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Dietary Effect of Squalene on Lipid Metabolism of Obese/Diabetes KK-A^y Mice and Wild-Type C57BL/6J Mice

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Abstract

The purpose of this study was to evaluate the influence of squalene (SQ) on plasma and hepatic lipid levels of obese/diabetic KK-A^y mice and wild-type C57BL/6J mice. SQ supplementation significantly increased the HDL cholesterol of KK-A^y mice, which was paralleled with no significant difference in the total and non-HDL cholesterol levels. The increase in HDL cholesterol was also found in the plasma of normal C57BL/6J mice, but the difference was not significant. SQ administration significantly increased neutral lipids (NL) in the liver of KK-A^y mice, while no significant difference was observed in the polar lipids and the total cholesterol levels. The increase in NL was primarily due to the increase in TAG. However, the cholesterol level significantly increased due to SQ intake in the liver of C57BL/6J mice, while no significant difference was found in other lipid levels. The present study suggests that SQ may effectively increase HDL cholesterol level, an important anti-atherosclerotic factor, especially in subjects with metabolic disorders.

Keywords

Squalene; KK-A^y Mice; C57BL/6J Mice; HDL Cholesterol, Atherosclerosis

1. Introduction

Squalene (SQ), a naturally occurring triterpenic hydrocarbon, is widespread in nature, especially among shark liver oil and olive oils. The shark *Centrophorus squamosus* has been reported to contain approximately 14% liver oil, mainly composed of SQ (nearly 80% of the oil) [1]. Although shark liver oil remains the richest natural source of SQ, its use is limited by persistent organic pollutants and shark resource protection [1]. SQ is also found in many vegetable oils in va-

rying concentrations. Among vegetable oils, oil from *Amaranthus* sp. is known to have the highest concentration of SQ (up to 73.0 g/kg oil) [2] [3]. The SQ content in olive oil is also high (5.64 g/kg oil) compared to other vegetable oils such as that derived from hazelnuts (0.28 g/kg oil), peanuts (0.27 g/kg oil), corn (0.27 g/kg oil) and soybean (0.10 g/kg oil) [4].

Olive oil intake has shown beneficial health effects [5] [6] [7] [8] and these effects have been recognized to partially derive from olive oil minor compounds, mainly phenolic compounds. In addition, due to the relatively high content in olive oil compared with other vegetable oils, SQ is also regarded as a contributing factor in the reduced risk of diseases associated with olive oil intake. To date, SQ has been reported to show anticancer, anti-inflammatory, antioxidant, skin protection, liver protection, and neuroprotective activities [9] [10] [11] [12]. In particular, many studies have been conducted on the relationship between the reduced risk of cancer due to olive oil intake and the role of SQ as a vital dietary cancer chemopreventive agent [9] [10] [11] [12] [13].

Furthermore, the higher intake of olive oil in Mediterranean countries compared to northern European countries is related to the low incidence of cardiovascular disease (CVD) [10]. CVD is the leading cause of morbidity and mortality worldwide. In many cases, CVD is caused by atherosclerosis, a chronic vascular disease that generally occurs in the aorta and muscular-type arteries, such as coronary arteries, brain arteries, renal arteries and carotid arteries [14]. Although the exact cause of atherosclerosis is still unknown, modification and deposition of lipids in the vascular wall can induce this event. Among types of lipid deposition, low density lipoprotein (LDL) cholesterol deposition, especially oxidized LDL, is regarded as a main cause. Thus, cholesterolemia is known as a major inducer of atherosclerosis, and much attention has been paid to the hypocholesterolemic activity of olive oil components, such as oleic acid and other minor compounds including SQ [10].

SQ is known as an important intermediate for the biosynthesis of phytosterol or cholesterol in plants, animals and humans [11], and its endogenous synthesis begins with the conversion of acetyl coenzyme A to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), followed by the reduction of HMG-CoA to mevalonate, mediating HMG-CoA reductase. Thus, the involvement of SQ in cholesterol synthesis is easily expected, and the possibility has been demonstrated for the use of SQ as a biomarker to evaluate endogenous cholesterol biosynthesis (Brown *et al*., 2014). Indeed, numerous studies from this viewpoint have explored this possibility of using several experimental approaches in both animals and humans [11].

Many studies on the effect of dietary SQ on blood cholesterol levels have been reported. Several researchers have demonstrated that dietary SQ can inhibit the activity of HMG-CoA reductase, due to negative feedback regulation of endogenous cholesterol synthesis [12] [13]. The inhibition of HMG-CoA reductase, the rate-limiting control step in the normal biosynthetic pathway from SQ to

cholesterol, can also reduce the levels of the series of intermediates between HMG-CoA and cholesterol, including mevalonate, geranyl pyrophosphate, and farnesyl pyrophosphate. However, the effect of SQ on plasma lipids in humans and animals is controversial. Some authors have reported the decrease in plasma cholesterol levels in human studies by SQ intake, while other researchers have reported the opposite effect [1] [10] [15] [16]. The varied effects of SQ are hypothesized to be due to differences in the experimental approaches and sexes [10] [15] [17].

The controversial effect of SQ on blood cholesterol level has been replicated in animal models. Several researchers have reported a reduction of blood lipid levels, including cholesterol in rats after SQ intake [18] [19] [20], while other researchers have found increases in the cholesterol levels in normal animals by SQ feeding [21] [22]. In addition, Chmelik *et al.* [23] found the increase in high density lipoprotein (HDL) cholesterol levels of C57Bl/6J SPF mice fed SQ, while the total cholesterol and LDL cholesterol levels decrease with SQ feeding. This specific increase in HDL cholesterol by SQ intake was also found in three different mouse models (wild-type, Apoa1- and Apoe-deficient) [17].

Many animal and human studies have revealed SQ as a promising agent in CVD prevention [10] [11] [15]. The effect of SQ has been explained via several different mechanisms, including the elimination of cholesterol as fecal bile acids [16], inhibition of HMG CoA reductase by dietary SQ due to negative feed-back regulation [24], inhibition of oxidized LDL uptake by macrophages [25], stimulation of reverse cholesterol transport [26], inhibition of isoprenaline-induced lipid peroxidation [18], and attenuation of homocysteine-induced endothelial dysfunction [11]. These mechanisms are basically dependent on the antioxidant activity of SQ and the involvement of SQ in cholesterol metabolism [1] [10] [11] [15].

However, SQ's role in plasma lipids is not yet clear, although hyperlipidemia, especially hypocholesterolemia, is regarded as a major risk factor for CVD. In the present study, we assessed the effect of SQ on the plasma lipid content of animal models. For animals, we used C57BL/6J genetic background mice. These mice have been widely used due to their higher predisposition to atherosclerosis development [27]. Furthermore, we compared the effect of SQ in normal and obese/diabetes mice. Obesity and diabetes are a major risk factor for CVD [28] [29]. Therefore, the comparison may help to elucidate the effect of SQ on the reduction of CVD risk.

2. Experimental Procedures

2.1. Materials

SQ was purchased from Wako Pure Chemicals, Ltd., Osaka, Japan. Dietary lipids, linseed oil and lard, were obtained from Summit oil Mill Co. Ltd., Chiba, Japan, and Junsei Chemical Co. Inc., Tokyo, Japan, respectively. All other chemicals and solvents used in the study were of analytical grade.

2.2. Animals and Diets

Obese/diabetic KK-A^y mice (male, four weeks old) and wild-type C57BL/6J mice (male, four weeks old) were obtained from the Japan CREA Co., Tokyo, Japan. The mice were housed individually in an air-conditioned room (23˚C ± 1˚C and 50% humidity) with a 12 h light/12 h dark cycle. After acclimation feeding of a normal rodent diet MF (Oriental Yeast Co., Ltd, Tokyo, Japan) for 1 week, the mice were randomly divided into 3 groups of seven and were fed experimental diets for four weeks (Table 1). The body weight, diet and water intake of each mouse was recorded daily.

2.3. Ethics

The research project was approved by the Ethical Committee at Hokkaido University, and all procedures for the use and care of animals for this research were performed under approval by the Ethical Committee of Experimental Animal Care at Hokkaido University.

2.4. Sample Collection

Mice were sacrificed under diethyl ether anesthesia after 12 h fasting on day 28. Blood samples were taken from the caudal vena cava of the mice. A portion of blood was used for blood glucose analysis, while the remaining part was stored for lipid analysis. Blood glucose was measured using a blood glucose monitor, namely, the Glutest Neo Sensor (Sanwa Kagaku Kenkyusyo Co. Ltd., Aichi, Japan). This sensor is an amperometric sensor with flavin adenine dinucleotide (FAD)-dependent glucose dehydrogenase and $Fe(CN)_6^{3-}$. Liver, spleen, small

Table 1. Composition ($g \cdot kg^{-1}$) of experimental diets.

Ingredient %	Control	1% Squalene	2% Squalene
β-corn starch	224.586	224.586	224.586
α-corn starch	84.9	84.9	84.9
Milk casein	260	260	260
Sucrose	130	130	130
Cellulose	50	50	50
L-cystine	3	3	3
AIN93G mineral mix	35	35	35
AIN93G vitamin mix	10	10	10
Choline bitartrate	2.5	2.5	2.5
Ter-butyl hydroquinone	0.014	0.014	0.014
Lard	130	130	130
Soybean oil	0	0	0
Linseed oil	70	70	70
Fish oil	0	0	0
Squalene	0	10	20

intestine, pancreas, kidney, muscle, brain, brown adipose tissue (BAT) and white adipose tissues (WAT) including epididymal, mesenteric, perirenal, retroperitoneal, and inguinal WAT were immediately excised and weighed. Each tissue removed was stored at −20˚C for lipid analysis. The liver parts were frozen immediately in liquid nitrogen for the determination of enzyme activity or stored in RNA later™ (Sigma Chemical Co., St. Louis, MO) for quantitative real time Polymerase Chain Reaction (PCR) analysis.

2.5. Blood Lipid Analysis

The blood plasma analysis was performed by the Analytical Center of Hakodate Medical Association (Hakodate, Japan). The analysis included measurement of the following parameters: total cholesterol, HDL cholesterol, non-HDL cholesterol, triacyglycerols (TAG) and phospholipids.

2.6. Hepatic Lipid Analysis

Total lipids (TL) was extracted from the liver with chloroform/methanol (2:1, v/v) [30]. The TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridge (Waters Japan, Tokyo, Japan) by elution with chloroform (70 mL) and methanol (50 mL). The neutral lipids (NL) and polar lipids fractions were eluted with chloroform and methanol, respectively. Both lipid contents in the liver (mg/g liver) were calculated from the TL level per liver weight. Total cholesterol and TAG were measured using enzymatic kits (Cholesterol E-test and Triglyceride E-test) obtained from Wako Pure Chemical Industries, Osaka, Japan.

The fatty acid composition of the TL was determined by gas chromatography (GC) after conversion of fatty acyl groups in the lipid to their methyl esters. The fatty acid methyl esters (FAME) were prepared according to the method of Prevot and Mordret [31]. Briefly, 1 mL of *n*-hexane and 0.2 mL of 2 N NaOH in methanol were added to an aliquot of total lipid (*ca*. 10 mg), vortexed and incubated at 50˚C for 30 min. After incubation, 0.2 mL of 2 N HCl in methanol solution was added to the solution and vortexed. The mixture was separated by centrifugation at 1000 g for 5 min. The upper hexane layer containing fatty acid methyl esters was recovered and subjected to GC. GC was performed on a Shimadzu GC-14B equipped with a flame-ionization detector and a capillary column [Omegawax 320 (30 m × 0.32 mm i.d.); Supelco, Bellefonte, PA]. The injection port and flame ionization detector were set at 250˚C and 260˚C, respectively, and the column temperature was held at 200˚C. The carrier gas was helium at a flow rate of 50 kPa. Fatty acid content in the lipid samples was expressed as a weighted percentage of the total fatty acids. Each fatty acid level of liver tissue (1 g) was calculated by comparing the peak ratio to that of the internal standard (17:0) and the total lipid content.

In a preliminary experiment, we found that SQ and docosahexaenoic acid (22:6n-3, DHA) could not be separated clearly on the chromatogram using the Omegawax-320 capillary column. Therefore, the prepared FAME sample was

submitted to thin layer chromatography (TLC) to remove SQ before GC analysis. SQ and FAME were clearly separated on a 0.25-mm silica gel TLC plate (Silica Gel 60 F254, Merck, Darmstadt, Germany) developed with hexane-diethyl ether-acetic acid (80:20:1, v/v/v).

2.7. Quantitative Real-Time PCR

Total RNA was extracted from the livers of mice using RNeasy Lipid Tissue Mini Kits (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The cDNA was synthesized from total RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems Japan Ltd., Tokyo, Japan). Quantitative real-time PCR analyses of individual cDNA were performed with ABI Prism 7500 (Applied Biosystems Japan Ltd., Tokyo, Japan) using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd., Tokyo, Japan). Gene expression was normalized to the reference gene GAPDH.

The mRNA analyses were performed on genes associated with lipid metabolism, which included liver X receptor (LXR), sterol regulatory element-binding protein 2 (SREBP2), hydroxymethylglutaryl-CoA synthase 1 (HMGCS1), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), farnesyl-diphosphate farnesyltransferase 1 (FDFT1), sterol 14α-demethylase (CYP51), cholesterol 7-alpha-monooxygenase (CYP7A1), carbohydrate-responsive-element-binding protein (ChREBP), fatty acid synthase (FASN), stearoyl-CoA desaturase-1 (SCD1), diacylglycerol O-acyltransferase 1 (DGAT1), diacylglycerol O-acyltransferase 2 (DGAT2), lipin 1. The gene-specific primers were Hs00172885_m1 (LXR), Hs01081784_m1 (SREBP2), Hs00940429_m1 (HMGCS1), Hs00168352_m1 (HMGCR), Hs00926054_m1 (FDFT1), Mm00490968_m1 (CYP51), Hs00167982_m1 (CYP7A1), Hs00975714_m1 (ChREBP), Hs01005622_m1 (FASN), Mm00772290_m1 (SCD1), Hs01020362_g1 (DGAT1), Hs01045913_m1 (DGAT2), Hs00299515_m1 (lipin 1), and Hs02786624_g1 (GAPDH; internal control), respectively.

2.8. HMG-CoA Reductase Analysis

Activity of HMG-CoA reductase, a key enzyme of hepatic cholesterol synthesis, was measured according to the procedure described by Rao and Ramakrlshnan (1975). This procedure is based on the formation of hydroxymate from the reaction of HMG-CoA and mevalonate with hydroxylamine. The resulting hydroxymate can be quantitatively measured using the colorimetric assay. The HMG-CoA and mevalonate concentration in the liver homogenate were collected separately by changing pH to avoid interference by mevalonate in the HMG-CoA analysis. Therefore, liver homogenate was reacted with freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG-CoA) at pH 5.5 for HMG-CoA and pH 2.1 for mevalonate, respectively. HMG-CoA reductase activity was calculated from the ratio of HMG-CoA concentration to mevalonate concentration.

2.9. Statistical Analysis

Results are expressed as mean ± SEM. Statistical significances between groups were evaluated by one-way ANOVA with *post hoc* comparisons (Scheffe's F-test). Differences with $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Food Intake, Water Intake, Weight Gain, Tissue Weights, Blood Glucose Levels, Serum and Hepatic Lipid Parameters

The weights of major tissues were not significantly different, except for a significant increase in the liver weight of KK-A^y mice fed SQ (1% and 2%) (Table 2).

Table 2. Weight gain (g) and tissue weight (g per 100 g body weight).

	KK-Ay mice				
	Control		SQ(1%)		SQ(2%)
Weight gain	14.47 ± 0.84	a	17.09 ± 0.74	a,b	18.13 ± 0.69
Tissue weight					
Liver	6.21 ± 0.19	a	9.07 ± 0.21	c	8.07 ± 0.31
Small intestine	3.48 ± 0.07		3.23 ± 0.12		3.40 ± 0.13
Kidney	1.80 ± 0.09		1.64 ± 0.08		1.56 ± 0.03
Brain	0.89 ± 0.03		0.81 ± 0.03		0.74 ± 0.05
Pancreas	0.69 ± 0.02		0.64 ± 0.02		0.68 ± 0.02
Heart	0.35 ± 0.01		0.33 ± 0.01		0.34 ± 0.01
Spleen	0.26 ± 0.03		0.22 ± 0.02		0.24 ± 0.01
Total WAT	11.82 ± 0.32		11.50 ± 0.38		12.28 ± 0.33
BAT	0.45 ± 0.03		0.43 ± 0.02		0.43 ± 0.02
Muscle	0.63 ± 0.01		0.57 ± 0.02		0.59 ± 0.02
	C57BL/6J mice				
	Control		SQ(1%)		SQ(2%)
Weight gain	6.48 ± 0.47		7.50 ± 0.38		6.75 ± 0.26
Tissue weight					
Liver	5.71 ± 0.17		5.80 ± 0.13		5.67 ± 0.05
Small intestine	3.20 ± 0.11		3.16 ± 0.17		2.92 ± 0.14
Kidney	1.26 ± 0.03		1.30 ± 0.02		1.31 ± 0.01
Brain	1.57 ± 0.21		1.42 ± 0.81		1.60 ± 0.02
Pancreas	0.94 ± 0.02		0.88 ± 0.04		1.10 ± 0.13
Heart	0.48 ± 0.01		0.47 ± 0.01		0.49 ± 0.02
Spleen	0.31 ± 0.02		0.31 ± 0.01		0.30 ± 0.01
Total WAT	7.16 ± 0.50		8.08 ± 0.44		7.83 ± 0.62
BAT	0.44 ± 0.02		0.46 ± 0.02		0.44 ± 0.03
Muscle	1.16 ± 0.02		1.12 ± 0.02		1.08 ± 0.03

Different letters (a, b,c) show significant differences at $P < 0.05$.

Significant increases in weight gain were also found in KK-A^y mice fed SQ (2%) (Table 2). Although a tendency in the decrease in plasma non-HDL cholesterol was found in KK-A^y mice, other lipid parameters increased (Table 3). A significant increase in HDL cholesterol was found. However, all plasma cholesterols levels (total cholesterol, HDL cholesterol and non-HDL cholesterol) increased in C57BL/6J mice, but the difference was not significant (Table 3). SQ intake significantly increased TL, NL and TAG in the liver of KK-A^y mice, while no significance was observed in the hepatic lipid levels of C57BL/6J mice, except for total cholesterol (Table 4). Total cholesterol level in the liver from C57BL/6J mice significantly increased due to SQ (2%) feeding, but levels in KK-A^y mice fed SQ (1% and 2%) were lower than those fed control diet (Table 4).

3.2. Fatty Acid Levels of Liver Lipids

SQ (1% and 2%) supplementation significantly all fatty acid contents in the liver from KK-A^y mice except for 20:4n − 6, resulting in a significant increase in the total fatty acid contents (Table 5). The increase in total fatty acids presented in Table 5 was consistent with the result in Table 4 showing the increasing effect of SQ on liver TL and NL. However, there was little difference in the fatty acid content in the liver of C57BL/6J mice (Table 5). This was also expected due to the TL and NL levels in the liver of C57BL/6J mice (Table 4).

Table 3. Plasma glucose and lipid levels (mg/dL).

	KK-Ay mice		
	Control	SQ(1%)	SQ(2%)
Total cholesterol	186.14 ± 6.22	197.57 ± 4.79	200.71 ± 9.20
HDL cholesterol	130.14 ± 6.44 a	152.86 ± 6.19 b	155.43 ± 5.23 b
Non-HDL cholesterol	56.00 ± 3.69	44.71 ± 4.51	46.71 ± 4.93
TAG	353.71 ± 50.80	471.14 ± 54.78	375.43 ± 87.51
Phospholipids	365.00 ± 15.94	377.29 ± 9.52	376.00 ± 15.33
Glucose	578.83 ± 35.51	499.14 ± 36.84	376.00 ± 15.33
	C57BL/6J mice		
	Control	SQ(1%)	SQ(2%)
Total cholesterol	120.71 ± 3.58	134.50 ± 3.21	134.71 ± 5.79
HDL cholesterol	85.43 ± 2.95	92.50 ± 3.25	93.29 ± 3.28
Non-HDL cholesterol	35.29 ± 1.34	42.00 ± 1.24	41.43 ± 3.05
TAG	83.57 ± 9.40	87.17 ± 15.67	86.00 ± 10.26
Phospholipids	269.14 ± 11.09	282.00 ± 5.38	284.00 ± 9.48
Glucose	130.14 ± 11.34	124.86 ± 7.85	113.83 ± 6.10

Different letters (a, b) show significant differences at $P < 0.05$.

Table 4. Hepatic lipid levels.

	KK-Ay mice					
	Control		SQ(1%)		SQ(2%)	
Total lipids (mg/g liver)	102.00 ± 11.30	a	269.00 ± 9.40	b	226.90 ± 15.0	b
NL (mg/g liver)	82.92 ± 8.66	a	258.38 ± 10.25	b	203.44 ± 18.62	b
Polar lipids (mg/g liver)	36.28 ± 1.65		38.99 ± 2.93		40.05 ± 1.18	
Total cholesterol (mg/g liver protein)	21.53 ± 2.72		14.26 ± 2.13		15.45 ± 3.56	
TAG (mg/g liver protein)	249.33 ± 23.12	a	526.99 ± 57.44	a, b	689.26 ± 163.98	b
	C57BL/6J mice					
	Control		SQ(1%)		SQ(2%)	
Total lipids (mg/g liver)	48.90 ± 2.50		54.70 ± 2.00		52.90 ± 1.80	
NL (mg/g liver)	20.01 ± 1.77		25.54 ± 3.77		23.91 ± 1.96	
Polar lipids (mg/g liver)	28.33 ± 0.69		30.17 ± 1.05		28.46 ± 0.32	
Total cholesterol (mg/g liver protein)	6.24 ± 0.76	a	8.77 ± 1.00	a, b	9.85 ± 0.74	b
TAG (mg/g liver protein)	39.88 ± 4.96		46.90 ± 8.44		56.11 ± 7.50	

Different letters (a, b) show significant differences at $P < 0.05$.

3.3. Gene Expression Related to Lipid Metabolism and HMG-CoA Reductase Activity

To determine the effect of dietary lipids on liver lipid metabolism, the related gene expressions were analyzed using real-time PCR. Although the analysis showed no significant effect of SQ on the gene expression of C57BL/6J mice (**Figure 1**), a significant difference was found in the HMGCR and CYP7A1 genes in the liver from KK-A^y mice fed SQ compared with the control (**Figure 2**). Furthermore, SQ (2%) supplementation significantly increased HMG-CoA reductase activity in the liver from KK-A^y mice, but not C57BL/6J mice.

4. Discussion

Epidemiological studies have revealed an inverse correlation between HDL cholesterol levels and the risk of cardiovascular disease and atherosclerosis. HDL cholesterol promotes reverse cholesterol transport and has several atheroprotective functions, such as anti-inflammation, anti-thrombosis, and antioxidation [32] [33]. In prospective epidemiologic studies, every 1-mg/dL increase in HDL is associated with a 2% to 3% decrease in CVD risk, independent of LDL cholesterol and TAG levels [34]. Furthermore, normal or high HDL levels appear to have anti-atherosclerotic, anti-inflammatory, antioxidant and anti-thrombotic properties, even in the presence of high LDL cholesterol [35]. In the present study, we found a significant increase in the plasma HDL cholesterol of obese/diabetic KK-A^y mice, with no significant difference in the total and non-HDL cholesterol levels (**Table 3**). The increase in HDL cholesterol was also

Table 5. Major fatty acid content in liver (mg/g liver).

	KK-Ay mice					
	Control		SQ(1%)		SQ(2%)	
C16:0	14.35 ± 1.08	a	39.16 ± 1.49	c	33.89 ± 2.60	b
C18:0	4.64 ± 0.18	a	8.05 ± 0.21	c	7.02 ± 0.21	b
C20:0	0.39 ± 3.69	a	1.38 ± 0.07	c	1.08 ± 0.07	b
C16:1	1.27 ± 0.20	a	3.15 ± 0.50	b	2.67 ± 0.35	b
C18:1n − 9	23.99 ± 2.68	a	79.37 ± 5.74	c	64.62 ± 7.67	b
C20:1n − 9	0.74 ± 0.09	a	2.60 ± 0.21	c	1.98 ± 0.21	b
C18:2n − 6	8.95 ± 0.74	a	24.66 ± 0.57	c	19.12 ± 1.39	b
C18:3n − 3	4.70 ± 0.51	a	16.73 ± 0.88	c	11.93 ± 0.64	b
C20:4n − 6	1.85 ± 0.07		1.98 ± 0.06		1.91 ± 0.10	
C20:5n − 5	1.26 ± 0.06	a	1.67 ± 0.10	b	1.43 ± 0.08	a, b
C22:6n − 3	2.22 ± 0.16	a	3.32 ± 0.25	b	3.82 ± 0.19	b
Total fatty acids	64.37 ± 5.31	a	181.63 ± 6.75	b	148.83 ± 12.14	b
	C57BL/6J mice					
	Control		SQ(1%)		SQ(2%)	
C16:0	7.03 ± 0.32		8.47 ± 0.75		8.16 ± 0.55	
C18:0	3.25 ± 0.10		3.49 ± 0.11		3.35 ± 0.05	
C20:0	0.14 ± 0.01		0.16 ± 0.01		0.14 ± 0.00	
C16:1	0.38 ± 0.05		0.65 ± 0.11		0.70 ± 0.11	
C18:1n − 9	6.43 ± 0.47		8.63 ± 0.93		8.48 ± 0.81	
C20:1n − 9	0.18 ± 0.01	a	0.25 ± 0.02	b	0.23 ± 0.02	a, b
C18:2n − 6	3.95 ± 0.13	a	4.86 ± 0.20	b	4.48 ± 0.18	a, b
C18:3n − 3	1.20 ± 0.06	a	1.76 ± 0.15	b	1.72 ± 0.20	a, b
C20:4n − 6	1.92 ± 0.08		1.99 ± 0.05		1.87 ± 0.05	
C20:5n − 5	0.99 ± 0.05		1.15 ± 0.06		1.11 ± 0.04	
C22:6n − 3	2.51 ± 0.13		2.56 ± 0.11		2.40 ± 0.10	
Total fatty acids	28.37 ± 1.21		34.42 ± 2.31		33.07 ± 1.75	

Different letters (a, b, c) show significant differences at $P < 0.05$.

found in the plasma of normal C57BL/6J mice, but the difference was not significant. This result suggests the anti-atherosclerosis effect of SQ, especially for subjects with metabolic disorders.

Various animal and human studies have shown that SQ could reduce the risk of atherosclerosis, and this effect of SQ has been explained by the involvement of SQ in lipid metabolism and by its protective action on lipoproteins and artery endothelial cells against oxidative damage [10] [11] [15]. However, there is a contradiction in the effect of SQ on blood lipid levels. Several researchers have shown a significant decrease in blood cholesterol levels in animal and human studies by SQ intake, while other researchers have reported opposite results [1] [10] [15] [16] [18] [19] [21] [22] [28]. Although there have been controversial

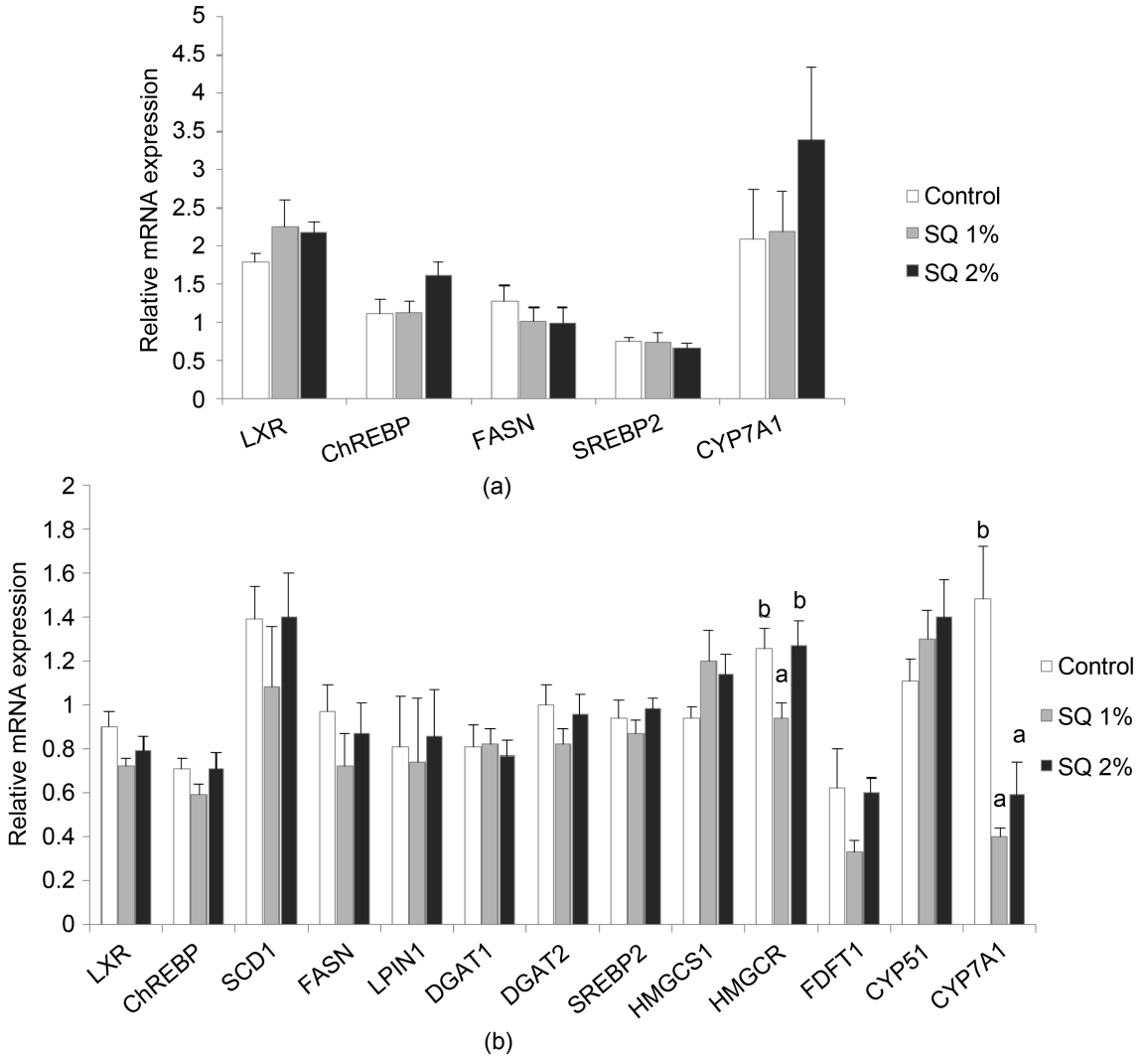

Figure 1. Effect of squalene administration on hepatic mRNA expression in KK-A^y mice (a) and C57BL/6J mice (b).

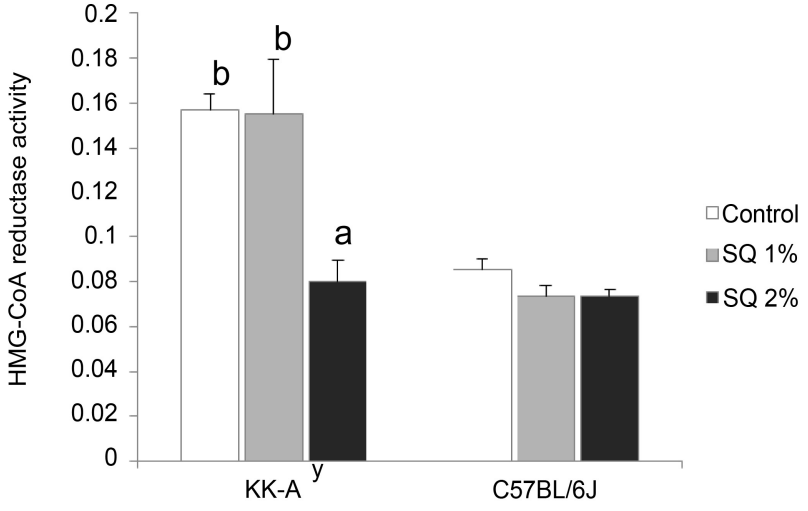

Figure 2. Effect of squalene administration on HMG-CoA reductase activity in liver of KK-A^y and C57BL/6J mice.

results in the effect of SQ on plasma lipid levels, recent studies have demonstrated the effect of SQ on blood HDL cholesterol level as important factor in atherosclerosis protection [11]. Administration of SQ for seven weeks (2.1 g/kg) to C57Bl/6J SPF mice showed a 60% increase in HDL cholesterol with no changes in total cholesterol [23]. Likewise, SQ administration for 11 weeks at a dose of 1 g/kg caused a specific increase in HDL cholesterol levels in three male mouse models (wild-type, *Apoa1*- and *Apoe*-deficient) with the C57BL/6J genetic background [17]. In a rat model, specific increase in HDL cholesterol has also been reported [18]. These studies have demonstrated that high HDL level would be independent of an anti-atherosclerotic factor [35]. The present study confirmed that the increase in HDL cholesterol level is a major effects of SQ in atherosclerosis protection.

HDL cholesterol biogenesis and its development are involved in various complex metabolic networks, such as upregulation of ATP-binding cassette transporter A1, apoA-I transcription and liver X receptor (LXR) [36]. Therefore, the increasing effect of SQ on HDL cholesterol would be related to these events; however, the mechanisms by which SQ elevate plasma HDL-cholesterol levels remain unclear. SQ is known to show a broad repertoire of biological action based on its antioxidant activity [11]. This effect could be exerted in HDL to prevent oxidative modifications of the apolipoprotein A-I (ApoA-I), other HDL proteins, and HDL lipids. The prevention by HDL of oxidative stress can make it more fluid and thus more functional. Further study will be needed.

In humans, orally administered SQ is well absorbed (60% - 85%). This, and the intestinal de novo synthesized SQ, are transported by chylomicrons into circulation and are rapidly taken up by the liver, where it is converted into cholesterol [17]. A significant increase in total hepatic cholesterol found in normal C57BL/6J mice may be reflected by the conversion of SQ to cholesterol in the liver (**Table 4**). However, cholesterol level decreased in the liver of obese/diabetic KK-A^y mice (**Table 4**). However, significant increase in NL by SQ intake was observed in KK-A^y mice, while no significant difference was observed in polar lipids (**Table 4**). The increase in NL resulted in higher TL levels in the liver. SQ administration to KK-A^y mice induced its accumulation in the liver. Moreover, SQ is eluted as the NL fraction in the separation of TL with the column chromatography used in the present study. However, the level of SQ measured was less than 20 mg/g liver in both groups. Therefore, the increase in NL found in **Table 4** was mainly due to the increase in TAG. The TAG increase due to SQ intake was strongly related to the higher level of total fatty acids found in **Table 5**.

As shown in **Table 4** and **Table 5**, squalene administration to KK-A^y mice induces TAG accumulation in the liver. To determine the effect of squalene, gene expression related to fatty acid (FASN and SCD1) and TAG (DGAT1 and DGAT2) synthesis was analyzed (**Figure 1**). However, no significant difference was found in these gene expressions, together with other kinds of genes related to lipid metabolism, except for HMGCR. It is difficult to explain the discrepancy

found between gene expression and TAG content in the liver of KK-A^y mice. One possibility is the involvement of TAG that originated from other tissues.

HMGCR is a gene related to cholesterol metabolism. Its expression was significantly decreased by squalene (1%) intake, but no significance was observed with administration of squalene (2%) (**Figure 1**), while a significant increase in HMG-CoA reductase activity was found in the liver of KK-A^y mice fed squalene (2%), but with no significance in squalene (1%) (**Figure 2**). Although HMG-CoA reductase activity and its gene expression, e.g., HMGCR, is known as a key factor in cholesterol synthesis, the changes in these factors would not greatly affect cholesterol concentrations in the liver (**Table 4**). This may be due to the compensation of other pathways of cholesterol metabolism and/or the effect of cholesterol supplementation from other tissues. CYP7A1 is known to catalyze the cholesterol catabolic pathway. This enzyme expression was decreased by squalene intake (**Figure 1**). Although the difference was not significant, this may affect the cholesterol level of the liver.

5. Conclusion

The present study showed that SQ supplementation to C57BL/6J background mice increased plasma HDL cholesterol level, which is an important and independent anti-atherosclerotic factor. It is noteworthy that this effect of SQ was found more clearly in an obese/diabetes mouse model compared with normal mice. Although more research is needed to clarify the effect of SQ on the lipid metabolism and dynamics related to atherosclerosis, the present study suggests the anti-atherosclerotic effect of SQ, especially for subjects with metabolic disorders.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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