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Long Term Feeding with Soy Isoflavone and L-Carnitine Synergistically Suppresses Body Weight Gain and Adiposity in High-Fat Diet Induced Obese Mice

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Objective: We investigated the efficacy of a 12-week supplementation of soy isoflavone with L-carnitine on the development of obesity in high fat-induced obese C57BL/6J mice, which are known as a good model of diet-induced obesity.

Methods: We measured body weights, adipose tissue mass, serum/liver lipid profiles and fat cell size/number in C57BL/6J mice fed diets containing either low fat (4%) or high fat (35%), or high fat supplemented with soy isoflavone powder containing 10% isoflavone and L-carnitine for 12 weeks.

Results: Body weight gain, abdominal adipose tissue and liver weight were lower by 31%, 78%, and 31.4%, respectively, in mice on high fat diet containing soy isoflavone+L-carnitine (SC mixture) compared with high fat diet group. Also, SC mixture improved serum lipid profiles such as total cholesterol (TC), triglycerides (TG), and liver lipid profiles such as total lipids and TG. As subsequent results, this SC mixture prevented high-fat diet from accumulating TG in the liver. The size of fat cell was also significantly decreased in SC mixture fed mice. At the end point of this experiment, our results showed that feeding with soy isoflavone for 12 weeks finally increased carnitine palmitoyltransferase 1 (CPT 1) activity through elevating the level of CPT1 expression.

Conclusions: This study suggests that long-term supplementation with dietary soy isoflavone and L-carnitine is more synergistically beneficial for the suppression of high-fat diet induced obesity by inhibiting liver TG accumulation and the gain in abdominal adipose tissue weight than that with soy isoflavone. The antiobesity effects of SC mixture might be attributed, at least in part, to the induction of fatty acid catabolism by soy isoflavone, genistein.

Key words: Soy isoflavone, Obesity, High-fat diet, Carnitine palmitoyltransferase 1, Lipid profile

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INTRODUCTION

Obesity can be defined as a condition of abnormal or excessive fat accumulation in adipose tissue to the extent that health may be impaired. Obesity is known to be a strong risk factor for hypertension, coronary heart disease, type 2 diabetes, and certain types of cancer.[1] Among the causes of obesity, high energy diet is strongly associated with the development of obesity. Obesity is rapidly increasing in industrialized countries and recognized as a major public health problem.

In recent years, isoflavones have been brought to many people's attention because of their several beneficial aspects, which provide protection against degenerative diseases such as menopausal symptoms, osteoporosis, cardiovascular disease and cancer.[2] Several studies in animals and humans have shown that the consumption of soybean have beneficial effects in variety of disorders including hypocholesterolemia as well as protection against cardiovascular disease, renal disease, bone resorption, certain forms of cancer, and menopausal symptoms.[2-8] Increasing evidence suggests that soybean may also have beneficial effect against obesity and diabetes.[9] There are many evidences that dietary isoflavones may play a beneficial role in lipid and glucose metabolism.[10-13] According to an observational study with postmenopausal women, higher daily isoflavone intake decreased body mass index (BMI) and induced the tendency toward lower fasting insulin concentration.[14] In a study of 48 nonhuman primates, soy protein containing isoflavones resulted in

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a significant improvement in plasma lipid, insulin sensitivity and glucose effectiveness. Considering that obesity is a disorder pertaining to energy imbalance and associated with abnormal insulin and lipid metabolism, it might be assumed that isoflavones would play a role in the control of body weight and fat accumulation.

The most abundant food sources of isoflavones are soybeans and soy products. Genistein is a major soy isoflavone and has been the focus of studies investigating the biological effects of isoflavones. There are many evidences that genistein has effects on lipid metabolism. In isolated rat adipocytes, genistein inhibited lipogenesis by restricting glucose conversion to total lipids. Also, it has an inhibitory effect on the proliferation of both preconfluent and postconfluent 3T3-L1 preadipocyte cells.[15,16] In the perfused liver, genistein decreased the incorporation of glucose into lipids and increased the release of fatty acids into the medium.[17] The liver triglyceride (TG) contents were also decreased by genistein.

Given that genistein promotes lipolysis and inhibits adipogenesis *in vitro*, it might be expected that genistein would act similarly in vivo and have an inhibitory effect on fat accumulation. Nogowski et al. determined the effects of genistein on the lipid metabolism of ovariectomized rats.[17] They reported that dietary genistein significantly decreased serum triglyceride levels, but increased the free fatty acid concentration. It hasalso been reported that dietary genistein exerts a hypolipidemic action by suppressing both nephritis-induced severe hypercholesterolemia and hypertriglyceridemia in rats with glomerulonephritis.[18] When Schleicher et al. studied rats with accessory sex gland carcinoma, those rats supplemented with genistein had decreased body weight compared with the controls.[19] All things considered, it might be expected that dietary genistein would have a beneficial effect on obesity. Although genistein has a lot of beneficial effects on several diseases such as mentioned above, there arefew works of conventional safety studies published for genistein per se. Even soybean and its constituents such as genistein, have been consumed at high levels in several Asian populations without apparent adverse effects, concern has been raised about potential adverse effects due to the estrogenic and other activities. In recent comprehensive safety studies with genistein in rats, the no observed adverse effect level (NOAEL) of genistein is considered to be 50 mg/kg/day based on the presence of mild hepatic effects at the high dose of 500 mg/kg/day. The no observed effect level (NOEL) is considered to be 5mg/kg/day based on the hormonally induced functional changes at higher doses.[20]

In our previous studies on antiobesity, we reported that genistein enhanced the expression of genes involved in fatty acid catabolism *in vitro* and *in vivo* tests.[10] But, for avoiding the possibility to occur to adverse effects caused by estrogenic effects of genistein, we decided to use the soy isoflavone powder extracted from soy embryo having total 10% genistein content for antiobesity studies.

L-cartinitine is an important component to generate energy by oxidizing fats, which is prepared inliver or kidney of human and contained generally in meat. When L-carnitine is not sufficient, the concentration of fatty acid in the mitochondria becomes low, and as a result, the generation of energy also becomes low. In addition, it is also reported that CPT-1 using L-carnitine as substrate works as a rate-limiting enzyme in the oxidation of fatty acid.[21]

However, the long-term effects of dietary isoflavone and L-carnitine on diet-induced obesity in vivo test have not yet been investigated in detail.

The purpose of this study is thus to determine whether complex supplementation of dietary isoflavone and L-carnitine may synergistically prevent the development of obesity in high fat-induced obese C57BL/6J mice, which are known as a good model of diet-induced obesity.[22]

METHODS

1. Animals and Diets

All animal experiments in this study were approved by the ethical committee for the animal experiments of the pharmaceutical & health research institute, Amorepacific Co. and carried out in the facility of the preclinical research lab. Male, 5 weeks old, C57BL/6J mice were obtained from Charles River Laboratory (Wilmington, USA). Because C57BL/6J (B6) inbred strains of mice develop their body weight to a comparable degree of obesity when fed a high-fat diet, they are usually used in the obesity study. Male mice were used to eliminate endogenous estrogen effect of female mice. The mice were housed individually and maintained at a controlled temperature (22±2℃), humidity (65±5%) and light cycle (12 h light/12 h darkness, lights on from 06:00 to 18:00). The mice were allowed to rest for one week in the animal facility before beginning the experimental feeding. The mice were randomly divided into five groups (n=7~8) and fed one of the following diets: a normal fat diet (NF), a high-fat diet (HF), a high-fat diet supplemented with 0.2% soy isoflavone powder containing 10% isoflavone content (HFS), a high-fat supplemented with 0.5% L-carnitine (HFC) or a high-fat

Table 1. Composition of experimental diet (AIN-93 modified diet for rodents)

Ingredients (g)	Control group	Treated groups			
	NF[1]	HF[2]	HFS	HFC	HFSC
Casein, lactic	200	200	200	200	200
L-cystine	3	3	3	3	3
Corn Starch	315	-	-	-	-
Maltodextrin	35	125	125	125	125
Sucrose	350	68.8	68.8	68.8	68.8
Cellulose	50	50	50	50	50
Soybean Oil	25	25	25	25	25
Lard	20	245	245	245	245
Mineral Mix	10	10	10	10	10
Dicalcium Phosphate	13	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5	5.5
Potassium Citrate	16.5	16.5	16.5	16.5	16.5
Vitamin Mix	10	10	10	10	10
Choline Bitarate	2	2	2	2	2
FD&C Yellow Dye #5	0.05	-	-	-	-
FD&C Blue Dye #1	-	0.05	0.05	0.05	0.05
Soy isoflavone powder[3]	-	-	1.55	-	1.55
L-Carnitine	-	-	-	3.87	3.87
Total	1055.05	773.85	775.40	777.72	779.27
Kcal	4057	4057	4057	4057	4057
Kcal/g	3.8	5.2	5.2	5.2	5.2

[1] AIN-93 Modified diet with 4% fat (10% fat calorie) content,
[2] AIN-93 Modified diet with 35% fat (60% fat calorie) content,
[3] Fat diet containing 0.2% soy isoflavone powder containing 10% isoflavones, NF normal diet, HF; High fat diet, HFS High fat diet plus soy isoflavone powder, HFC; High diet plus L-carnitine, HFSC; High fat diet plus soy isoflavone powder plus L-carnitine

diet supplemented with 0.2% soy isoflavone powder plus 0.5% L-carnitine (HFSC). The experimental diets were manufactured based on AIN-93M. The normal fat diet contained 10 % fat calorie of total energy compared with 60% fat calorie of total energy in the high-fat diets. The detailed composition of the diets is listed in Table 1. Soy isoflavone powder and L-carnitine (more than 99% purity) were purchased from Fujicco Co LTD (Osaka, Japan) and Lonza. Ltd (Basel, Switzerland) respectively. The animals were allowed *ad libitum* access to water and food. The experimental diets were maintained for 12 weeks.

2. Body Weight and Food Intake

Body weight was measured weekly throughout the study. The total amount of food intake for each mouse was recorded at least three times every week for 12 weeks, so total at least 36 observations were achieved.

3. Sample Collection

On the final day of the experiment, blood samples were taken for the determination of the serum lipid level of the animals via the orbit venous plexus after anesthetizing them with ketamine hydrochloride following over-night starvation. Subsequently, the liver was quickly removed, weighed and frozen in liquid nitrogen in separated portions for RNA isolation and lipid analysis, respectively, or stored in 10% formalin for histochemical study. Abdominal and back fat tissues were also quickly removed and weighed. Abdominal fat tissues were stored in 10% formalin to determine the number and size of fat cells.

4. Serum and Liver Lipid Analysis

Serum was prepared by centrifugation of the blood sample at 3000 rpm for 20 min and then stored at 80℃ until the analysis was performed. Serum total cholesterol, glutamic oxaloacetic transaminase (GOT), and γ-glutamic pyruvic transaminase (γ-GPT) were measured using a fully automated dry chemistry system (SPOTCHEM, Daiichi Kagaku Co., Japan). Serum free fatty acids were measured by enzymatic, colorimetric methods using a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany). The serum triglyceride was determined using an assay kit (Sigma Diagnostics, St. Louis, MO, USA). Serum leptin was measured by radioimmunoassay kit (Linco ML82K, St. Charles, MO).

Total lipids from the liver were extracted according to the Bligh and Dyer procedure using a 2:1 chloroform: methanol solution.[23] The TG and total cholesterol levels in the liver lipid extracts were measured by a spectrophotometric method using a specific, commercially available enzymatic kit (YD Diagnostics, Korea).

5. Determination of Fat Cell Size/Number

Freshly isolated white adipose tissue from each group was fixed overnight in 10% formalin, dehydrated, and embedded in paraffin for subsequent sectioning. Sections(8 μm) were stained with hematoxylin and eosin, and cell size was analyzed using Image J (National Institutes of Health, Bethesda, MD) Software. At least 300 cells from each animal were measured.

6. Histopathological Investigation in Liver

Small pieces of liver tissue from sacrificed animals were preserved in 10% formal saline. Light microscopy was performed after slides were routinely stained with haematoxylin and eosin (H&E)

7. Western Blot Analysis

Mouse liver was homogenized in Proprep solution (Intronbio, Korea) with a glass homogenizer. Fifty micrograms of total protein were analyzed under reducing conditions on 8% sodium dodecyl sulfate/polyacrylamide gel and blotted onto PVDF membrane. The blot was blocked by 5% non-fat milk for 3 hours. After blocking, the blot was incubated with anti-CPT1 (1 μg/ml, alpha diagnostics) followed by horseradish peroxidase-conjugated anti-rabbit IgG1 (1:1000, Amersham BioScience). The reaction products were detected by chemiluminescence with the ECL kit (Amersham BioSciences) according to the manufacturer's instructions. Anti-actin (0.5 μg/ml, Santa Cruz) was used to assess equal loading of the protein.

8. Determination of CPT1 Activity

The CPT1 activity in liver was determined from the tissue homogenates using slight modification of the method developed by McGarry[24] et al and Zierz and Engel.[25] This method measures the rate of palmitoylcarnitine formation from palmitoyl-CoA and carnitine. A 10 μl aliquot of the 20-fold diluted tissue homogenates was preincubated for 10 min at 30℃ in a microcentrifuge tube. The reactions were initiated when 90 μl of reaction mixture was added to the preincubated tissue homogenates at 30℃ for 10 min. The substrate was 0.2mM[^{3}H]-carnitine (0.5μCi) and the incubation mixture (pH 7.4) included 117 mM Tris-HCl, 0.28 mM reduced glutathione, 4.4 mM ATP, 4.4 mM MgCl2, 16.7 mM KCl, 2.2 mM KCN, 40 mg/l rotenone, 0.1% BSA, and 50 μM palmitoyl CoA. The reaction was quenched with 60μl 1.2 mM ice-cold HCl. The formed [^{3}H]-palmitoylcarnitine was extracted with water-saturated butanol and determined by liquid scintillation counting.

Glycerol-3-Phosphate Dehydrogenase(GPDH) and FAS Enzymatic Activity

GPDH (EC 1.1.1.8) activity was measured by a spectrophotometric method for the determination of oxidized NADH during GPDH-catalyzed reduction of DHAP,[26,27] as modified by Ref.[28] Protein content was determined by bicinchoninic acid (BCA) method using BSA as a standard (Pierce Chemical, Rockford, IL)

The activity of the FAS was determined with the method of Linn.[29] Briefly, frozen tissue samples (~100 mg) were crushed in liquid nitrogen and then homogenized in 0.3 ml of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, and protease inhibitor cocktail. Fat-depleted infranatants were obtained after centrifugation at 800 g and 4℃ for 10 min. A second centrifugation was performed on the infranatant at 100,000 g and 4℃ for 1 h. Then 60 μl of the supernatant was added to 220 μl of 100 mM phosphate buffer (pH 6.5) containing 85 μM acetyl-CoA and 126 μM NADPH in the final concentration. The reaction was started by adding malonyl CoA at a final concentration of 115 μM. The oxidation of NADPH was followed at 340 nm and 37℃ for 20 min.

9. Statistics

In this study, significance of differences was determined by using two-way analysis of variance (ANOVA) using SAS software version 8 (SAS Institute, Cary, NC, USA).

Significance of differences within the four groups were determined by Duncan's multiple range test and the accepted level of significance was $p<0.05$. Results were expressed as mean±standard deviation(S.D)

RESULTS

1. Food Consumption, Body Weight, and Abdominal and Back Fat Tissue Weights

The mice fed on the high fat diet containing 35% lard for 12weeks had significantly higher body weight and significantly heavier visceral adipose tissues (e.g., abdominal and back adipose tissue) than the mice fed on the normal diet (Table 2). Food consumption (g/day) during the whole experimental period did not differ significantly between the normal diet group and high fat diet group(2.62±0.17 in the normal diet versus 3.10±0.71 in the high fat diet group), but only HFSC group differed significantly from other 3 high fat diet groups (HF, HFS and HFC groups)(Table 2).

During the entire period of study, the body weights of the C57BL/6J mice were regularly measured. As shown

Table 2. Effects of soy isoflavone powder and L-carnitine on body weight, fat weight, food intake and energy intake of mice fed a high fat diet for 12weeks

	Control group	Treated groups			
	NF	HF	HFS	HFC	HFSC
Initial Weight (g)	19.15 ± 0.36^{a}	19.59 ± 0.55^{a}	19.16 ± 0.26^{a}	19.44 ± 0.31^{a}	19.28 ± 0.34^{a}
Final Weight (g)	26.26 ± 1.85^{c}	38.14 ± 2.36^{a}	30.65 ± 1.79^{b}	36.25 ± 3.10^{a}	26.41 ± 2.17^{c}
Weight gain (g/day)	0.12 ± 0.05^{c}	0.25 ± 0.09^{a}	0.19 ± 0.10^{bc}	0.23 ± 0.11^{ab}	0.14 ± 0.08^{c}
Food intake (g/day)	2.62 ± 0.17^{ab}	3.10 ± 0.71^{a}	2.62 ± 0.62^{ab}	2.56 ± 0.29^{ab}	2.48 ± 0.34^{b}
Energy intake (Kcal/day/mouse)	9.93 ± 0.59^{c}	16.2 ± 3.68^{a}	13.62 ± 3.24^{ab}	13.33 ± 1.57^{ab}	12.88 ± 2.23^{b}
Abdominal fat (g)	0.42 ± 0.13^{c}	2.31 ± 0.26^{a}	0.86 ± 0.23^{b}	2.07 ± 0.50^{a}	0.49 ± 0.04^{c}
Abdominal fat content % (g/b.w)	1.63 ± 0.58^{c}	6.06 ± 0.64^{a}	2.82 ± 0.78^{b}	5.75 ± 0.60^{a}	1.86 ± 0.20^{c}
Back fat (g)	0.19 ± 0.06^{b}	1.48 ± 0.38^{a}	0.39 ± 0.10^{b}	1.33 ± 0.26^{a}	0.20 ± 0.01^{b}
Back fat content % (g/b.w)	0.73 ± 0.28^{b}	3.90 ± 1.05^{a}	1.29 ± 0.34^{b}	3.71 ± 0.91^{a}	0.77 ± 0.06^{b}

Mean±S.D of 6 mice per group. Values with different alphabet within the same raw are significantly different at P<0.05 by Duncan's multiple range test. NF normal diet, HF high fat diet, HFS high fat diet plus soy isoflavone powder, HFC; high diet plus carnitine, HFSC; high fat diet plus soy isoflavone powder plus carnitine

in Table 2, the high-fat diet induced the larger increase in body weight as compared with the normal control diet, with the final weight being 38.14±2.36 g in the high-fat diet group compared with 26.26±1.85 g in the normal fat diet group. Feeding with diets containing 0.2 % soy isoflavone powder containing 10% isoflavone and 0.5% L-carnitine (HFSC) markedly reduced high-fat diet-induced body weight gain by 31%.

To examine the effects of isoflavone and L-carnitine on visceral fat accumulation, we analyzed the distribution of fat in two individual fat fads. Consumption of the high-fat diet for 12 weeks resulted in significant increases in abdominal and back fat tissue weights compared to those of the normal fat diet group. SC (soy isoflavone powder 0.2% containing 10% isoflavone and 0.5% L-carnitine) significantly reduced the final weight of abdominal/back fat by 78%, 86% respectively. It was more powerful effect on suppressing fat accumulation than soy isoflavone powder alone. These results suggested that the combined supplementation with isoflavone and L-carnitine is more useful for the suppression of body fat accumulation.

2. Serum Lipid Profiles, GPT & GOT, and Leptin

Table 3 shows the effects of the high-fat diet containing soy isoflavone powder and L-carnitine on the blood lipid profile. The serum concentration of triglyceride was unchanged in NF, HFS and HFC groupscompared with the high fat control diet group, but, HFSC group was significantly different fromhigh fat control group. Plasma FFA levels tended to be increased in 0.2% soy isoflavone powder treated group and combined treated group with 0.2% soy isoflavone powder and 0.5% L-carnitine. The high-fat diet induced significantly higher serum total cholesterol level compared with the normal fat diet group. However, in contrast to the high-fat fed mice, those mice fed with 0.2% soy isoflavone powder and 0.2% soy isoflavone powder +0.5% L-carnitine mixture showed significantly suppressed elevation of serum total cholesterol. In addition, plasma HDL- and LDL-cholesterol levels were also significantly decreased in HFS and HFSC groups as compared with HF group. These results that plasma TC was markedly increased in the HF mice, while plasma TG and FFA levels remained unchanged, were agreed with the previous studies, which showed that long-term

Table 3. Effects of soy isoflavone powder and L-carnitine on plasma components in C57BL/6J mice

	Control group	Treated groups			
	NF	HF	HFS	HFC	HFSC
Triglyceride (mg/dl)	165.72 ± 48.68^{ab}	196.51 ± 66.04^{a}	140.23 ± 37.84^{ab}	184.72 ± 55.75^{ab}	112.76 ± 31.48^{b}
Total Cholesterol	139.14 ± 15.85^{bc}	184.71 ± 17.25^{a}	127.30 ± 14.85^{c}	171.36 ± 23.35^{ab}	140.09 ± 11.68^{b}
HDL-Cholesterol	94.62 ± 12.51^{c}	140.38 ± 10.24^{a}	105.15 ± 14.25^{bc}	132.29 ± 24.78^{ab}	112.63 ± 11.56^{b}
LDL-Cholesterol	41.74 ± 8.52^{a}	43.59 ± 8.02^{a}	21.77 ± 2.56^{c}	38.64 ± 10.81^{a}	26.97 ± 3.89^{b}
GOT (IU/L)	482.79 ± 171.51^{a}	513.17 ± 139.34^{a}	410.70 ± 149.53^{a}	422.22 ± 111.68^{a}	431.20 ± 123.02^{a}
GPT	152.50 ± 80.72^{b}	334.17 ± 119.58^{a}	241.50 ± 98.93^{ab}	92.67 ± 42.96^{b}	147.50 ± 72.50^{b}
Free Fatty Acid (uEq/L)	1654.51 ± 484.59^{a}	1768.86 ± 309.43^{a}	1883.30 ± 420.64^{a}	1766.21 ± 431.13^{a}	2038.52 ± 279.45^{a}
Leptin (ng/ml)	5.739 ± 0.713^{c}	27.386 ± 4.993^{a}	3.839 ± 0.808^{c}	21.464 ± 3.843^{b}	3.979 ± 1.400^{c}

Mean±S.D of 6 mice per group. Values with different alphabet within the same raw are significantly different at P<0.05 by Duncan's multiple range test. NF normal diet, HF high fat diet, HFS high fat diet plus soy isoflavone powder, HFC; high diet plus carnitine, HFSC; high fat diet plus soy isoflavone powder plus carnitine.

high fat diet feeding did not elevate plasma lipids but induced fatty liver and increased parametrical adipose tissue weight.[30-32] To investigate the effect of high fat diet with soy isoflavone powder and L-carnitine on acute liver failure in all of groups, we analyzed serum GOT and GPT levels. The administration of SC mixture effectively reduced the serum GPT level near to that of normal fat diet group, but, there was no significant difference in the serum concentration of GOT among the groups.

Leptin has been acknowledged as an adipocyte derived signal molecule, which may limit food intake and increase energy expenditure via interacting with specific receptors.[33] Plasma leptin concentrations among high fat diet groups were significantly lower in HFS and HFSC groups (3.839±0.808, 3.979±1.400 ng/ml, n=6, respectively) compared to HF and HFC groups (27.386±4.993, 21.464±3.843 ng/ml, n=6 respectively)

3. Liver Weight, Liver Lipid Profile and Histopathological Investigation

The high-fat diet induced a significant increase in the liver weights of the mice compared with the normal fat diet (Table 4). The weight of the liver was increased by 34.7% in the case of high-fat diet supplementation alone. The relative liver weights were 16.1%, 31.4% lower respectively in HFS, HFSC groups than in the high-fat diet control group.

To determine the extent of the lipid accumulation in liver, the total lipid, TG and cholesterol contents were measured. As shown in Table 4, the high-fat diet resulted in a fatty liver with the accumulation of TG and total cholesterol. The liver TG concentration of the mice fed a high-fat diet for 12 weeks was approximately 2.8-fold greater than that of the mice fed a normal fat diet (6.56 vs. 18.44 μmol/g wet liver tissue). Supplementing the high-fat diet with soy isoflavone powder and L-carnitine significantly decreased the accumulation of TG caused by the high-fat diet.

As expected, the high-fat diet resulted in increased liver cholesterol concentration compared with that in the normal fat diet group. However, the liver total cholesterol concentrations were not significantly different among the high fat diet groups.

The total lipid content in the liver was also increased in the high-fat diet group compared with the normal fat diet group. Supplementation with soy isoflavone powder and L-carnitine reduced the amount of hepatic lipid compared to HF group. In HFS group, the liver lipid level was also significantly decreased but HFC group did not show difference statistically compared to HF group.

Histological examination revealed steatosis in the livers of the mice belonging to the high-fat diet group supplemented with 0.2% soy isoflavone powder and 0.5% L-carnitine and the mice belonging to the normal fat diet group (Fig. 1). Most of the hepatocytes in the high-fat diet control group contained large globule of lipids. However, the histological sections of hepatocyte in HFS and HFSC groups showed mild fat and glycogen accumulation. These results suggested that the combined treatment with soy isoflavone powder containing isoflavone and L-carnitine suppressed effectively the vacuolation of lipid in hepatocytes.

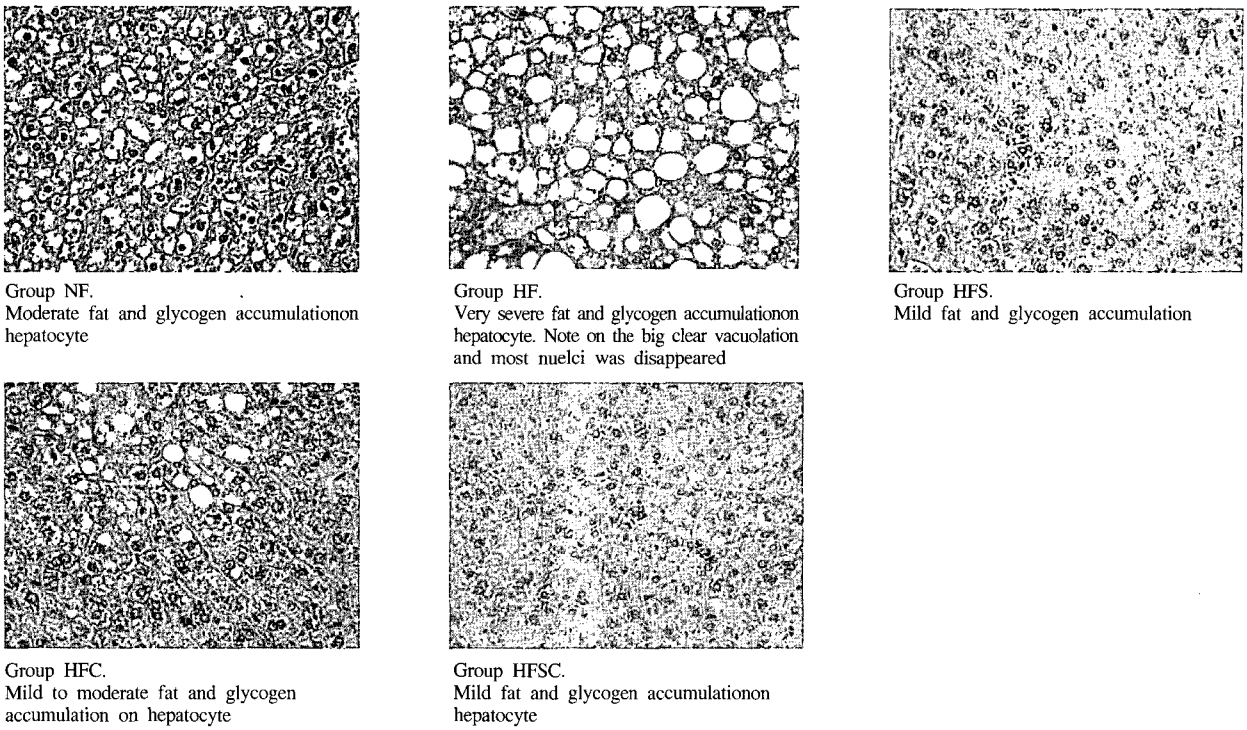
4. Fat Cell Size and Number

As shown in Table 5, the average size of fat cells in HF, HFS and HFC groups was significantly larger than that of the normal fat diet group, whereas that of fat cells in HFSC group was not significantly different compared to normal diet group. The adipocytes of the HF mice were more voluminous than those of the NF group mice (Fig.2). As in the results mentioned above, the HF mice had significantly lower numbers of adipocytes than the NF group by 40% within a known area. The addition of soy isoflavone powder to the high-fat diet

Table 4. The effects of soy isoflavone powder and L-carnitine on liver weight, liver triglyceride, cholesterol and total lipid of mice fed a high fat diet for 12 week

	Control group	Treated groups			
	NF	HF	HFS	HFC	HFSC
Liver weight	0.92 ± 0.10^{bc}	1.24 ± 0.19^{a}	1.04 ± 0.07^{b}	1.16 ± 0.12^{ab}	0.85 ± 0.07^{c}
Triglyceride (umol/g wet tissue)	6.56 ± 0.62^{d}	18.44 ± 1.17^{a}	11.89 ± 1.03^{b}	16.75 ± 1.06^{ab}	8.56 ± 1.22^{c}
Total Cholesterol (umol/g wet tissue)	12.88 ± 0.41^{b}	58.09 ± 4.28^{a}	53.97 ± 3.92^{a}	54.73 ± 3.68^{a}	51.54 ± 4.57^{a}
Total Lipid (mg/g wet tissue)	51.92 ± 3.84^{d}	142.46 ± 16.23^{*a}	87.36 ± 6.02^{b}	121.10 ± 7.99^{ab}	65.76 ± 4.44^{c}

Mean±S.D of 6 mice per group. Values with different alphabet within the same raw are significantly different at P<0.05 by Duncan's multiple range test. NF normal diet, HF; high fat diet, HFS high fat diet plus soy isoflavone powder, HFC; high diet plus carnitine, HFSC; high fat diet plus soy isoflavone powder plus carnitine



Group NF.
Moderate fat and glycogen accumulationon hepatocyte

Group HF.
Very severe fat and glycogen accumulationon hepatocyte. Note on the big clear vacuolation and most nuelci was disappeared

Group HFS.
Mild fat and glycogen accumulation

Group HFC.
Mild to moderate fat and glycogen accumulation on hepatocyte

Group HFSC.
Mild fat and glycogen accumulationon hepatocyte

Fig. 1. Histological section of liver of male C57BL/6 mice treated with test materials (X160).
Frozen sections were stained with Hematoxylin & Eosin (H&E) to demonstrate stored lipids.

Table 5. The effects of dietary soy isoflavone powder and L-carnitine on fat cell size and the number from abdominal fat pad

	Control group	Treated groups			
	NF	HF	HFS	HFC	HFSC
Fat Cell Size (m)	55.73 ± 3.67^{d}	89.89 ± 3.76$^{a1)}$	63.23 ± 1.77^{c}	72.20 ± 2.56^{b}	58.26 ± 3.26^{cd}
Fat Cell Number (/ mm2)	22.50 ± 2.68^{c}	13.50 ± 1.93^{a}	19.86 ± 1.52^{bc}	17.25 ± 1.61^{b}	21.74 ± 3.02^{c}

Values are means S.D for six mice in each group.
[1] Values within the same row not sharing a common superscript letter are significantly different at $p<0.05$.

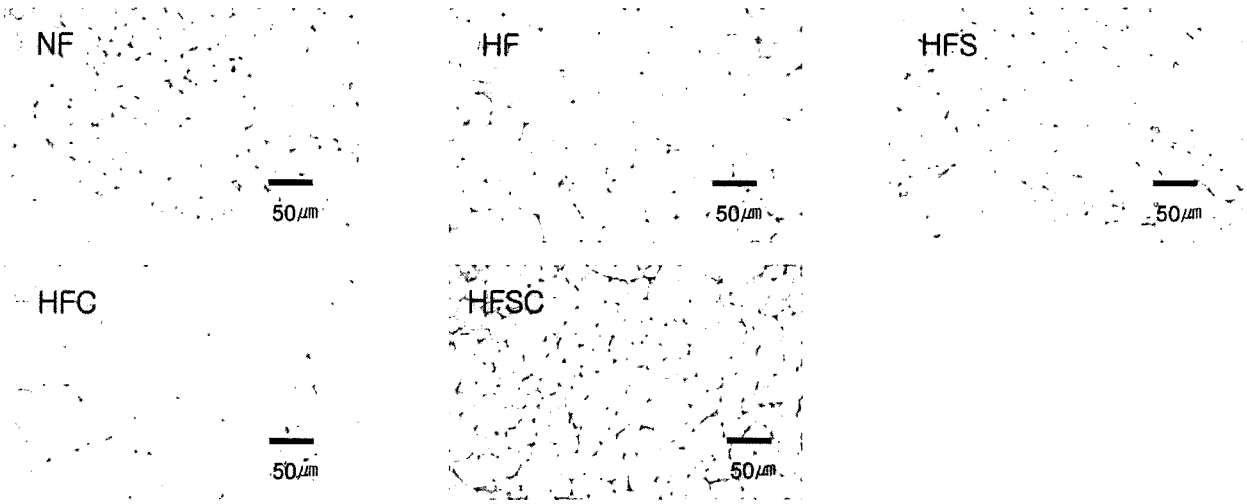


Fig. 2. Paraffin-embedded sections of abdominal white adipose tissue after treatment for 12 weeks were stained with hamatoxylin and eosin.
The size of at least 300 cells per sample from 6 different mice per group was determined using Image J software (National Institutes of Health)

resulted in increased fat cell numbers, due to the consequent decrease in the mean cell volume. The average number of cell numbers in the HFS, HFC and HFSC groups were 19.86±1.52, 17.25±1.61 and 21.74±3.02 (/mm^2) respectively, whereas the corresponding value was 13.50±1.93 (/mm^2) in the HF mice.

5. Carnitine Palmitoyl Transferase-1 (CPT1) Expression Level and Activity in the Liver

The expression level and activity of carnitine palmitoyl-transferase 1 (CPT 1), which are the major genes involved in fatty acid metabolism, were measured in the liver of mice euthanized after experimental period of 12 weeks (Fig 3 & Table 6). As shown in Fig 3, the expression levels of CPT1 were higher in HFS and HFC groups than in NF and HF groups. Similarly to this result, the activity of CPT1 was also about 48% and 28% ($P<0.05$) higher in HFS and HFC groupsthan in NF group, respectively. On the other hand, HFSC and HF groups were similar to normal diet group.

6. Glycerol-3-Phosphate Dehydrogenase (GPDH) and Fatty Acid Synthase (FAS) Enzymatic Activity

In this study, we measured the activity of GPDH and FAS, which reflect the promotion of differentiation, to assess whether supplementation of soy isoflavone powder and L-carnitine affect adipogenesis on adipose tissues in C57BL/6J mice. As shown in Figure 4, the activities of FAS were significantly declined in HF group because of external diet fat supply compared to NF group. In high fat diet groups, however, the values were unaffected

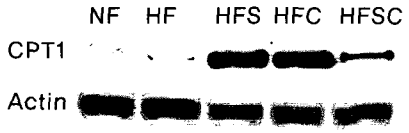


Fig. 3. CPT1 expression in liver of male C57BL/6Jmice fed HF diets supplemented with 0.2% soy isoflavone powder (S) and 0.5% L-carnitine (C).

Representative samples illustrating protein levels of CPT1 in liver measured by western blot. NF normal diet, HF high fat diet, HFS high fat diet plus soy isoflavone powder, HFC; high diet plus carnitine, HFSC; high fat diet plus soy isoflavone powder plus carnitine

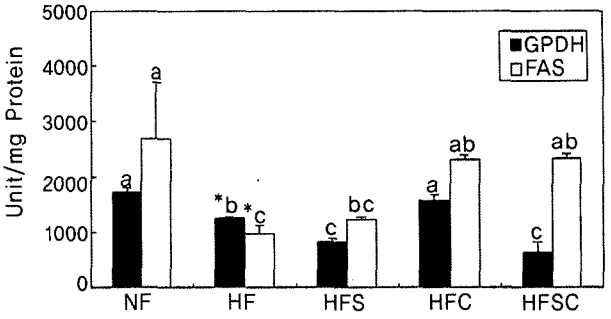


Fig. 4. CPT1 expression in liver of male C57BL/6Jmice fed HF diets supplemented with 0.2% soy isoflavone powder (S) and 0.5% L-carnitine (C)

Representative samples illustrating protein levels of CPT1 in liver measured by western blot. NF normal diet, HF high fat diet, HFS high fat diet plus soy isoflavone powder, HFC; high diet plus carnitine, HFSC; high fat diet plus soy isoflavone powder plus carnitine

by diet, which were of borderline significance (P=0.21). In contrast, GPDH activity of HF group was suppressed by about 26% compared to NF group. Especially in HFSC and HFS groups, the values were significantly lower than that in HF group.

DISCUSSION

In our previous studies, we found that genistein, one of the major soy isoflavone, increasedCPT1 enzyme activity by increasing CPT1 transcription and combined treatment of L-carnitine and genistein additively increased CPT1 activity in HepG2 cells.[10,11] L-carnitine also plays a major role in fatty acid oxidation. L-carnitine levels in hepatocytes modulate hepatic CPT1 activity.[34]

In this study, we examined the long-term effects of soy isoflavone powder (containing 10% isoflavone) and L-carnitine treatment on the development of obesity in C57BL/6J mice. To characterize the antiobesity effect of these two substances, C57BL/6J mice have been used since C57BL/6J mice are genetically susceptible to become obese if the weights of those are raised on a high-fat diet.[22] As high-fat diets leading to excessive energy intake are strongly linked to increasing cases of obesity,[35] this study using high-fat diet induced obese

Table 6. CPT-1 activities in Liver (nmol/minute/mg protein)

	Control group	Treated groups			
	NF	HF	HFS	HFC	HFSC
CPT-1	0.624 ± 0.0924^c	0.735 ± 0.0823^{bc}	0.9238 ± 0.0629^a	0.8024 ± 0.0649^b	0.6340 ± 0.0537^c

Mean±S.D of 6 mice per group. Values with different alphabetwithin the same raw are significantly different at $P<0.05$ by Duncan's multiple range test. NF normal diet, HF high fat diet, HFS; high fat diet plus soy isoflavone powder, HFC; high diet plus carnitine, HFSC; high fat diet plus soy isoflavone powder plus carnitine

animals would appear to constitute a useful model for further studies designed to characterize the effects of soy isoflavone powder(containing 10% isoflavone) and L-carnitine on human obesity.

At the end point of the experiment, this study has demonstrated that SC mixture is beneficial for the suppression of diet-induced obesity. First, average body weight gain in HFSC group was significantly lower than that in high fat-fed mice by 31%. In addition, feeding with soy isoflavone powder alone, and SC mixture resulted in a decrease in abdominal/back fat mass in the high-fat diet-induced obese mice compared to the control high-fat diet group, as well as reduced the final liver weight by 16%, 31% respectively compared with that of high fat diet control group.

Second, in the case of a high-fat diet, excessively ingested fat may be converted to FFAs, which may be oxidized as fuel or reesterified for storage in adipose tissue.[36] If excess FFAs are taken up by the liver, they may be converted to TG resulting in hepatosteatosis unless they are oxidized by mitochondria or peroxisomes. Therefore,it is likely that reduced fatty acid oxidation would aggravate the accumulation of TG in the liver, as well as promote its accumulation in adipose tissues, thus leading to obesity. The present study showed that the liver weight was greater in HF group than in NF group, probably due to the accumulation of lipids in the liver. On the other hand, the supplementation with SC mixture synergistically suppressed hepatosteatosis, and improved hepatic triglyceride and total cholesterol. As we expected, histological analysis of the liver tissues revealed a distinctive pattern of fat droplets accumulation, indicating a shift of the liver toward fat storage (Fig 1). These results indicate that SC mixture prevents the obesity and fatty liver induced by feeding a high fat diet.

Third, our resultsshowed that abdominal adipose tissue weight of mice supplemented with SC mixture was much less than those of mice in HF group by 78% (Table 2). The diameter and the number of adipose tissueswere also measured in each group. Increase in adipose tissue mass can be the result of an increase in adipose cell size (hypertrophy) and increase in cell number (hyperplasia), or a combination of both of these processes. The diameters of adipose tissues in the high fat diet group were significantly larger than those in the normal fat diet group, which suggests that the increased size of the abdorminial fat pad maybe result from the enlargement of adipose tissues. However, the diametersof the adipocyte in the soy isoflavone powder containing isoflavone, and SC mixture treated mice were significantly smaller than those of the adipocyte in the high-fat diet group(Fig 2).

There would be some mechanisms involved in the antiobesity effect of soy isoflavone as we previously reported that genistein and L-carnitine enhanced the expression of genes involved in fatty acid catabolism, and regulated positively CPT1 activity in the liver.[10,12] CPT1 is the rate-limiting enzyme in the process of β -oxidation. We investigated the underlying mechanism of the beneficial effects of soy isoflavone and L-carnitine on antiobesity. We thought that soy isoflavone had a function to potently activate energy expenditure through activating β-oxidation of fatty acid in the liver and L-carnitine might play a role as a substrate to boost CPT1 activity, considering the observation that body weight gain in HFSC group mice was significantly lowered than that in high fat fed mice by 31%. As shown in Fig 3, the hepatic CPT1 expression was increased in the soy isoflavone powder- and L-carnitine- fed mice but decreased in SC mixture-fed mice. Also the level of expression was similar to that in normal control mice. The CPT1 activity was significantly elevated by 1.3 fold and higher than that inthe high fat control mice, whereas that of SC mixture fed mice was similar to normal control mice (Table 6). According to these results, the increase in CPT1 expression by feeding soy isoflavone powder and L-carnitine might have influence on regulating CPT1 activity. It suggested that the body weight and fat mass in SC mixture fed mice has been already significantly decreased at the end of this experiment, thus CPT1 activity and expression in the liver subsequently were down regulated by homeostasis and the levels were similar to those in normal control group.

The transcription of CPT 1 is known to be regulated by a number of factors, including peroxisome proliferator-activated receptor-α (PPAR-α), glucocorticoids, and insulin[37] Recently, it was reported that genistein improved the lipid and glucose metabolism by acting as an antidiabetic PPAR agonist.[38] In our previous study, genistein also induced the expression of PPAR-α at both mRNA and protein levels in HepG2 cells and genistein activated the transcriptional activity of PPARα in reporter gene analysis.[12] In addition, the activation of PPAR-α increased fatty acid β-oxidation and reduced the level of circulating or cellular lipids.[39] This report agreed with our serum lipid concentration data as shown in Table 3. Serum lipid concentration was significantly decreased in HFSC group *in vivo* compared with HF group. All things considered, it is possible that SC mixture might produce an antiobesity effect by increasing CPT 1

expression through its role as a PPAR agonist. Leptin has been believed to regulate body weight homeostasis and energy balance,[40,41] and circulating leptin level was found to be proportional to adipose tissue mass.[42] This present study also found significant reductions of serum leptin concentration in mice fed diets supplemented with soy isoflavone powder, and SC mixture. This study emphasized visceral adipose tissue and measured the wet weights of abdominal and back adipose tissuesbecause visceral-fat obesity is frequently accompanied by hyperlipidemia, hypertention, and glucose intolerance, which are causes of cardiovascular diseases.[43]

In this study, we measured the activity of GPDH and FAS, which reflect the promotion of differentiation, to assess whether the supplementation of soy isoflavone powder and L-carnitine affect adipogenesis on adipose tissue in C57BL/6J mice. As shown in Fig 4, the activity of FAS was significantly increased in HFSC group compared with in HF group, whereas that of GPDH was statistically lower in HFSC group than in HF. According to the previous study, soy isoflavone seems to have a direct effect on lipid metabolism in adipose tissues by affecting both lipogenesis and lipolysis.[15]

CONCLUSION

Our results indicate that long-term supplementation with dietary soy isoflavone and L-carnitine is more synergistically beneficial for the suppression of high-fat diet induced obesity by inhibiting liver TG accumulation and the gain in abdominal adipose tissue weight than that with soy isoflavone. The antiobesity effects of SC mixture might be attributed, at least in part, to the induction of fatty acid catabolism by soy isoflavone, genistein.

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