tube, and the plasma was obtained by centrifuging the blood at 15.000 rpm for 15 min at 4 ℃. The plasma triglyceride concentration was measured using a kit based on a lipase-glycerol phosphate oxidase method (Asan Pharmaceutical, Seoul, Korea). Plasma total-cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and free fatty acid (FFA) concentrations were determined using a commercial kit (Asan Pharmaceutical, Seoul, Korea). Low-density lipoprotein-cholesterol (LDL-C) was calculated using the following equation; LDL-C = TC - HDL-C - TG/2.18 [23]. Free fatty acid (non-esterified fatty acid, NEFA) was also measured using NEFA-Wako (Wako Pure Chemical Industries, Osaka, Japan). Plasma glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and blood urea nitrogen (BUN) levels were measured using a commercial kit (Asan Pharmaceutical, Seoul, Korea). Plasma adiponectin, leptin, glucose, tumor necrosis factor-α (TNF-α), insulin levels were analyzed using enzyme-linked immunosorbent assay kits (Shibayagi, Gunma, Japan).

3-4. Hepatic lipid profiles

Hepatic lipids were extracted using the method developed by Folch et al [24] and the dried lipid residues were dissolved in 1 ml ethanol. High-density lipoprotein cholesterol (HDL-C), TC, TG and FFA concentrations in the hepatic lipid extracts were measured using the same enzymatic kits that were applied for the plasma analysis.

3-5. Oil-red O staining

The tissues were sliced (7 μm) and stained in 60 % of the oil red O stock solution (0.5 g oil red O in 100 mL isopropanol) for 30 min, then briefly washed with 60 % isopropanol, and then with distilled water for microscopic observation and photography.

3-6. Hematoxylin and eosin staining

Tissue samples of liver and epididymal fat pads were fixed with 4 % buffered formalin and embedded in paraffin. Standard 4-μm-thick sections were stained with hematoxylin and eosin, viewed with an optical microscope (Olympus Optical, Tokyo, Japan) and photographed at a final magnification of 200×. The average size of adipocytes was measured by using Image J software (National Institute of Mental Health, Bethesda, USA)

3-7. RNA isolation from tissue samples

Livers and skeletal muscles were dissected, weighed, frozen in liquid nitrogen, and stored at -70 ℃ until use. The tissue samples (in the amount of 100 mg) were homogenized in easy blue reagent (iNtRON, Gyeonggi, Korea). Phase separation of RNA was performed by adding one-fifth volume of chloroform and centrifugation at 12,000 *g* for 10 min. Isopropyl alcohol (0.5 ml) was added to the aqueous phase to precipitate total RNA, then RNA pellet was washed twice with 75 % ethanol. The RNA sample was dried and dissolved in TE buffer. RNA concentration was determined by measuring absorbance at 260 and 280 nm using an Optizen 2120UV spectrophotometer (Mecasys, Daejeon, Korea).

3-8. Reverse transcription–polymerase chain reaction analysis

Reverse transcription (RT)–polymerase chain reaction (PCR) assays for mRNA levels in skeletal muscle and liver tissue were performed using a Maxime™ RT-PCR PreMix kit (iNtRON, Gyeonggi, Korea) according to the manufacturer's protocol. RT was performed at 45 ℃ for 30 minutes. PCR was carried out as follows: 5 minutes at 94 ℃, 30 cycles of (94 ℃ for 1 minute, 56 ℃ for 1 minute, and 72 ℃ for 5 minutes), and a 5-minute incubation at 72 ℃. The β-actin gene was used as an internal control. The sequences of primes used in this study are shown in Table 2.

Table 2. Primer sequences

Target	Primer	Sequence(5'-3')	Amplicon size (bp)
PPARα	F	TGACCAGCCTGATGGAGTTA	239
	R	ACTGATAGTGGGATTCTTATTGGG	
SCD-1	F	CACATCAACTTCACCACGTTCTTC	74
	R	GAAACTTTCTTCCGGTCGTAAGC	
CPT1(muscle)	F	CAATGGTGGGATGAGAGAAC	175
	R	AACCCAGAACTTTAACCTCAA	
CPT1(liver)	F	AACCTTGGCTGCGGTAAGACTA	184
	R	AGTGGGACATTCCTCTCTCAGG	
ACO	F	CCAACAAGGTGACATGCTGTGT	184
	R	ATTCGCATTGTGAGAGCAGTTC	
UCP3	F	GTTTTGCTGATCTCCTCACCTTT	242
	R	GAACTGCTTGACAGAGTCATAGAGG	
SREBP-1c	F	GATGCCAACCAGATTCCCTAAG	210
	R	TCAGTTGTTTCTTTGCCTTCCA	
ACC-1	F	TCTATTCGGGGTGACTTTC	109
	R	CTATCAGTCTGTCCAGCCC	
ACC-2	F	GGAACTCACGCAGTTGACCAGG	299
	R	CACATAAACCTCCAGGGACGCC	
FAS	F	CTGGACTCGCTCATGGGTG	111
	R	CATTTCCTGAAGTTTCCGCAG	
DGAT	F	AGACTAGGAGGAGTGTGCAGGC	212
	R	CGCTTCTTCCAAGGGAACTATG	
AGPAT	F	GCATTTCAGGATCTCGTTCACA	197
	R	ATCAACCCAACGAGAGCACTTT	
GPAT	F	TGATCAGCCAGGAGCAGCTG	508
	R	AGACAGTATGTGGCACTCTC	
UCP2	F	GCATTGCAGATCTCATCACTTTCC	222
	R	AGCCCTTGACTCTCCCCTTG	
β-Actin	F	GCTCTTTTCCAGCCTTCCTT	259
	R	TGATCCACATCTGCTGGAAG	

PPARα, peroxisome proliferator-activated receptor α; SCD-1, Stearoyl-CoA desaturase-1; CPT-1,carnitine palmitoyl-transferase-1; ACO, acyl CoA oxidase; UCP3, uncoupling protein3; SREBP-1c, sterol regulatory element-binding protein-1c; ACC1, acetyl-CoA carboxylase1; ACC2, acetyl-CoA carboxylase2; FAS, fatty acid synthase; DGAT, diacylglycerol O-acyltransferase; AGPAT, acylglycerol-3-phosphate-O-acyltransferase; GPAT, glycerol-3-phosphate-acyltransferase; UCP2, uncoupling protein2.

3-9. Protein extraction and Western blot analysis

Liver tissues (100-150 μg) were homogenized in an extraction buffer (100 mM Tris–HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM sodium pyrophosphate, 50 mM NaF, 100 mM orthovanadate, 1 % Triton X-100, 1 mM PMSF, 2 μg/mL aprotinin, 1 μg /mL pepstatin A, and 1 μg /mL leupeptin). Tissue homogenates were centrifuged at 13,000 *g* for 20 min at 4 ℃ and the resulting supernatants were used for Western blot analysis. The protein concentrations of homogenates were measured by the Lowry assay (Bio-Rad, Hercules, CA, USA). Protein samples (50 μg /lane) were separated by 8 % SDS–PAGE and were transferred onto the nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). After blocking (5 % nonfat dry milk in 10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl and 0.1 % Tween 20) for 2 hours at room temperature, the membrane was incubated overnight at 4 ℃ with primary antibodies (1:1,000 dilution). Antibodies against acetyl-CoA carboxylase (ACC), phospho-ACC at Ser 79, AMP-activated protein kinase α (AMPKα) and phospho-AMPK α at Thr 172 were purchased from Cell Signaling Technology (Beverly, USA). After the membrane was incubated with 1:5,000-diluted secondary antibody for 1 hour, immune reactive signals were detected chemiluminescence (ECL) detection system (Fuji Film, Tokyo, Japan). The densities of bands were measured by science lab 2.0 software (Fuji Film, Tokyo, Japan).

3-10. Statistical analysis

Numerical data are expressed as means ± standard deviation values. The significance of differences was examined using ANOVA, followed by Tukey's test. Differences between groups were considered statistically significant at $P<0.05$.

Ⅳ. Results

4-1. Effect of LE treatment on body weight, food efficiency ratio, and fat-pad weights

The body weight and food intakes are shown in Table 3. During the 6 weeks experimental period, the amount of food efficiency ratio as well as body weight changes was measured every 2 days. The final body weight and weight gain of the HD group were 30.1 % and 65.9 % greater, respectively, than those of the ND group. LE administration suppressed HD-induced body weight increase in a dose-dependent manner. The LE (400 mg/kg) and tea catechin supplementation significantly reduced body weight gain compared to no treatment (HD group) by 47 %, respectively. The food intake and food efficiency ratio (FER) of the obese rats given LE were significantly lower than the values for the rats fed HD-only, suggesting that LE regulates food intake and energy metabolism. In addition, the epididymal, abdominal, visceral, and brown fat-pad weights were significantly higher in the HD group than the value for the ND group, and these increases were lower by LE treatment (Table 3). In agree with these results, the size of adipocytes from the epidydimal white adipose tissue of the obese rats fed LE (400 mg/kg) were 72.9 % smaller than those for the HD group (Fig. 2).

Table 3. Effect of LE on body weight, food efficiency ratio, and fat-pad weights in HD-induced obese rats

Group	ND	HD	HD+Tea	HD+LE100	HD+LE200	HD+LE400
Initial body weight (g)	453.33±30.95	553.25±35.12$^{\#\#\#}$	553.00±41.79	553.20±38.40	553.20±41.32	553.20±33.80
Final body weight (g)	560.40±16.94	729.13±77.27$^{\#\#\#}$	631.13±42.13**	692.20±24.53	665.60±34.88*	634.00±16.18*
Body weight gain (g)	106.00±19.89	175.88±83.91$^{\#}$	93.29±41.32**	139.00±42.34	112.40±27.26**	93.50±24.38***
Food intake(g/rat/day)	27.02±1.88	19.88±1.24$^{\#\#\#}$	16.60±1.66*	17.88±1.78	17.57±1.56	17.06±0.99*
FER (%)	9.55±1.54	17.48±3.82$^{\#}$	11.50±1.83*	15.02±2.99	14.76±3.97	10.71±2.85*
Whole fat (g)	32.61±1.77	76.79±5.17	59.73±4.17	71.94±3.26	66.38±2.99	60.13±2.77
Abdominal (g)	14.73±3.44	39.35±8.37$^{\#\#\#}$	29.88±4.74	36.48±2.64	33.59±3.94	31.45±4.38
Epididymal (g)	10.94±2.39	23.39±7.15$^{\#}$	19.72±9.13	22.06±6.06	21.74±4.27	19.15±4.08
Visceral (g)	6.50±1.18	13.39±5.09$^{\#}$	9.54±2.80	12.86±4.24	10.51±3.71	8.99±2.56
Brown (g)	0.44±0.08	0.67±0.09$^{\#\#\#}$	0.59±0.02	0.55±0.08	0.54±0.05	0.54±0.06

Data are mean ± SD (n ≥ 8). $^{\#}P<.05$, $^{\#\#\#}P<.001$ versus the ND group; $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ versus HD groups. FER, food efficiency ratio.

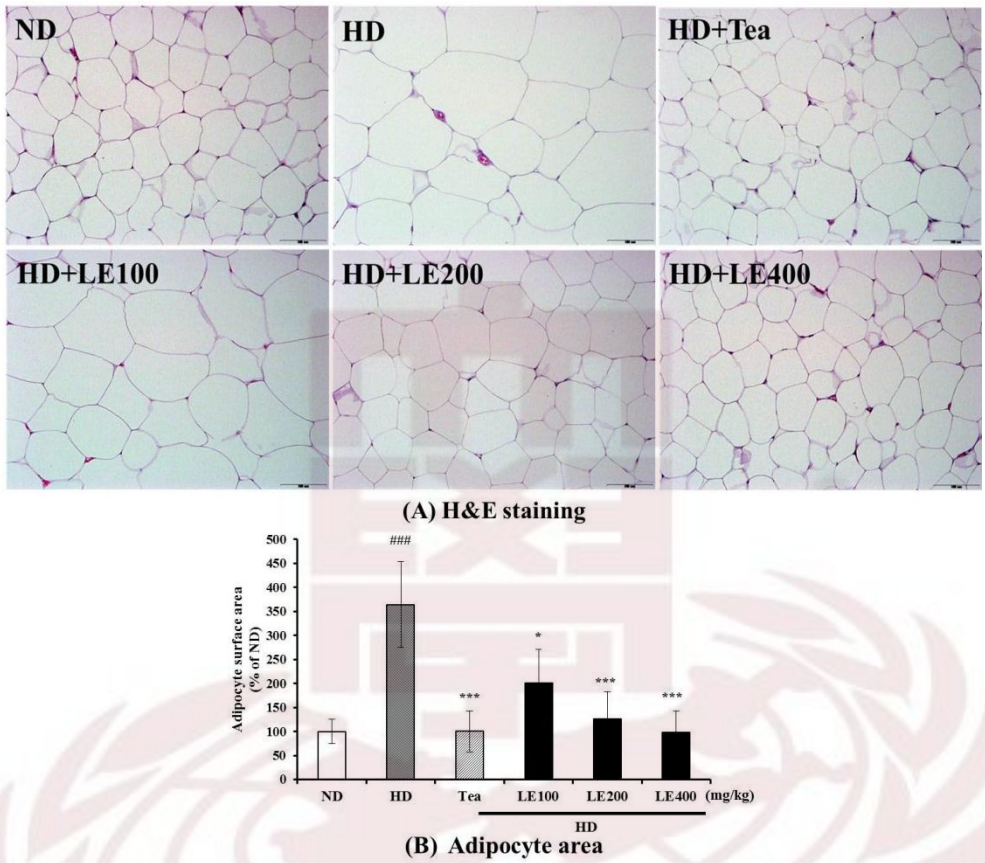

Figure 2. Histological analysis of epididymal adipose tissue (a) and adipocyte surface area (b) of the rats fed the experimental diets for 6 weeks. All sections were stained with hematoxylin and eosin; magnification, X 200. Magnification bar = 100 μm. Mean surface area for epididymal white adipocytes was measured using Image J software. Data are mean ± SD (n ≥ 8). $^{###}P<.001$ versus the ND group; $^{*}P<.05$, $^{***}P<.001$ versus HD group.

4-2. Effect of LE treatment on lipid parameters in blood

Rats in the HD group exhibited significantly higher TG, TC, LDL-C and FFA levels, and lower HDL-C and HDL-C/TC ratio, compared to rats in the ND group (Fig. 3). However, LE (400 mg/kg) or tea catechin treatment led to a reversal of the aforementioned parameters to the levels similar to those of the ND group. LE treatment (400 mg/kg) resulted in 39.6 %, 38.1 %, 85.5 % and 27.2 % decreases in serum TG, TC, LDL-C and FFA, respectively, and a 65.7 % and 49.3 % increase in HDL-C and HDL-C/TC ratio, respectively, compared with no treatment (HD group). These results indicate that oral administration of LE suppresses the accumulation of body fat, resulting in improved lipid profiles in serum.

Serum glucose, insulin, leptin and TNF-α levels were also increased by high fat diet (Fig. 4). Conversely, the adiponectin level was significantly lower in the HD group. However, LE or tea catechin supplemented showed decreased TNF-α, leptin, glucose and insulin levels, and increased adiponectin level in blood compared to the HD group. To evaluate the effect of LE on hepatic and renal function, we determined the plasma GOT, GPT and BUN level in LE treated obese rat (Table 4). Although serum levels of GOT, GPT and BUN of the HD group were significantly higher than those of the ND group, and tea catechin or LE treatment reduced the serum GOT and BUN levels to values of the ND groups, they all were within normal range. These results indicate that LE supplementation did not affect hepatic and renal functions.

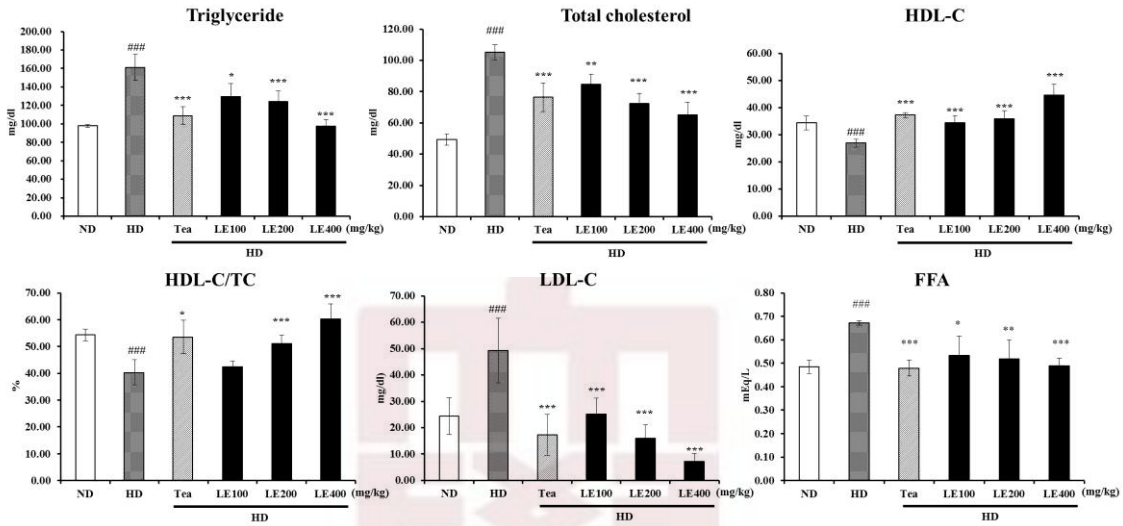

Figure 3. Effect of LE on lipid levels in blood. FFA, free fatty acid; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol. Values are mean ± SD (n ≥ 8). $^{\#\#}P<.01$, $^{\#\#\#}P<.001$ versus the ND group; $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ versus HD group.

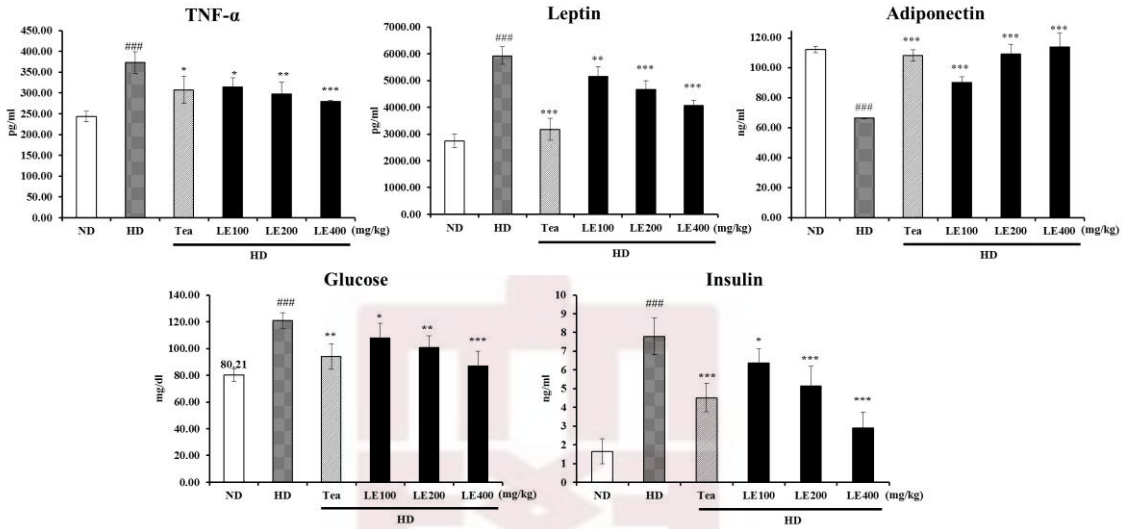

Figure 4. Effect of LE on the level of tumor necrosis factor-α, leptin, adiponectin, glucose, and insulin in bood. Values are mean ± SD (n ≥ 8). $^{###}P$<.001 versus the ND group; $^{*}P$<.05, $^{**}P$<.01, $^{***}P$<.001 versus HD groups.

Table 4. Effect of LE on the liver and renal functions in HD-induced obese rats

Group	ND	HD	HD+Tea	HD+LE100	HD+LE200	HD+LE400
GPT(IU/L)	53.72±0.52	56.35±1.84$^{\#}$	54.83±1.30	57.35±2.10	54.38±6.68	54.96±5.16
GOT(IU/L)	76.00±0.58	77.60±0.48$^{\#\#}$	75.44±0.56***	76.69±1.07	76.58±0.88*	76.41±0.70*
BUN(mg/dl)	16.43±0.23	19.71±0.93$^{\#\#\#}$	15.98±0.73***	16.7±1.21***	15.59±1.34***	15.42±0.36***

Data are mean ± SD (n ≥ 8). $^{\#}P$<.05, $^{\#\#}P$<.01, $^{\#\#\#}P$<.001 versus the ND group; $^{*}P$<.05, $^{***}P$<.001 versus HD groups. GPT, glutamatepyruvate transaminase; GOT, glutamic oxaloacetic transaminase; BUN, blood urea nitrogen.

4-3. Effect of LE treatment on hepatic lipid profiles

Liver weights and hepatic lipid levels are shown in Figure 5. Liver weight was about 1.5 times greater in the HD group than that in the ND group. In addition, hepatic TG, TC and FFA levels in the HD group were significantly higher than ND group. Conversely, HDL-C and HDL-C/TC ratio levels in liver were significantly lower than those in the ND group. LE-treated group (400 mg/kg) resulted in 38.3 %, 33.5 %, and 49.1 % decreases in hepatic TG, TC and FFA, respectively, and 114.4 % and 163.1 % increase in HDL-C and HDL-C/TC ratio, respectively, compared with the HD group. In consistent with these findings, H&E sections from the rats fed HD-only revealed the presence of a large number of circular lipid droplets which were also detected by Oil-red-O staining (Fig. 6). These lipid droplets were strikingly reduced in both size and number in the liver of LE or tea catechin-treated rats.

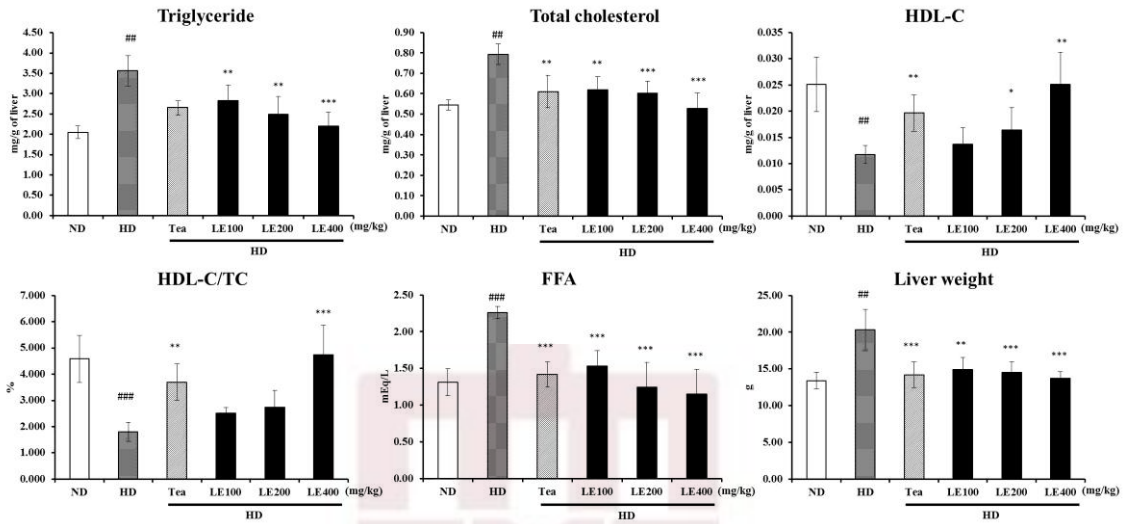

Figure 5. Effect of LE on liver weight and lipid levels in liver. Values are mean ± SD (n ≥ 8). $^{\#\#}P<.01$, $^{\#\#\#}P<.001$ versus the ND group; $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ versus HD groups.

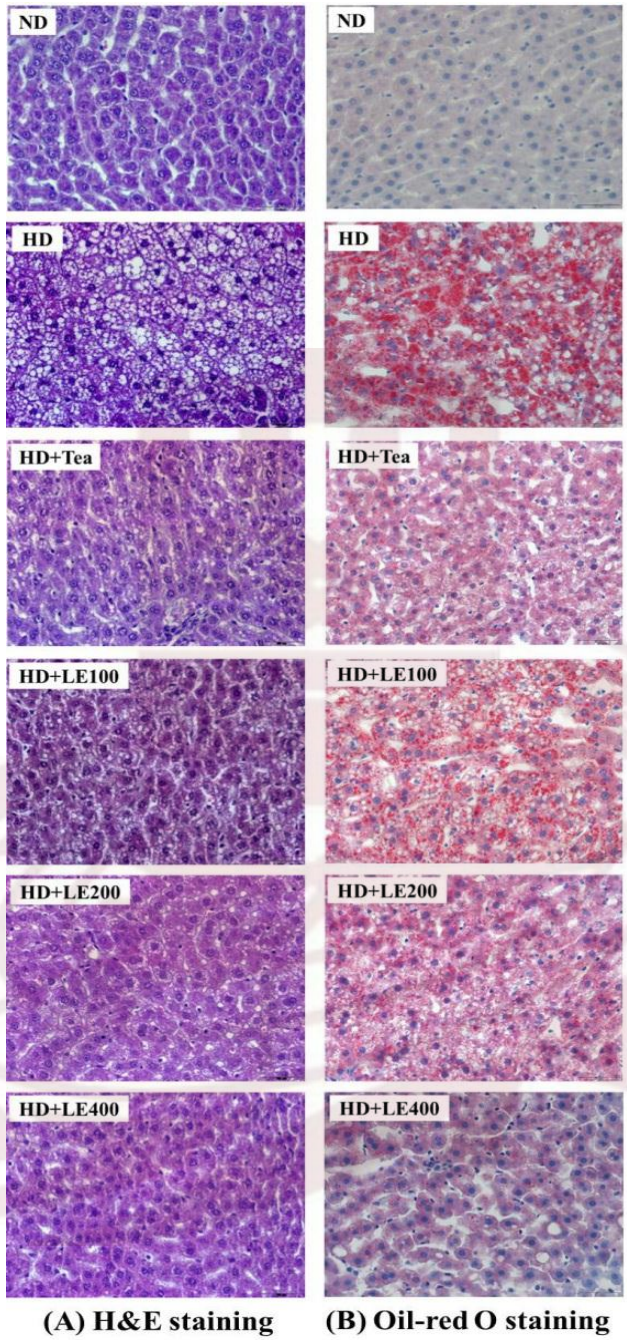

Figure 6. Effect of LE on lipid accumulation in liver. (A) H&E staining (B) and Oil Red O staining

4-4. Effect of LE treatment on the expression of genes related to lipid metabolism in skeletal muscle

RT-PCR was performed to measure the mRNA expression of fatty acid oxidation-related genes such as acetyl-CoA carboxylase 2 (ACC2), carnitine palmitoyl-transferase 1 (CPT-1) and lipolytic genes such as peroxisome proliferator-activated receptor α (PPARα), acyl CoA oxidase (ACO), uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3) in skeletal muscle which is an important organ for energy expenditure (Fig. 7). Compared with control rats (HD group), LE treated rats showed significantly induced mRNA expression of PPARα, ACO, CPT1, UCP2 and UCP3, which were highly reduced in HD rats. Tea catechin as a positive control also showed similar effects of LE treatment (200 mg/kg). These results indicate that oral administration of LE enhanced the expression of fatty acid oxidation-related genes, and lipolytic genes in a dose-dependent manner.

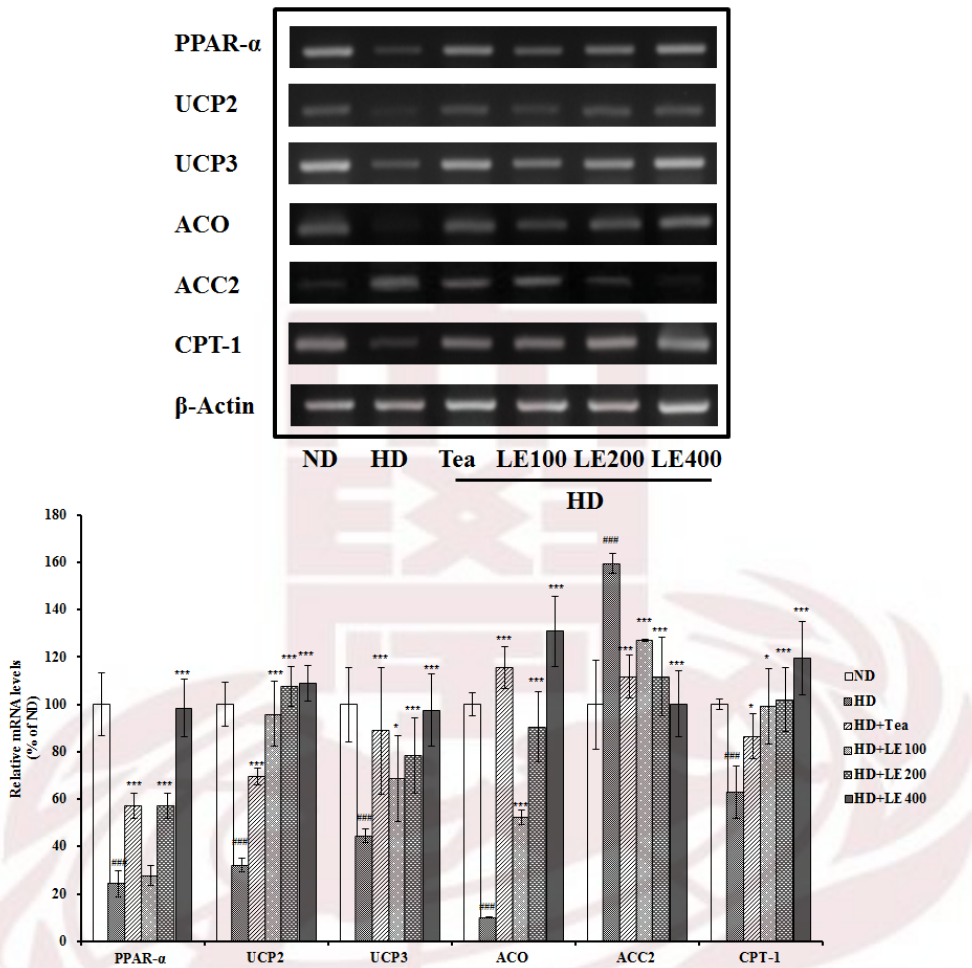

Figure 7. Effect of LE on the gene expressions related to lipid metabolism in skeletal muscle. Rats were treated with LE or tea catechin for 6 weeks. The level of PPARα, UCP2, UCP3, ACO, ACC2 and CPT-1 mRNA were determined by RT-PCR analysis. Actin mRNA was used as an internal control. Relative fold intensities were analyzed using band densities obtained from RT-PCR. All RNA levels were normalized to that of Actin mRNA. Results shown are mean and standard deviation (n≥8). $^{###}P<.001$ versus the ND group; $^{*}P<.05$, $^{***}P<.001$ versus HD groups.

4-5. Effect of LE treatment on the expression of genes related to lipid metabolism in liver

Expressions of lipogenesis-related genes (SREBP-1c, ACC, FAS, SCD-1 and GPAT, AGPAT, DGAT), a fatty acid oxidation-related gene (CPT-1 and PPARα) in liver were measured by RT-PCR (Fig. 8). mRNA expressions of SREBP-1c, ACC, FAS, SCD-1, GPAT, AGPAT and DGAT in the HD group were significantly higher than those in the ND group. However, tea catechin or LE (400 mg/kg) supplementation significantly suppressed the mRNA expressions to near-normal levels. Conversely, mRNA expressions of PPARα and CPT1 in high fat diet rats were significantly rescued by LE treatment. Tea catechin also showed similar effects to LE (400 mg/kg).

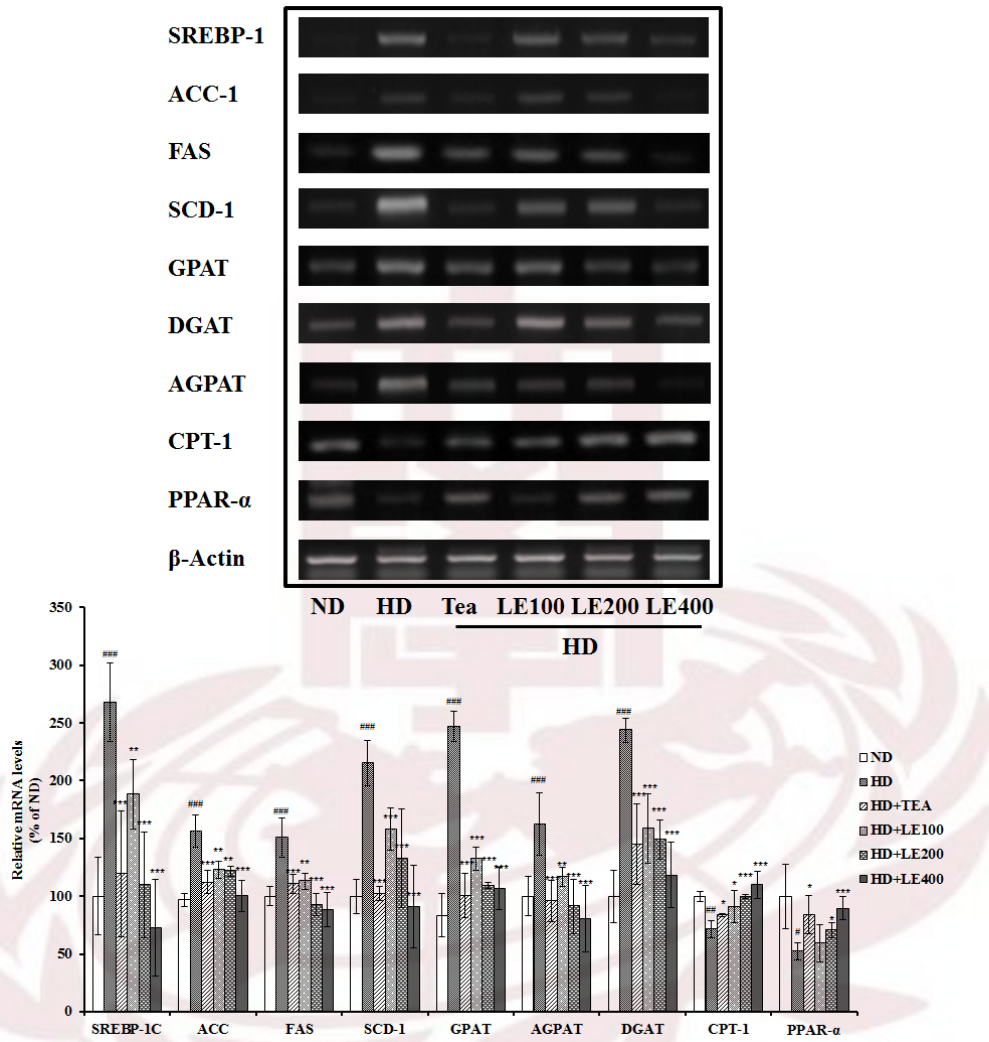

Figure 8. Effect of LE on the gene expressions related to lipid biosynthesis in liver. Rats were treated with LE or tea catechin for 6 weeks. The level of SREBP-1, ACC, FAS, SCD-1, GPAT, DPAT, AGPAT, CPT-1 and PPARα mRNA were determined by RT-PCR analysis. Actin mRNA was used as an internal control. Relative fold intensities were analyzed using band densities obtained from RT-PCR. All RNA levels were normalized to that of Actin mRNA. Results shown are mean and standard deviation ($n \geq 8$). $^{\#}P<.05$, $^{\#\#}P<.01$, $^{\#\#\#}P<.001$ versus the ND group; $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ versus HD groups.

4-6. Effect of LE treatment on the activity of AMPK and ACC in liver

To determine whether LE supplementation affect the activity of AMPK, which are one of the major regulators in lipid metabolism, western immunoblotting analysis were performed using the whole tissue extract prepared from the liver of LE-treated rats. As shown in Figure 9, feeding the rats with the HD significantly lowered the phospho-AMPK/AMPK and phosphor-ACC/ACC ratio in the liver tissue. ACC phosphorylation is regulated by AMPK activity. Therefore, it appears that high level of AMPK phosphorylation resulted in phosphorylation of ACC. As shown in Figure 9, the reduction of phospho-AMPK/AMPK and phospho-ACC/ACC ratio induced by high fat diet was almost completely recovered by LE treatment.

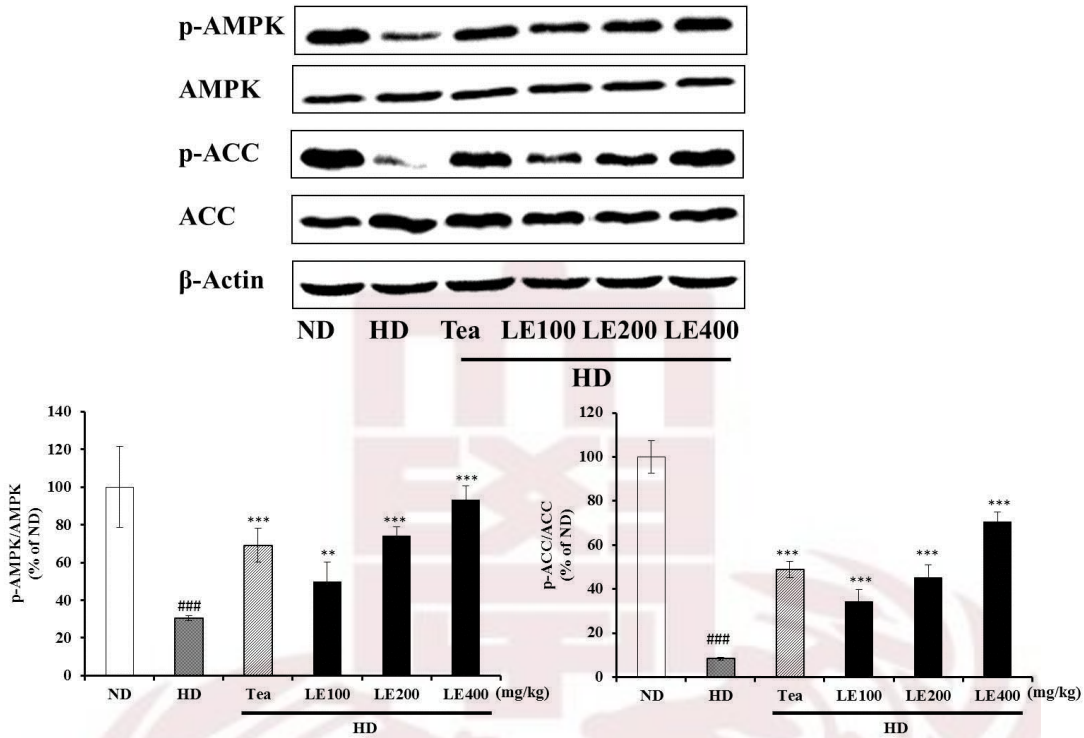

Figure 9. Effect of LE on the phosphorylation of AMPK and ACC in liver. LE treatment enhanced phosphorylation in a dose-dependent manner. Rats were treated with LE or tea catechin for 6 weeks. Relative fold intensities were analyzed using band densities obtained from Western immunoblotting. All levels were normalized to that of β-actin. Total proteins were extracted by cell lysis buffer and resolved by SDS-PAGE. Results shown are mean and standard deviation ($n \geq 6$). $^{###}P<.001$ versus the ND group; $^{**}P<.01$, $^{***}P<.001$ versus HD groups.

Ⅴ. Discussion

Obesity has become a major worldwide health problem [3], not least because it is associated with many diseases, particularly diabetes, hypertension, osteoarthritis, and heart disease. As a result, obesity has been found to reduce life expectancy, and causes huge economic and social problems [4-8]. Thus, the quest for possible natural products that aid in weight loss has been intensified. Intrigued by recent reports indicating that *Laminaria japonica* Areshoung possesses various biological functions including anti-inflammatory [19], anti-tumor [20], anti-therosclorosis [21] and anti-diabetic activity [22], we sought to investigate whether *Laminaria japonica* Areshoung has anti-obesity effect.

In this study, anti-obesity activity of LE was investigated by measuring body weight gain, food intake, and lipid profiles in LE-treated obese rats. High-energy diets are widely used in nutritional experiments as a strategy to induce overweight conditions and fat deposition in animals [25]. We also observed that final body weight, body-weight gain, and total fat-pad weight in the HD group were greater by 30.1 %, 65.9 %, and 135.5 %, respectively, compared to the ND group. Administration of LE (400 mg/kg) or tea catechin for 6 weeks remarkably decreased the body weight gain by more than 21 % compared with that of the HD group (Table 3). The LE significantly and dose-dependently decreased food intake and food efficiency ratio. In addition, the epididymal, abdominal, visceral, and brown fat-pad weights of the obese rats receiving LE (200 or 400 mg/kg) proved significantly lower compared to that of the HD group (Table 3)

In general, a high-fat diet significantly increases the TC and TG levels in serum and liver [26]. Our data also showed that rats in the HD group exhibited significantly higher TG, TC, LDL-C and FFA levels, and lower HDL-C and HDL-C/TC ratio. However, the administration of LE or tea catechin reduced these parameters to near normal levels in serum and liver (Fig. 3 & 5). These results indicate that oral administration of LE suppresses the accumulation of body fat in a dose-dependent manner, resulting in improved lipid profiles in serum and liver. Notably, LE treatment did not show any renal or hepatic toxicity (Table 4).

Leptin, a key fat-derived regulator of appetite and energy expenditure, has levels in blood that usually correlate positively with the extent of the TG stores in adipocytes [27-28]. In our

study, the plasma leptin level was decreased by LE or tea catechin treatment (Fig. 4). Moreover, our histological examinations revealed that the sizes of the adipocytes were significantly reduced in LE-treated rats (Fig. 2). These results suggest that the decreased plasma leptin levels after LE supplementation might be attributable to decreased lipid accumulation in white adipose tissue. In addition, HD is known to increase the synthesis of fatty acids in the liver and the delivery of free fatty acids to the liver [29-30], and decrease β-oxidation of free fatty acids, resulting in fat accumulation in the liver [30]. Our histological examination also showed that macrovesicular steatosis in liver tissues of the HD group (Fig. 6). However, LE supplementation noticeably attenuated the extent of steatosis, suggesting that LE may regulate lipid storage and mobilization in adipocyes by modulation of the leptin level.

It is noticeable that LE (400 mg/kg) treatment significantly reduced the serum glucose and insulin levels in obese rats (Fig. 4). Considering the increase in blood glucose and insulin in animals on the high-fat diet is a strong indicator of obesity-induced insulin resistance and progression to type-2 diabetes, these results suggest that LE has protective effect against the development of obesity-induced insulin resistance.

It has been reported that TNF-α secretions are elevated by the accumulation of fat in adipocytes and that TNF-α induces insulin resistance in obese animal models [31-35]. The TNF-α level were greater in the HD group than in the ND group. LE (400 mg/kg) treatment reduced the serum TNF-α level to values similar to those of the ND group. Tea catechin also showed similar effects to LE at 100 mg/kg (Fig. 4). It seems possible that the lowered TNF-α levels are due to the decreased fat mass by LE treatment.

To gain insight into the molecular mechanism underlying the anti-obesity effects of LE described above, we determined the expression of genes involved in obesity and lipid metabolism. PPARα is known to regulate lipid metabolism through a ligand-dependent transcriptional activation of the expression of genes involved in the fatty acid oxidation pathway. Activation of PPARα expression is known to increase expression of CPT-1, ACO, UCP2 and UCP3 to elevate energy expenditure, subsequently resulting in anti-obesity actions [36-39]. The expression levels of PPARα in the skeletal muscle showed significant increase in the rats fed with LE or tea catechin, compared to the HD group (Fig. 7). The expression of downstream target genes of PPARα, such as ACO, CPT1, UCP2 and UCP3 were significantly increased by

LE administration. Furthermore, LE lowered the mRNA expression of ACC2, which are related to fat intake by the skeletal muscle. acetyl-CoA carboxylase (ACC2) is a key regulator of mitochondrial fatty acid (FA) uptake via carnitine palmitoyltransferase 1 (CPT1) in the skeletal muscle. In this study, LE may lead to loss of body fat by increasing fatty oxidation in the skeletal muscle through the mRNA expression of ACC2 (Fig. 7). These results suggest that the anti-obesity effect of LE is likely due to the increase of fatty oxidation and thermogenesis in the liver and skeletal muscle through PPARα and its downstream target genes.

To maintain lipid homeostasis, adipocytes perform two reciprocal biochemical processes, called lipogenesis and lipolysis. These two processes are regulated by hormones, lipid metabolites and nutritional conditions [40]. Regarding these signals, lipogenic transcription factors, including SREBP1c, play key roles in lipid metabolism in adipose tissue by regulating the gene expressions of enzymes for fatty acid synthesis and uptake, and TG synthesis [40-41]. FAS, ACC1 and SCD1, a downstream factor of SREBP1c, are enzymes for de novo fatty acid synthesis and energy homeostasis [42]. Fatty acids are critical substrates for biosynthesis of triglycerides. Several genes, especially GPAT, AGPAT, and DGAT, are involved in catalyzing the several steps of triglyceride synthesis in turn [43-48]. We demonstrated that the mRNA levels of lipogenesis-related genes such as SREBP1c, FAS and ACC1, which were increased in the liver of rats fed with HD, were down-regulated when they were fed with LE (200 mg/kg) or tea catechin (Fig. 8). Similarly, the mRNA levels of GPAT, DGAT1 and AGPAT were decreased in the liver from rats treated with LE or tea catechin, compared with the HD group. These results suggest that the anti-obesity effects of LE are possibly due to the suppression of the mRNA expression levels of genes involved in fatty acid and TG synthesis.

To further investigate the molecular mechanism, we determined the effect of LE on the activity of AMPK. Both leptin and adiponectin stimulate fatty acid oxidation via the phosphorylation of AMPK and ACC [49-50]. AMPK is a phylogenetically conserved serine/threonine protein kinase which has been proposed to act as a ‘metabolic master switch’ mediating the cellular adaptation to environmental or nutritional stress factors [51]. Once activated, AMPK leads to a concomitant inhibition of energy-consuming biosynthetic pathways, such as fatty acid and sterol synthesis, and activation of ATP-producing catabolic pathways, such as fatty acid oxidation. Phosphorylated AMPK phosphorylates (inactivates) ACC and

lowers the intracellular malonyl-CoA level, which is the substrate for fatty acid synthesis and, at the same time, the inhibitor of CPT-1, the rate-limiting enzyme of mitochondrial fatty acid oxidation [52]. Therefore, phosphorylated AMPK increases fatty acid oxidation through a decrease in myocardial malonyl-CoA levels and an increase in CPT-1 activity [53-55]. In the present study, the protein levels of phospho-AMPK and phospho-ACC, which were reduced in the liver of rats fed with HD, were up-regulated when they were fed with LE (100 mg/kg) or tea catechin. Tea catechin also showed parallel effects to LE at 200 mg/kg (Fig. 9). Thus, it appears that LE stimulates β-oxidation of fatty acid by activation of AMPK pathway.

Ⅵ. Conclusion

Taken together, LE treatment significantly reduced body weight gain, food intake, lipid levels in serum and liver and size and number adipocyte in high fat induced-obese rats. LE appears to have anti-obesity effects by modulating lipid metabolism through the decreased activity in lipogenesis as well as the increase in fatty acid oxidation. These results demonstrate that LE supplementation could exert beneficial effect against the development of obesity.

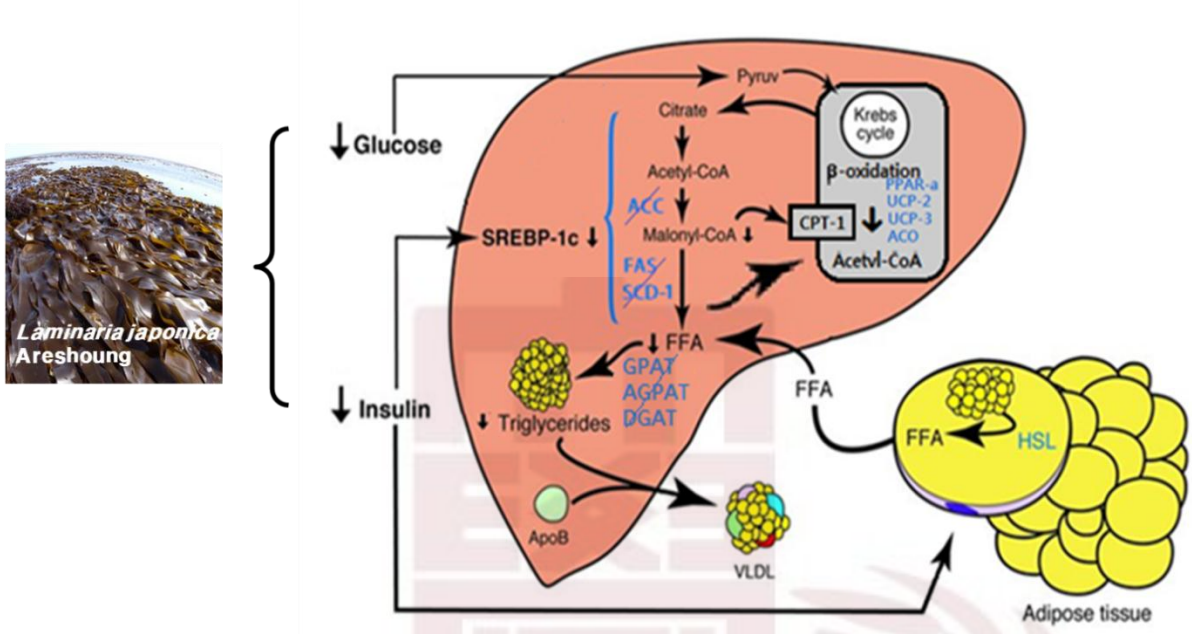

Figure 10. Role of *L. japonica* in the transcriptional regulation of lipogenic genes in liver. The diagram shows the major metabolic intermediates in the pathways for synthesis of fatty acids and triglycerides. *L. japonica* decreased mRNA expression ACC, FAS, SCD-1, GPAT-1, AGPAT and DGAT regulating enzymes of lipogenesis in the liver, and mRNA expression of SREBP-1c, which controls the expression of these enzymes (Jay D, 2002) [56].

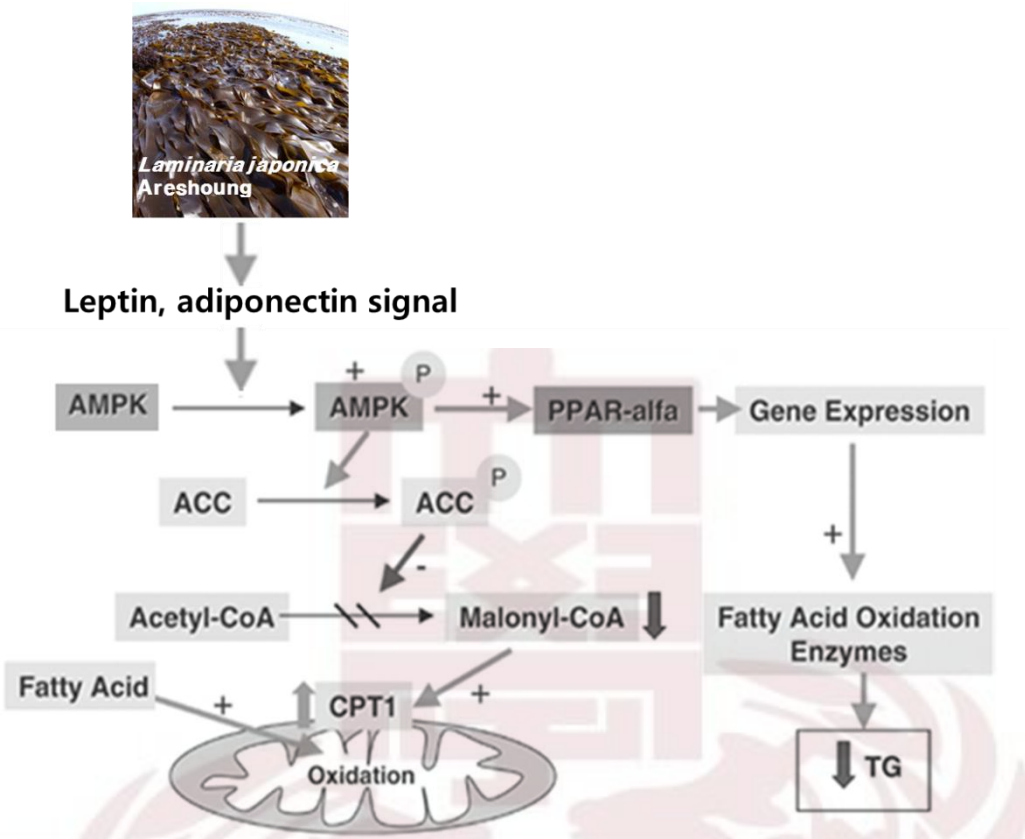

Figure 11. Potential stimulatory effect of *L. japonica* on fatty acid oxidation in the liver. *L. japonica* could activate AMPKK/AMPK in the liver. Activation of AMPK phosphorylates and inhibits ACC activity. Malonyl-CoA synthesis is inhibited, through the action of leptin and adiponectin resulting in activation of CPT1, thereby increasing mitochondrial import and fatty acid oxidation in the liver(Marx J, 2003) [57].

Acknowledgments

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국문초록

비만이란, 신체 에너지의 섭취와 소비의 불균형으로 발생되는 과도한 에너지가 체지방으로 축적되어 나타나는 일종의 대사성 질환으로서 전 세계적으로 심각한 건강상의 문제로 대두되고 있다. 비만의 원인은 일반적으로 과다한 열량섭취, 운동부족이 주요 원인으로 꼽히고 있으며 개인차가 심하여 유전적인 요인, 체질 등 여러 원인이 제시되고 있다. 비만은 심혈관계 질환, 당뇨, 고혈압, 고지혈증, 담석증 등의 신체적 질환뿐만 아니라, 체중 증가로 인한 용모 손상을 비롯하여 그로 인한 스트레스를 야기하는 등 정신적 건강까지 영향을 미치므로 이에 대한 대책 마련이 시급한 실정이다.

비만의 예방과 치료를 위한 일환으로 행동양식의 관리(식사요법, 운동요법, 행동조절요법), 의학적인 기술의 적용(치아고정, 위 절제술), 사회적 또는 정신적인 측면에서의 관리 등 다방면에서 복합적으로 이루어지고 있지만 최근에는 인공적으로 합성된 항 비만 약제를 복용하는 방법이 많이 이용되고 있다. 그러나 이러한 약물들은 대부분 부작용을 가지고 있어 부작용을 최소화할 수 있고 향상된 항 비만 기능을 가진 천연물질을 이용한 비만 치료제 개발이 절실히 요구되고 있다.

본 연구에서는 체중 조절 효과가 우수하고, 부작용이 없는 천연소재를 개발하고자 다시마 주정 추출물을 고지방 식이로 비만을 유도한 렛드에 100, 200, 400 mg/kg의 농도로 6주간 경구 투여하면서 체중, 혈중 지질 및 지방 대사에 미치는 영향 등을 검토하여 다시마 추출물의 항 비만 효과와 작용기전을 살펴보았다.

그 결과 대부분의 지표에서 비만한 대조군에 비해 다시마 추출물 투여 군에서 개선되는 결과를 보여주었다. 이들은 체중뿐만 아니라 혈당 수치에서도 유의적인 감소를 나타내었으며 체중감소 작용 기전은 간 에서 metabolic sensor에 해당하는 단백질인 AMPK의 활성화와 그로 인한 지방산 산화촉진, 그리고 간과 근육에서 SREBP-1, FAS, SCD1, GPAT, AGPAT, DGAT 등 lipogenesis gene들의 발현을 억제하여 지방합성 억제, PPAR-α, CPT-1, ACO, UCP2, UCP3 등 지방산 산화 및 에너지 소모에 관여하는 gene들의 발현 증가 등에 기인하는 것으로 사료된다.

결론적으로 다시마 추출물은 고지방식이 유도 비만 동물모델에서 체중 감소 활성을 나타내었고, 이로 인해 혈당치 및 지질 프로파일이 개선되는 활성을 보여주어서 향후 비만 치료제 혹은 예방 목적으로 개발될 수 있을 것으로 기대된다.