Research Article

Dietary docosahexaenoic acid-rich diacylglycerols ameliorate hepatic steatosis and alter hepatic gene expressions in C57BL/6J-*Lep*$^{ob/ob}$ mice

Hye-Jin Kim[1], Ki-Teak Lee[2], Yong Bok Park[3], Seon-Min Jeon[1] and Myung-Sook Choi[1]

[1] Department of Food Science and Nutrition, Kyungpook National University, Daegu, Korea
[2] Department of Food Science and Technology, Chungnam National University, Daejon, Korea
[3] School of Life Science and Biotechnology, Kyungpook National University, Daegu, Korea

We examined the effect of docosahexaenoic acid (DHA)-enriched structured lipids-diacylglycerol (SL-DG), which were synthesized using soybean oil (SO) and algae oil (AO), on hepatic lipid metabolism and the mRNA expression of genes involved in hepatic steatosis of C57BL/6J-*Lep*$^{ob/ob}$ compared to the SL-triacylglycerol (TG). The animals were fed a high-fat (10% lard and 10% test oils) and high-cholesterol (0.2% cholesterol) diet for 12 weeks. Mice fed SL-DG showed a lower total white adipose tissue weight and plasma triglyceride concentration than the SO group. Reduction of hepatic triglyceride content in the SL-DG group was related with the suppression of hepatic enzyme activities for fatty acid and triglyceride synthesis along with fecal triglyceride excretion compared to the SL-TG. SL-DG also lowered hepatic cholesterol levels by suppressing cholesterol regulating enzyme activity compared to the SO group. Moreover, SL-DG lowered the mRNA expressions of sterol regulatory element binding protein-1 and its target genes than TG-form oils (SO, AO and SL-TG) in the liver. Thus, the current results suggest that DHA-enriched SL-DG oil used in this study is beneficial for ameliorating hepatic steatosis in obese animal model by improving hepatic fatty acid and cholesterol metabolic enzyme activity and their gene expression.

Keywords: C57BL/6J-*Lep*$^{ob/ob}$ / Diacylglycerol / Docosahexaenoic acid / Hepatic steatosis / Structured lipid

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1 Introduction

Obesity is at the forefront of global health issues and it directly contributes to many chronic illnesses. Recently, attention has focused on the excessive accumulation of triglycerides in the liver as part of this syndrome [1]. Since excessive fat accumulation in the liver may be a predisposing condition to hepatic inflammation, steatohepatitis, and liver cirrhosis, which can lead to death, efforts to prevent fatty liver are needed.

Several dietary components show promise in the prevention or improvement of obesity, one of which is dietary oil that is rich in diacylglycerols (DG). The present objectives are to examine the scientific knowledge concerning DG with n-3 and/or n-6 fatty acids in order to assess evidence that supports the effect on substrate oxidation rates, body weight and fat mass, and blood lipids, as well as to elucidate potential mechanisms of its action. The 1,3-DG oil is believed to have the ability to increase β-oxidation, enhance body weight loss, suppress body fat accumulation, and lower serum triacylglycerol concentration postprandially [2]. In addition, docosahexaenoic acid (DHA) has attracted a great deal of attention because of its various physiological functions within the human body. DHA reduces or inhibits risk factors that are involved in various diseases such as cardiovascular diseases [3], and can have a positive effect on diseases such as hypertension, arthritis, arteriosclerosis and thrombosis [4].

Correspondence: Dr. Myung-Sook Choi, Department of Food Science and Nutrition, Kyungpook National University, 1370 Sank-Yuk Dong Buk-Ku, 702-701, Daegu, Korea
E-mail: mschoi@knu.ac.kr
Fax: +82-53-950-6229

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; **ACC**, acetyl-CoA carboxylase; **ACL**, ATP-citrate lyase; **AO**, algae oil; **CPT**, carnitine palmitoyl transferase; **DHA**, docosahexaenoic acid; **DG**, diacylglycerol; **FAS**, fatty acid synthase; **G6PD**, glucose-6-phosphate dehydrogenase; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **HMG-CoA**, 3-hydroxy-3-methylglutaryl-coenzyme; **ME**, malic enzyme; **PAP**, phosphatidate phosphohydrolase; **SCD-1**, stearoyl-CoA desaturase; **SREBP-1c**, sterol regulatory element binding protein-1c; **PPAR**γ, peroxisome proliferator activated receptor γ; **SL**, structured lipids; **SO**, soybean oil; **TG**, triacylglycerol; **WAT**, white adipose tissue

Among the animal models, genetically obese Lep^{ob}/Lep^{ob} (*ob/ob*) mice can develop obesity, glucose intolerance, insulin resistance, and fatty livers (excess fat that is deposited in the liver) due to an inherited deficiency of the appetite-suppressing hormone, leptin [5]. Reflecting the plural activity of leptin, the *ob/ob* mouse represents a complex model of obesity-related steatosis. The expanded white adipose tissue (WAT) mass in *ob/ob* mice promotes adipose tissue lipolysis that releases long-chain fatty acids (LCFA). This event can lead to an increase in circulating LCFA that partly can be delivered to the liver. In addition, sterol regulatory element-binding protein (SREBP)-1c is activated and can accumulate in *ob/ob* hepatocyte nuclei that promote fatty acid synthase activity. The increased hepatic lipid synthesis and its storage coincide with expanded adipose tissue storage that contributes to hepatic steatosis and obesity [6].

There have been no comparative studies regarding the effect of n-3 fatty acid enriched diacylglycerol- and triacylglycerol-structured lipids (SL-DG and SL-TG), synthesized based on soybean oil (SO) and algae oil (AO), on obese animal models. The chemical structure of SL-DG that we designed would be expected to exert a beneficial effect that could be attributable to its DG structure itself in addition to the presence of linolenic acid and DHA. Accordingly, the effect of SL-DG was compared with SL-TG, which included approximately the same n-6/n-3 fatty acid ratio in ameliorating hepatic steatosis. We examined its influence on hepatic lipid metabolism, such as lipogenesis, fatty acid oxidation, and cholesterol synthesis. *Ob/ob* mice were chosen as they are characterized by hyperphagia, excessive body weight gain and lipid deposition in hepatic and adipose tissue. Furthermore, we examined its influence on the mRNA expression of various genes that are involved in hepatic lipid metabolism.

2 Materials and methods

2.1 Test oils

Commercially-available SO was used, and AO was purchased from Martek Biosciences Corporation (Columbia, MD). SL-TG was synthesized by enzymatic interesterification with SO and AO [7]. SL-DG was produced by glycerolysis with SL-TG and glycerol catalyzed by lipase. Each test oil was analyzed for its fatty-acid profile and acylglycerol moieties, as shown in Table 1.

2.2 Animals and diets

Four-week-old, male *ob/ob* mice (strain C57BL/6J Lep^{ob}/Lep^{ob}) were purchased from Jackson Laboratory (Bar Harbor, ME). The animals were housed in a room with controlled temperature (20 – 23°C) and lighting (12-h light/dark cycles) and they were fed a pelletized commercial non-purified diet for 1 week after arrival. The mice were then randomly divided into four groups, SO, AO, SL-TG and SL-DG (n = 10) and provided with experimental diets for 12 weeks. The diets contained (%) casein, 20; fat, 20 (10% lard and 10% test oil); corn starch, 15; sucrose, 34.8; cellulose, 5; mineral mixture (AIN-76-MX) [8], 3.5; vitamin mixture (AIN-76-VX) [9], 1; D,L-methionine, 0.3; choline bitartrate, 0.2; and cholesterol, 0.2. The mice were allowed free access to food and water. Food consumption and body weight were measured daily and weekly, respectively. At the end of the experimental period, the mice were anesthetized with Ketamin-HCl after withholding food for 14 h. Blood samples were taken from the inferior vena cava in order to determine the plasma leptin and lipids concentrations. The organs and adipose tissues were removed and rinsed with physiological saline. All samples were stored at –70°C until analyzed. The current study protocol was approved by the Ethics Committee at Kyungpook National University for animal studies.

Table 1. Acylglycerol and fatty acid composition of test oils (%)

	Soybean oil	Algae oil	SL-TG	SL-DG
Acylglycerol species				
Triacylglycerol	100	100	100	47.5
1,2-Diacylglycerol	–	–	–	16.5
1,3-Diacylglycerol	–	–	–	25.8
Monoacylglycerol	–	–	–	10.2
Fatty acid				
14:0	0.1	13.3	5.1	5.2
16:0	15.0	31.4	20.2	19.2
16:1	0.1	0.6	0.3	0.2
18:0	4.9	0.6	2.5	2.3
18:1	28.1	0.7	13.5	15.0
18:2	48.1	0.4	31.1	33.8
18:3	3.7	0.5	3.5	3.6
20:4	–	0.2	0.1	0.1
20:5	–	3.2	1.4	1.3
22:5	–	14.0	6.6	5.9
22:6	–	35.1	15.7	13.4
SFA	20.0	45.3	27.8	26.7
MUFA	28.2	1.3	13.8	15.2
PUFA	51.8	53.4	55.4	52.7
P/S[a)]	2.6	1.2	2.0	2.0
n-6	48.1	0.6	31.2	33.9
n-3	3.7	52.8	27.2	24.2
n-6/n-3	13.0	0.0	1.2	1.4

a) Polyunsaturated fatty acid/saturated fatty acid ratio.

2.3 Fat pad weights

Fat pads, including the epididymal WAT, perirenal WAT and interscapular WAT were dissected from each animal according to defined anatomical landmarks and the weights were measured. The fat pads were then immediately frozen in liquid nitrogen and stored at –70°C.

2.4 Blood analyses

The plasma cholesterol concentration was determined using a commercial kit (Sigma Chemical, St. Louis, MO) based on a modification of the cholesterol oxidase method of Allain *et al*. [10]. The plasma triglyceride concentrations were measured enzymatically using a kit from Sigma Chemical, a modification of the lipase-glycerol phosphate oxidase method [11]. Glucose levels were measured with a glucose analyzer (Arkra, Japan) using the glucose oxidase method. Plasma phospholipids and free fatty acid (non-esterified fatty acid, NEFA) were measured using commercial assay kits (phospholipids B-, NEFA-Wako; Wako Pure Chemical Industries, Osaka, Japan).

2.5 Hepatic and fecal lipids measurement

The hepatic lipids were extracted using the procedure developed by Folch *et al*. [12]. The dried lipid residues were dissolved in 1 mL of ethanol for cholesterol and triglyceride assays. Triton X-100 and a sodium cholate solution (in distilled H_2O) were added to 200 μL of the dissolved lipid solution to produce a final concentration of 5 g/L and 3 mmol/L, respectively. The hepatic cholesterol, triglyceride, phospholipid and free fatty acids were analyzed with the same enzymatic kit that was used in plasma analysis.

The feces from each group were collected daily for last five days and analyzed for lipids, by the method described previously with slight modifications [12]. Briefly, the feces were dried and extracted in ice-cold chloroform and methanol (2:1, v/v) for 24 h at 4 C. After centrifugation at 900 × *g* for 10 min, the supernatant was collected, dried at 50°C, and dissolved with ethanol. The cholesterol, triglyceride, phospholipids and free fatty acid levels of the feces were estimated using the same method that was used for the liver.

2.6 Enzyme analyses

The livers were prepared according to Hulcher [13] with slight modifications in order to measure the malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PD), phosphatidate phosphohydrolase (PAP), carnitine palmitoyl transferase (CPT), fatty acid β-oxidation, 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase, and acyl CoA:cholesterol acyltransferase (ACAT) activities. ME activity was measured according to the method of Ochoa [14] by monitoring the production of NADPH at 340 nm, where activity was represented by the formation of NADPH nmol/min/mg protein. G6PD activity was assayed by spectrophotometric methods according to the procedures described by Pitkanen [15], where the activity was expressed as the reduced NADPH nmol/min/mg protein. PAP activity was determined using the method of Walton [16]. CPT activity was analyzed according to the method of Markwell [17]. The results were expressed as nmol/min/mg protein. The fatty acid β-oxidation was determined using the method of Lazarow [18] by monitoring the reduction of NAD to NADH at 340 nm, where the activity was expressed as the reduced NAD nmol/min/mg protein. The HMG-CoA reductase activity was measured in the microsomes with [^{14}C]-HMG-CoA as the substrate based on a modification of the method of Shapiro [19], where the activity was expressed as the synthesized mevalonate pmol/min/mg protein. The ACAT activity in the microsomes was determined by the rate of incorporation of [^{14}C]-oleoyl-CoA into cholesterol ester fractions, as described by Erickson [20], where the activity was expressed as the synthesized cholesteryl oleate pmol/min/mg protein. The protein concentrations were measured according to the method of Bradford [21] using BSA as the standard.

2.7 RNA extraction and messenger RNA expression analysis

Total RNA was extracted from liver using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA samples were quantified spectrophotometrically. Complementary DNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Fermentas, Burlington, ON, Canada), random hexamers, deoxyribonucleoside triphosphates, and 5 μg of total RNA. After first-strand complementary DNA synthesis, the RNA expression was quantified by a real-time quantitative PCR using SYBR green PCR reagents (Applied Biosystems, Foster City, CA) and the SDS7000 sequence-detection system (Applied Biosystems). Gene–specific mouse primers were used for acetyl-CoA carboxylase (ACC), 5′-taaggtcttcctcaccactg–3′ (forward) and 5′–tctcgaatggtattcaaagg–3′ (reverse); ATP-citrate lyase (ACL), 5′–cagcaagcactgtcagaata–3′ (forward) and 5′–ttaaaacttgcattcccttc–3′ (reverse); stearoyl-CoA desaturase-1 (SCD-1), 5′–taagctcagtctcactcctt–3′ (forward) and 5′–aaaagatttctgcaaaccaa–3′ (reverse); sterol regulatory element binding protein-1c (SREBP-1c), 5′–acagatgtgtctatggaggg–3′ (forward) and 5′–aaaagacaaggggctactct–3′ (reverse); peroxisome proliferator activated receptor γ (PPARγ), 5′–agagtctgctgatctgcgagc–3′ (forward) and 5′–ttcctgtcaagatcgccctc–3′ (reverse); fatty acid synthase (FAS) , 5′–gtgaagaagtgtctggactgtgtcat–3′ (forward) and 5′–ttttcgctcacgtgcagttta–3′ (reverse) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′–tgcagtggcaaagtggagat–3′ (forward) and 5′–ttgaatttgccgtgagtgga–3′ (reverse). The relative quantitation values were calculated by analyzing the changes in the SYBR green fluorescence during PCR, according to the manufacturer's instructions. The C_t values obtained were the threshold cycles at which a statistically significant increase in SYBR green emission intensity occurred. Using the $2^{-\Delta\Delta Ct}$ method, the fold changes were calculated; transcripts of GAPDH were also amplified from the samples in

Table 2. Comparative effect of soybean oil and algae oil SL on weight of adipose tissue in *ob/ob* mice[a)]

	SO	AO	SL-TG	SL-DG
Initial body weight (g)	32.9 ± 0.8	32.9 ± 0.8	32.5 ± 0.8	32.7 ± 0.9
Final body weight (g)	58.0 ± 1.7	58.0 ± 1.3	59.7 ± 0.9	59.7 ± 1.0
Body weight gain (g/d)	2.2 ± 0.1	2.1 ± 0.1	2.3 ± 0.1	2.1 ± 0.1
Epididymal WAT (g)	2.7 ± 0.2[a]	3.2 ± 0. 2[b]	3.0 ± 0.1[ab]	3.0 ± 0.2[ab]
Perirenal WAT (g)	3.6 ± 0.1[a]	2.9 ± 0.3[b]	3.4 ± 0.1[ab]	3.3 ± 0.2[ab]
Interscapular WAT (g)	3.4 ± 0.2[a]	3.6 ± 0.3[a]	2.7 ± 0.1[b]	2.4 ± 0.1[b]
Total WAT (g)	9.6 ± 0.3[a]	9.1 ± 0.4[ab]	9.0 ± 0.2[ab]	8.5 ± 0.4[b]

a) Mean ± SEM (n = 10). [ab]Means not sharing a common letter are significantly different among groups at $p < 0.05$ as determined by a one-way ANOVA test.

Table 3. Comparative effect of soybean oil and algae oil SL on the concentration of plasma, hepatic and fecal lipids in *ob/ob* mice[a)]

	SO	AO	SL-TG	SL-DG
Plasma lipids (mmol/L)				
Total cholesterol	9.8 ± 0.5[a]	12.3 ± 0.7[b]	10.3 ± 0.5[a]	11.4 ± 0.4[ab]
Triglyceride	1.0 ± 0.1[ab]	0.9 ± 0.0[ab]	1.1 ± 0.1[a]	0.9 ± 0.0[b]
Free fatty acid	6.9 ± 0.3[a]	5.5 ± 0.6[b]	6.2 ± 0.4[ab]	5.4 ± 0.4[b]
Phospholipid	9.2 ± 0.3[a]	9.6 ± 0.3[a]	9.7 ± 0.4[a]	10.6 ± 0.3[b]
Glucose	14.0 ± 0.4	14.7 ± 1.0	14.0 ± 0.8	14.0 ± 0.6
Hepatic lipids (μmol/g)				
Cholesterol	382.3 ± 25.0[a]	219.9 ± 8.2[c]	310.9 ± 13.9[b]	327.2 ± 12.7[b]
Triglyceride	262.3 ± 11.9[a]	280.1 ± 7.3[a]	229.0 ± 5.5[b]	202.3 ± 6.6[c]
Free fatty acid	73.7 ± 1.2	75.2 ± 2.3	73.1 ± 1.3	74.6 ± 0.9
Phospholipid	209.4 ± 11.2[a]	233.7 ± 18.8[ab]	248.2 ± 18.6[ab]	271.6 ± 16.1[b]
Fecal lipids (μmol/g)				
Cholesterol	114.6 ± 22.7	106.2 ± 22.8	107.7 ± 12.6	120.2 ± 13.7
Triglyceride	44.5 ± 0.5[a]	44.3 ± 2.0[a]	44.3 ± 4.4[a]	77.1 ± 14.3[b]
Free fatty acid	73.3 ± 3.9	70.1 ± 2.9	75.1 ± 4.4	81.8 ± 6.1

a) Mean ± SEM (n = 10). [abc]Means not sharing a common letter are significantly different among groups at $p < 0.05$ as determined by a one-way ANOVA test.

order to assure normalized real-time quantitative RT-PCR detection [22].

2.8 Statistical analysis

All data were presented as mean ± SEM. Significant differences among the groups were determined by a one-way ANOVA using SPSS. Duncan's multiple-range test was performed if differences were identified between groups at $p < 0.05$.

3 Results

3.1 Body weight and fat pad weight

No significant differences were observed in initial body weight, final body weight and body weight gain of the mice in the four diet groups (SO, AO, SL-TG and SL-DG).

In order to examine the effect of diet on body-fat accumulation, we analyzed the distribution of WAT as shown in Table 2. There was a significant reduction in the weight of the epididymal WAT in the SO group compared with the AO group, whereas the weight of perirenal WAT was significantly lower in the AO group than in the SO group. In particular, the quantity of interscapular WAT, which is normally detected in higher amounts in the *ob/ob* mice compared to the normal mice, was significantly lower in the SL-TG and SL-DG groups than in the SO and AO group. Overall, the total WAT weight was significantly lower only in the SL-DG group than in the SO group.

3.2 Plasma, hepatic and fecal lipids

The supplementation of SL-DG significantly lowered the plasma triglyceride level compared to the SL-TG group, although it did not significantly alter compared to the precursor oil groups (AO and SO). Plasma total cholesterol concentration was significantly higher in the AO group than in the SO group (Table 3). In the SL-DG group, the plasma free fatty acid level was significantly lowered, whereas the phospholipid level was significantly elevated compared to the SO group. The plasma glucose level did not differ among the

Table 4. Comparative effect of soybean oil and algae oil SL on activities of hepatic G6PD, ME, CPT, β-oxidation, PAP, HMGR and ACAT in *ob/ob* mice[a)]

	SO	AO	SL-TG	SL-DG
Fatty acid synthesis (nmol/min/mg protein)				
G6PD	10.0 ± 0.3 [a]	8.4 ± 0.1 [b]	8.7 ± 0.4 [b]	7.5 ± 0.1 [c]
ME	32.7 ± 1.7 [a]	31.5 ± 2.4 [a]	32.9 ± 2.3 [a]	23.2 ± 3.2 [b]
Fatty acid oxidation (nmol/min/mg protein)				
CPT	21.8 ± 1.6	23.4 ± 0.6	23.8 ± 1.8	24.6 ± 0.6
β-oxidation	31.3 ± 2.3 [a]	44.6 ± 0.6 [b]	57.0 ± 1.8 [c]	63.9 ± 0.6 [c]
TG synthesis (nmol/min/mg protein)				
PAP	19.3 ± 0.4 [a]	17.5 ± 1.1[ab]	17.3 ± 0.8[ab]	16.3 ± 0.5 [b]
Cholesterol-regulating (μmol/min/mg protein)				
HMGR	367.9 ± 27.6 [a]	297.5 ± 13.5 [b]	390.0 ± 24.6 [a]	304.4 ± 11.2 [b]
ACAT	387.6 ± 28.3 [a]	313.5 ± 13.8 [b]	286.1 ± 21.1 [b]	266.0 ± 9.3 [b]

a) Mean ± SEM (n = 10). [abc]Means not sharing a common letter are significantly different among groups at $p < 0.05$ as determined by a one-way ANOVA test.

groups. Hepatic cholesterol content was significantly lower in the AO group than in the other groups, however, the two structured lipid groups (SL-TG and SL-DG) resulted in a significant reduction in the hepatic triglyceride content. When the two structured lipid groups were compared, the hepatic triglyceride level was significantly lower in the SL-DG group. In addition, hepatic cholesterol contents were significantly lowered in the DHA-containing oil groups (AO, SL-TG and SL-DG) than DHA-deficient oil group (SO). The hepatic free fatty acid level did not differ among the groups, however, similar to plasma, the hepatic phospholipid level was significantly higher in the SL-DG group than in the SO and AO groups. Fecal triglyceride was significantly higher in the SL-DG group than in the other groups, although the fecal cholesterol and free fatty acid levels did not differ among the groups.

3.3 Hepatic G6PD, ME, CPT, β-oxidation and PAP activity

The activity of the hepatic lipogenic enzymes, G6PD, ME and PAP, were significantly lower in the SL-DG group than in the SO group (Table 4). Although, the groups exhibited no significant difference with regard to CPT activity, fatty acid β-oxidation activity was significantly elevated in the SL-TG and SL-DG groups than in the SO group.

3.4 Hepatic HMG-CoA reductase and ACAT activity

Hepatic HMG-CoA reductase activity was significantly lower in the AO and SL-DG groups than in the SO and SL-TG groups. Hepatic ACAT activity was significantly lower in the AO, SL-TG and SL-DG groups by 19, 26 and 31%, respectively, compared to the SO group (Table 4).

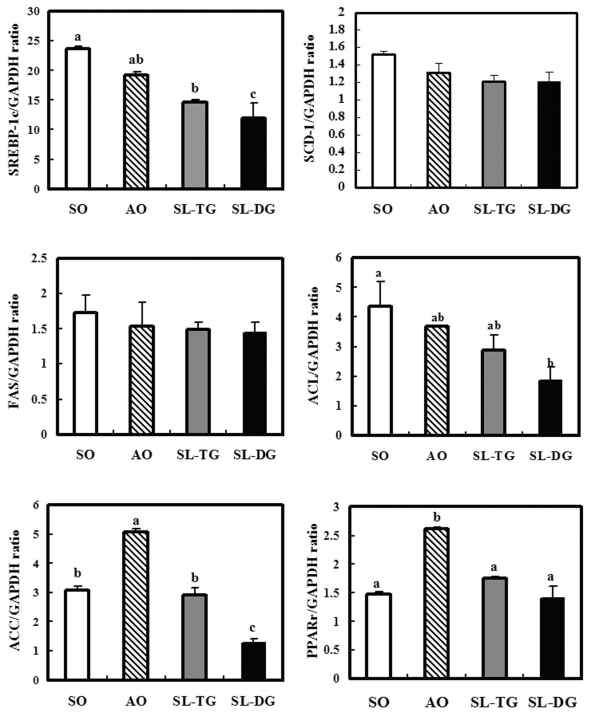

Figure 1. Comparative effect of soybean oil and algae oil SL on the hepatic mRNA expression of SREBP-1c, SCD-1, FAS, ACL, ACC and PPARγ in *ob/ob* mice. Mean ± SEM (n = 10). [abc]Means not sharing a common letter are significantly different among groups at p <0.05 as determined by a one-way ANOVA test.

3.5 mRNA expression of hepatic lipid-regulating enzymes

To elucidate the mechanism by which SL-DG reduces hepatic lipid content in *ob/ob* mice with fatty liver, the mRNA levels of the hepatic key lipogenic transcription factors,

SREBP-1c and PPARγ were examined, along with other related genes using a real-time PCR analysis (Fig. 1). The mRNA expression of SREBP-1c was significantly lower in the SL-TG and SL-DG groups compared to the SO group. Especially, the SREBP-1c mRNA levels were significantly reduced in the SL-DG group than SL-TG group. In addition, the mRNA levels of ACL was lower in the SL-DG group compared to the SO group. Expression of ACC mRNA exhibited the lowest level in the SL-DG group among the groups. Meanwhile PPARγ mRNA level was reduced in the SO, SL-TG and SL-DG groups than AO group.

4 Discussion

In this study, we observed the effect of dietary SL-DG on the fatty livers of genetically obese animal model. The buildup of fat in the liver is regulated by the integrated activity of cellular enzymes that catalyze lipid uptake, synthesis, oxidation, and export. In addition to genetic factors, many environmental factors such as diet and toxins can also modulate fat accumulation. Our results suggest that dietary DG might be beneficial for ameliorating the hepatic steatosis in *ob/ob* mice. We also observed the lipid-lowering action of SL-DG with regard to hepatic cholesterol regulating enzyme activity and body fat pads weight when compared to the SO.

The fatty acid composition of dietary oils markedly affects the physiologic action of each oil. For instance, dietary n-3 polyunsaturated fatty acid (PUFA) has been reported to reduce body fat deposition by inducing the expression of genes that are involved in lipid metabolism and thermogenesis, thereby increasing total body heat generation [23]. Thus, certain dietary oils, due to their constituent fatty acids, show a beneficial effect on lipid metabolism and therefore on obesity. It seems that DHA can suppress the development of obesity and hepatic fat accumulation [24]. In current study, total WAT weights tended to lower in the n-3 fatty acid containing oil-fed groups (AO and SL-TG) compared to the n-3 fatty acid deficient oil-fed group (SO). Especially, we observed that fat deposition was more efficiently lowered by the ingestion of DHA with the DG form (SL-DG) than by the TG form (SL-TG). These findings indicate that the molecular species composition of constituent fatty acid and the acylglycerol structure affect the accumulation of body fat in *ob/ob* mice. In fact, the position of the fatty acid on the glycerol skeleton is responsible for metabolic differences of DG oil [2]. When comparing DG and TG oils, studies have reported differential changes in body compartment volumes and, more specifically, in adipose tissue mass in experimental animals [2, 25].

It has been determined that abnormal patterns of postprandial serum lipid levels are related to abdominal obesity [26]. It is hypothesized that the 1,3-DG is less readily resynthesized into chylomicrons TG, but directly transported to the portal vein for β-oxidation. In particular, 1,3-DG, in contrast to dietary TG, has been associated with positive physiological results in the areas of weight control, fat deposition, and lipid metabolism [2]. When TG was replaced by varying amounts of DG, a significant decrease in serum triglyceride concentration was observed with diets supplying 50% or more of total fatty acids as DG. Accordingly, plasma triglyceride levels were also significantly lower in the mice that were fed DG oil (SL-DG) compared to those fed TG oil (SL-TG group) in the current study. It is possible that the retarded assembly of chylomicrons and decreased transport of TG into chylomicrons might be one of the mechanisms underlying the plasma TG-lowering effect, and then it could be implicated in a decrease of body fat mass in the SL-DG group.

Hepatic triglyceride content was significantly lower in the SL-DG and SL-TG groups. These two structured oils were prepared by interesterifying SO, which was rich in n-6 fatty acid (almost linoleic acid) and AO rich in n-3 fatty acid (almost DHA). It is plausible that the improvement of the n-6/n-3 ratio in the SL-DG and SL-TG groups could be attributed to preventing an accumulation in hepatic triglycerides. The addition of fish oil ameliorated the hepatic steatosis induced by a 0.1% cholesterol diet, whereas the n-6-rich safflower oil did not show the same effect [27]. Despite that both SL-TG and SL-DG contain DHA, the suppression of hepatic triglyceride accumulation was more prominent in the *ob/ob* mice that were fed the SL-DG diet. This suggests that additional mechanisms can be responsible for altering lipid metabolism by SL-DG compared to the SL-TG. The SL-DG also markedly lowers the activity of hepatic fatty acid synthesis enzymes, G6PD and ME. These lipogenic enzymes are involved in supplying NADPH for fatty acid biosynthesis. Thus, the reduction of hepatic ME and G6PD activity can limit the availability of the long chain fatty acids that are required for hepatic triglyceride synthesis. Changes in hepatic fatty acid biosynthesis also modify the plasma triglyceride concentration, since it alters the hepatic triglyceride synthesis and, in turn, this affects the production of very low-density lipoproteins (VLDL) by the liver. Interestingly, hepatic fatty acid oxidation enzyme (β-oxidation) activity also was increased in the DHA-containing oil supplemented groups, AO, SL-TG and SL-DG, compared to the SO group. The supplement of very long-chain n-3 PUFA in fish oils lead to enhance hepatic fatty acid oxidation, diminish triglyceride synthesis, and decrease VLDL-triglycerides [28]. Increased fatty acid oxidation can result to limit the fatty acid supply which in available for hepatic triglyceride synthesis. In addition, reduced hepatic lipogenesis may retard VLDL secretion in n-3 fatty acid fed animals [29]. In the present study, the SL-DG group exhibited the lowest activity in hepatic fatty acid synthesis enzyme, but the highest activity in hepatic fatty acid oxidation enzyme. Our results imply that the acylgly-

cerol structure has a significant effect on lipid catabolism and accumulation in the liver. Murata *et al.* [30] reported that increased intake of dietary DG also resulted in elevating the activity of other hepatic enzymes that are involved in the β-oxidation pathway, such as CPT, acyl-CoA dehydrogenase, acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 2,4-dienoyl-CoA reductase. Furthermore, enzymes involved in fatty acid synthesis, such as fatty acid synthase, G6PD, and ME were shown to be lower in rats fed DG than in those fed TG for 14 days [30]. Meng *et al*. [25] examined the inhibitory effect of 1,3-DG on diet-induced lipid accumulation in the liver and abdominal adipose tissues of rats. The increase in hepatic fat accumulation induced by high TG feeding for 21 days was also suppressed when this was replaced with the same amount of DG containing oils [31]. In the current study, SL-DG tended to lower PAP activity, a rate-limiting enzyme in triglyceride synthesis. Furthermore, SL-DG appeared to facilitate to increase the excretion of triglyceride into the feces. Consequently, the simultaneous action of SL-DG on the down-regulation of the hepatic lipogenic enzyme activity and the up-regulation of the hepatic fatty acid oxidation and the fecal triglyceride excretion all seemed to contribute to the ameliorating hepatic steatosis in *ob/ob* mice.

In addition, not only SL-DG oil lowered hepatic triglyceride levels but also it decreased the cholesterol levels to higher extent than SO in *ob/ob* mice. These results were accompanied by the remarkable inhibition of hepatic cholesterol-regulating enzyme activity. The two key enzymes involved are HMG-CoA reductase and ACAT, the enzymes that catalyze the rate-limiting step in cholesterol biosynthesis and esterification, respectively. The present results suggest that SL-DG-fed mice exhibited to lower cholesterol biosynthesis through the partial inhibition of hepatic HMG-CoA reductase. The lower ACAT activity with the SL-DG supplement may have led to less cholesteryl ester being available for VLDL packing. These results were consistent with our previous studies that dietary DG intake lowered the hepatic cholesterol-regulating enzyme, HMG-CoA reductase and ACAT, activities [32]. Moreover, the suppression of hepatic HMG-CoA reductase activity, following intake of DHA by rats, was consistent [33]. Therefore, inhibition of cholesterol synthesis by DHA seemingly induced lower hepatic cholesterol content in the SL-DG groups. Although the underlying mechanism is still not clear, we suggest that the dietary DG intake attributed to the reduction of *de novo* SREBP-1c-mediated cholesterol synthesis.

The present study showed that the structure of acylglycerols as well as the fatty acid composition of dietary oil affected hepatic lipid metabolism and its related gene expressions. PUFA are a part of the negative regulators of hepatic lipogenesis, which is apparently mediated by the repression of SREBP-1c [34]. SREBP are transcription factors involved in the transcription of genes related to cholesterol and lipid synthesis. The crucial role of SREBP-1c has been shown by studies, in which deficiency of SREBP-1c prevented hepatic steatosis in *ob/ob* mice, while the overexpression of SREBP-1c induced hepatic steatosis [35]. SREBP-1c binds with sterol-regulatory elements in the promoters of many genes including ACL, ACC, FAS, and SCD-1. PUFA suppress the transcription of many lipogenic genes. Moreover, n-3 PUFA suppresses triglyceride synthesis, VLDL secretion, and serum triglycerides that are different from n-6 PUFA [28]. Kim *et al*. [36] suggested that the feeding of fish oil down-regulated hepatic SREBP-1c mRNA in C57BL/6J mice. DHA is known to suppress hepatic fatty acid synthesis by decreasing the levels of mature SREBP-1c and SREBP-1c mRNA. We observed that the expression of SREBP-1c mRNA was up-regulated by feeding SO that was DHA-free oil, whereas it was markedly suppressed by SL-DG oil in *ob/ob* mice. SL-DG also decreased hepatic triglyceride production in *ob/ob* mice. The mRNA expressions of SREBP-1c and its target enzymes, FAS, ACL and SCD-1, were also determined. ACL mRNA expression was suppressed along with SREBP-1c mRNA in *ob/ob* mice by SL-DG although mRNA expressions of FAS and SCD-1 were not altered significantly. This suggests that the SL-DG structure used in this study potently regulates the hepatic lipogenic pathway at the transcription level. These results indicate that SL-DG might be useful in preventing the formation of fatty liver in obese animals; this effect could be closely related to the down-regulation of the SREBP-1c mRNA expression as well as its target genes in the liver.

Similar to SREBP-1c, PPARγ mediates the development of hepatic steatosis in the mouse models [37]. PPARγ is predominantly expressed in adipose tissues and to a lesser extent in many other tissues including the liver. Several murine models of obesity and diabetes, including *ob/ob*, A-ZIP, aP2/DTA, and KKAy, develop fatty livers with high levels of PPARγ in the liver [37, 38]. These results suggest that PPARγ might be implicated in the pathophysiology of fatty liver that can be observed in animals. Liver PPARγ likely increases the transcription of genes involved in hepatic fatty acid synthesis (including FAS, ACC, SCD) and fatty acid uptake (including FAT/CD36, fatty acid translocase) [39]. Thus, it is likely that liver PPARγ contributes to the regulation of lipid synthesis, transport, and storage within the hepatocytes, causing the development of hepatic steatosis. In the present study, PPARγ mRNA expression was increased in the AO group compared to the other groups. Some studies indicate that PUFA are better activators of PPARγ, especially DHA activates the PPAR [40]. SL-DG significantly lowered the hepatic PPARγ expression compared to the AO concomitant with suppression of the hepatic triglyceride accumulation in *ob/ob* mice. A decrease in PPARγ expression by SL-DG feeding seemingly resulted in an improvement of hepatic steatosis.

The present investigation is the first study that suggests benefits of DHA-esterified DG structure. DHA in algae oil is effective for altering body fat mass and hepatic lipid metabolizing enzymes activities. Especially, these effects were more predominant when provided with DG form. In summary, dietary SL-DG can mediate to lower body fat mass and hepatic lipid levels in *ob/ob* mice that were accompanied by stimulating hepatic lipid metabolizing enzyme activities. In addition, this functional oil was apparently beneficial for ameliorating the hepatic steatosis in $Lep^{ob/ob}$ mice via the down-regulation of the hepatic SREBP-1c and PPARγ mRNA expression. The combined effect of DHA and the structural difference in DG suggests that structured lipids can be further developed as potent functional oils that can enhance triglyceride metabolism and retard fatty liver formation.

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5 References

[1] Boer, M.-Den., Voshol, P.-J., Kuipers, F., Havekes, L.-M., Romijn, J.-A., Hepatic steatosis a mediator of the metabolic syndrome, lessons from animal models, *Arterioscler. Thromb. Vasc. Biol.* 2003, *24*, 644 – 649.

[2] Rudkowska, I., Roynette, C.-E., Demonty, I., Vanstone, C.-A., *et al*., Diacylglycerol: Efficacy and mechanism of action of an anti-obesity agent, *Obes. Res*. 2005, *13*, 1864 – 1876.

[3] Nordøy, A., Marchioli, R., Arnesen, H., Videbaek, J., N-3 polyunsaturated fatty acids and cardiovascular diseases, *Lipids* 2001, *36*, S127 – S129.

[4] Horrocks, L.-A., Yeo, Y.-K., Health benefits of docosahexaenoic acid (DHA), *Pharmacol. Res*. 1999, *40*, 211 – 225.

[5] Zhang, Y., Proenca, R., Maffei, M., Barone, M., *et al*., Positional cloning of the mouse obese gene and its human homologue, *Nature* 1994, *372*, 425 – 432.

[6] Clandinin, M.-T., Cheema, S., Pehowich, D., Field, C.-J., Effect of polyunsaturated fatty acids in obese mice, *Lipids* 1996, *31 (Suppl.2)*, 13 – 22.

[7] Kim, N.-S., Lee, K.-T., Synthesis of diacylglycerol-enriched functional lipid containing DHA by lipase-catalyzed in solvent-free system, *Korean J. Food Sci. Technol.* 2005, *37*, 584 – 589.

[8] American Institute of Nutrition: Report of the American Institute of Nutrition. ad hoc committee on standards for nutritional studies, *J. Nutr*. 1977, *107*, 1340 – 1348.

[9] American Institute of Nutrition: Report of ad hoc committee on standards for nutritional studies, *J. Nutr.* 1980, *110*, 1717 – 1726.

[10] Allain, C.-C., Poon, L.-S., Chan, C.-S., Richmond, W., Fu, P.-C., Enzymatic determination of total serum cholesterol, *Clin. Chem*. 1974, *20*, 470 – 475.

[11] McGowan, M.-W., Artiss, J.-D., Strandbergh, D.-R., Zak, B., A peroxidase-coupled method for the colorimetric determination of serum triglycerides, *Clin. Chem*. 1983, *29*, 538 – 542.

[12] Folch, J., Lees, M., Sloan-Stanley, G.-H., A simple method for isolation and purification of total lipids from animal tissues, *J. Biol. Chem*. 1957, *226*, 497 – 509.

[13] Hulcher, F.-H., Oleson, W.-H., Simplified spectrophotometric assay for microsomal 3-hydroxy-3-methylglutaryl CoA reductase by measurement of coenzyme A, *J. Lipid Res.* 1973, *14*, 625 – 631.

[14] Ochoa, S., in: Colowick, S. P., Kaplan, N. O. (Eds.), *Malic enzyme: malic enzymes from pigeon and wheat germ, Methods in Enzymology*, Academic Press, New York 1995, pp. 323 – 326.

[15] Pitkanen, E., Pitkanen, O., Uotila, L., Enzymatic determination of unbound D-mannose in serum, *Eur. J. Clin. Chem. Clin. Biochem*. 1997, *35*, 761 – 766.

[16] Walton, P.-A., Possmayer, F., Mg_2-dependent phosphatidate phosphohydrolase of rat lung: development of an assay employing a defined chemical substrate which reflects the phosphohydrolase activity measured using membrane-bound substrate, *Anal. Biochem*. 1985, *151*, 479 – 486.

[17] Markwell, M.-A.-K., McGroarty, E.-J., Bieber, L.-L., Tolbert, N.-E., The subcellular distribution of carnitine acyltransferases in mammalian liver and kidney, *J. Biol. Chem*. 1973, *248*, 3426 – 3432.

[18] Lazarow, P.-B., Assay of peroxisomal β – oxidation of fatty acids, *Methods Enzymol*. 1981, *72*, 315 – 319.

[19] Shapiro, D.-J., Nordstrom, J.-L., Mitschelen, J.-J., Rodwell, V.-W., Schimke, R.-T., Micro assay for 3-hydroxy-3-methylglutaryl-CoA reductase in rat liver and in L-cell fibroblasts, *Biochim. Biophys. Acta* 1974, *370*, 369 – 377.

[20] Erickson, S.-K., Schrewsbery, M.-A., Brooks, C., Meyer, D.-J., Rat liver acyl-coenzyme A:cholesterol acyltransferase: its regulation in vivo and some of properties *in vitro*, *J. Lipid Res*. 1980, *21*, 930 – 941.

[21] Bradford, M.-M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem*. 1976, *72*, 248 – 254.

[22] Livak, K.-J., Schmittgen, T.-D., Analysis of relative gene expression data using Real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method, *Methods* 2001, *25*, 402 – 408.

[23] Jump, D.-B., Clarke, S.-D., Regulation of gene expression by dietary fat, *Annu. Rev. Nutr.* 1999, *19*, 63 – 90.

[24] Nakatani, T., Kim, H.-J., Kaburagi, Y., Yasuda, K., Ezaki, O., A low fish oil inhibits SREBP-1 proteolytic cascade, while a high-fish-oil feeding decreases SREBP-1 mRNA in mice liver: relationship to anti-obesity, *J. Lipid Res.* 2003, *44*, 369 – 379.

[25] Meng, X., Zou, D., Shi, Z., Duan, Z., Mao, Z., Dietary diacylglycerol prevents high-fat diet-induced lipid accumulation in rat liver and abdominal adipose tissue, *Lipids* 2004, *39*, 37 – 41.

[26] Mekki, N., Christofilis, M.-A., Charbonnier, M., Atlan-Gepner, C., *et al*., Influence of obesity and body fat distribution on postprandial lipemia and triglyceride-rich lipoproteins in adult women, *J. Clin. Endocrinol. Metab.* 1999, *84*, 184 – 191.

[27] Lambert, M.-S., Avella, M.-A., Berhane, Y., Shervill, E., Botham, K.-M., The differential hepatic uptake of chylomicron remnants of different fatty acid composition is not mediated by hepatic lipase, *Br. J. Nutr.* 2001, *85*, 575 – 582.

[28] Harris, W.-S., Lu, G.-P., Rambjor, G.-S., Walen, A.-I., Ontko, J.-A., Influence of n-3 fatty acid supplementation on the endogenous activities of plasma lipoprotein lipase, *Am. J. Clin. Nutr.* 1997, *66*, 254 – 260.

[29] Wong, S.-H., Nestel, P.-J., Trimble, R.-P., Storer, G.-B., Illman, R.-J., Topping, D.-L., The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver, *Biochim. Biophys. Acta* 1984, *792*, 103 – 109.

[30] Murata, M., Ide, T., Hara, K., Reciprocal responses to dietary diacylglycerol of hepatic enzymes of fatty acid synthesis and oxidation in the rat, *Br. J. Nutr.* 1997, *77*, 107 – 121.

[31] Taguchi, H., Omachi, T., Nagao, T., Matsuo, N., *et al*., Dietary diacylglycerol suppresses high fat diet-induced hepatic fat accumulation and microsomal triacylglycerol transfer protein activity in rats, *J. Nutr. Biochem.* 2002, *13*, 678 – 683.

[32] Kim, H.-J., Lee, K.-T., Lee, M.-K., Jeon, S.-M., Choi, M.-S., Diacylglycerol-enriched structured lipids containing CLA and capric acid alter body fat mass and lipid metabolism in rats, *Ann. Nutr. Metab.* 2006, *50*, 219 – 228.

[33] Ramaprasad, T.-R., Srinivasan, K., Baskaran, V., Sambaiah, K., Lokesh, B.-R., Spray-dried milk supplemented with alpha linolenic acid or eicosapentaenoic acid and docosahexaenoic acid decreases HMG-CoA reductase activity and increases biliary secretion of lipids in rats, *Steroids* 2006, *71*, 409 – 415.

[34] Sekiya, M., Yahagi, N., Matsuzaka, T., Najima, Y., *et al.,* Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression, *Hepatology* 2003, *38*, 1529 – 1539.

[35] Yahagi, N., Shimano, H., Hasty, A.-H., Matsuzaka, T., *et al*., Absence of SREBP-1 ameliorates fatty livers but not obesity or insulin resistance in Lep(ob)/Lep(ob) mice, *J. Biol. Chem.* 2002, *277*, 19353 – 19357.

[36] Kim, H.-J., Takahashi, M., Ezaki, O., Fish oil feeding decreases mature SREBP-1 by down-regulation of SREBP-1c mRNA in mouse liver, *J. Biol. Chem.* 1999, *274*, 25892 – 25898.

[37] Gavrilova, O., Haluzik, M., Matsusue, K., Cutson, J.-J., *et al*., Liver PPARgamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass, *J. Biol. Chem.* 2003, *278*, 34268 – 34276.

[38] Memon, R.-A., Tecott, L.-H., Nonogaki, K., Beigneux, A., Moser, A.-H., Up-regulation of PPAR-alpha and PPAR-gamma messenger ribonucleic acid expression in the liver in murine obesity:troglitazone induces expression of PPAR-gamma-responsive adipose tissue-specific genes in the liver of obese diabetic mice, *Endocrinology* 2000, *141*, 4021 – 4031.

[39] Rahimian, R., Masih-Khan, E., Lo, M., van Breemen, C., Hepatic over-expression of PPAR-gamma2 in the *ob/ob* mouse model of non-insulin dependent diabetes mellitus, *Mol. Cell. Biochem.* 2001, *24*, 29 – 37.

[40] Forman, B.-M., Chen, J., Evans, R.-M., Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for PPARalpha and delta, *Proc. Natl. Acad. Sci.* 1997, *94*, 4312 – 4317.