Dietary Effect of Squalene and Farnesol on the Lipid Metabolism of Obese/diabetes KK-A^y Mice and Wild-type C57BL/6J Mice

（肥満/糖尿病 KK-*Ay*マウスと野生型 C57BL/6J マウスの脂質代謝に及ぼすスクワレンとファルネソールの効果）

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Content

List of abbreviations and acronyms

ABCA1	ATP-binding cassette transporter A family member 1
ABCG1	ATP-binding cassette sub-family G member 1
ABCG5	ATP-binding cassette sub-family G member 5
ABCG8	ATP-binding cassette sub-family G member 8
ALA	α-Linolenic acid
ApoA-1	Apolipoprotein A1
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
BAT	brown adipose tissue
CETP	cholesteryl ester transfer protein
ChREBP	carbohydrate-responsive-element-binding protein
COX-2	cyclooxygenase-2
CRP	C-reactive protein
CVD	cardiovascular diseases
CYP51	sterol 14-alpha-demethylase
CYP7A1	cholesterol 7-alpha-monooxygenase
DGAT1	diacylglycerol O-acyltransferase 1
DGAT2	diacylglycerol O-acyltransferase 2
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
FA	fatty acids
FAME	fatty acid methyl ester
FASN	fatty acid synthase
FDFT1	farnesyl-diphosphate farnesyltransferase 1
GC	gas chromatography
GSH	glutathione

GSSG	oxidized glutathione
HDL	high density lipoprotein
HDL-C	HDL cholesterol
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HMGCS1	hydroxymethylglutaryl-CoA synthase 1
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC-ELSD	high performance liquid chromatography with evaporative light scattering detection
IL-1β	interleukin 1 beta
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LDL-C	LDL cholesterol
LPL	lipoprotein lipase
LXR	liver X receptor
MUFA	monounsaturated fatty acid
NAFLD	nonalcoholic fatty liver disease
NL	neutral lipid
NR1H2	nuclear receptor subfamily 1 group h member 2
NR1H3	nuclear receptor subfamily 1 group h member 3
PL	phospholipid
PUFA	polyunsaturated fatty acid
qRT-PCR	quantitative real time Polymerase Chain Reaction
SCD1	stearoyl-CoA desaturase-1
SFA	saturated fatty acid
SL	sphingolipid
SOD	superoxide dismutase
SREBP1	sterol regulatory element-binding protein 1
SREBP2	sterol regulatory element-binding protein 2

SM	sphingomyelin
SQ	squalene
SRB1	scavenger receptor class B member 1
TAG	triacylglycerol
TC	total cholesterol
TL	total lipid
TLC	thin layer chromatography
TNFα	tumor necrosis factor alpha
VLDL	very low density lipoprotein
WAT	white adipose tissue
WHO	World Health Organization

General introduction

Cardiovascular diseases (CVD) are a group of disorders of the heart and blood vessels. They include: coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism. A report from world health organization (WHO) indicated that in 2016, an estimated 17.9 million people died from CVD, representing 31% of all global deaths. CVD have become the number 1 cause of death globally [1]. Over the past few decades it was established that about 80% of the future CVD can be attributed by known major cardiovascular risk factors, i.e. old age, male sex-non-modifiable risk factors, hypertension, high cholesterol, smoking, diabetes mellitus and obesity- modifiable major risk factors [2].

Atherosclerosis is a chronic inflammatory disease that arises from an imbalance in lipid metabolism and a maladaptive immune response driven by the accumulation of cholesterol-laden macrophages in the artery wall [3]. Atherosclerosis development is the underlying cause of CVD. Numerous pathophysiologic observations in humans and animals led to the formulation of the response-to-injury hypothesis of atherosclerosis, which initially proposed that endothelial dysfunction was the first step in atherosclerosis [4]. Then the endothelial dysfunction can alter the normal homeostatic properties of the endothelium through compensatory responses. As the normal homeostatic properties of the endothelium are altered, the permeability and adhesiveness of the endothelium with respect to leukocytes or platelets are increased. If this continues indefinitely, an intermediate lesion with smooth-muscle cells was formed. Inflammatory response then can increase the numbers of macrophages and lymphocytes which multiply within the lesion [4-7]. At the same time, the activation of macrophages and lymphocytes leads to the release of hydrolytic enzymes, cytokines, chemokines, and growth factors [8], which can induce further damage and eventually lead to focal necrosis. At last, a so-called advanced, complicated lesion is

formed through cycles of accumulation of mononuclear cells, migration and proliferation of smooth-muscle cells, and formation of fibrous tissue [8]. This complicated lesion may then intrude into the lumen and alter the flow of blood.

Cells attempt to respond to excessive lipid accumulation by pathways that promote the removal of cholesterol and other lipids from the cell [3]. Several macrophage transporters, including ATP-binding cassette transporter A family member 1 (ABCA1), ATP-binding cassette sub-family G member 1 (ABCG1) and scavenger receptor class B member 1(SRB1) facilitate this efflux of lipids in cholesterol-laden macrophages [9]. ABCA1 promotes cholesterol efflux to lipid-poor Apolipoprotein A1 (ApoA-1)- the building block of high-density lipoprotein (HDL), whereas ABCG1 promotes efflux to mature HDL particles. This illustrates that HDL act as an important role in the treatment of atherosclerosis.

HDL is short for high-density lipoprotein. Each bit of HDL cholesterol (HDL-C) is a microscopic blob that consists of a rim of lipoprotein surrounding a cholesterol center. Measurement of the HDL-C in many individuals became possible due to the advances in precipitation of Apolipoprotein B (ApoB)-containing lipoproteins [10]. First compelling report on the protective effect of HDL-C against coronary heart disease has been done in the Framingham Heart Study in 1964 [11]. These observational data formed the basis for the widely acknowledged concept of HDL-C as the good cholesterol and led to the idea that HDL might have properties to protect against coronary heart disease. Now several reports have indicated the strong inverse correlation between plasma HDL-C and the risk of CVD [12-15]. In the mice experiments, a same atheroprotective effect of HDL-C has also been reported [16-19]. In addition, a report on rabbit demonstrated that infusions of HDL reduced plaque size [20].

Thus far, two metabolic pathways of clearance of cholesteryl ester in HDL have been known. One is direct uptake by the liver or steroidogenic tissues via the HDL

receptor- SRB1. This pathway is selective and the smaller ApoA-1 containing HDL particle is dissociated and recycled after removal of cholesteryl ester [21]. Another pathway is transfer to ApoB-containing lipoproteins by the plasma protein cholesteryl ester transfer protein (CETP). In this pathway, cholesteryl ester from the HDL particle can be depleted to form a triacylglycerol (TAG)-enriched particle. This TAG-enriched particle can be modified by hepatic lipase and endothelial lipase to form a smaller HDL particle, which is susceptible to faster catabolism [22]. *In vivo*, these two HDL metabolism pathways are regulated by several factors which include：apolipoproteins, enzymes, lipid transfer proteins, cellular lipid transporters, and cell surface receptors. On the other hand, several medical and environmental factors can also influence HDL metabolism, including obesity, type 2 diabetes, inflammation and smoking-factors reduce HDL-C concentrations and oestrogen, thyroid hormone, exercise and alcohol use-factors increase HDL-C concentrations [23].

On the other hand, low density lipoprotein (LDL) cholesterol (LDL-C) has also been regarded to be strongly related to CVD incidence. High level of blood LDL-C induces the accumulation of oxidized cholesterols in the blood, which is the major risk factor of atherosclerosis. It is widely recognized that higher LDL-C and lower HDL-C levels are strongly linked to the increase in CVD risk. Therefore, much attention has been paid to food factors which can reduce LDL and total cholesterol (TC) levels and increase HDL levels. From this viewpoint, marine lipids, especially lipids rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been interested.

The protective ability of EPA and DHA on CVD has been made clear by the epidemiological studies on Greenland Eskimo [24-29]. After that, the detail mechanism of EPA and DHA on the protection for CVD has been exclusively studied from the nutritional, molecular biological, medical, and nutraceutical viewpoints.

EPA and DHA, two important n-3 long-chain polyunsaturated fatty acids (PUFA), play essential role in the health benefits including down-regulation of cholesterol

synthesis and up-regulation of cholesterol decomposition [30-32]. They are incorporated in many parts of the body and are thought to have anti-inflammatory and anti-oxidative effects [33]. A report has shown that the expression of 1040 genes was changed by EPA and DHA intake. Some of genes related with inflammatory and atherogenesis-related pathways were decreased, involved nuclear transcription factor κB signaling, hypoxia signaling, scavenger receptor activity, eicosanoid synthesis and adipogenesis [34]. Inflammatory markers such as interleukin 6 (IL-6) trigger C-reactive protein (CRP) are associated with the risk of the development of CVD [35]. The same study also showed a significant reduction in high-sensitivity CRP (66.7%, $P < 0.01$) by administration of EPA and DHA [35]. There are also many reports about the benefit effect of EPA and DHA on major coronary events and myocardial infarction [36-40], although the results are conflicting. What is more, it is thought that EPA and DHA play a role in atherosclerosis and peripheral arterial disease through improve plaque stability and vascular permeability and decrease endothelial activation [41]. Not only EPA and DHA, their metabolites has also been reported to be potent lipid mediators which are beneficial in the prevention or treatment of several diseases [42].

Squalene (SQ, 2,6,10,15,19,23‑hexamethyltetracosa‑2,6,10,14,18,22‑hexaene), a naturally occurring triterpenic hydrocarbon, is widespread in nature and recognized as marine lipids, because SQ is found to be very rich in shark liver oil. The shark *Centrophorus squamosus* has been reported to contain around 14% liver oil in which mainly is SQ (nearly 80% of the oil) [43]. Although shark liver oil remains the richest natural source of SQ, there is a limitation for its use due to persistent organic pollutants and shark resource protection [43]. On the other hand, SQ is also found in many vegetable oils in different concentrations. Among the vegetable oils, oil from *Amaranthus* sp. is known to have the highest concentration of SQ (up to 73.0 g/kg oil) [44; 45]. SQ content in olive oil is also high (5.64 g/kg oil) as compared with other kinds of general vegetable oils such as 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) [46]. Because of effects in both

in vitro and *in vivo* models, SQ as a functional food, nutritional supplement, or potential drug has attracted many attentions [47; 48]. For example, SQ has been proven to exert different biological actions on the immune system, act as potential therapeutic strategy for cancer, atherosclerosis protection effects, decrease hepatic steatosis, neuronal upkeep effects and enhance glucose-stimulated insulin secretion [49].

Here, the atherosclerosis protection effects of SQ will be introduced. SQ, an intermediary metabolite in cholesterol biosynthesis, possibility has been demonstrated as a biomarker to evaluate endogenous cholesterol biosynthesis [50-52]. What is more, SQ can also affect the lipoproteins, the transporter of cholesterol. In a study on C57Bl/6J SPF mice, SQ increased level of HDL-C for 60% [53]. This specific increase in HDL-C by SQ intake has been also found in three different types of mouse models at a dose of 1 g kg^{-1} (wild-type, ApoA-1- and ApoE-deficient) [54]. The protective role of SQ against atherosclerosis has also been shown in Apolipoprotein E (ApoE)-deficient mice [55]. Many studies have been carried out in several cell lines for understanding the molecule mechanism involved. The oxidized LDL uptake was inhibited by reducing CD36 expression in U937 monocytic cells and macrophages incubated with SQ [56]. SQ also significantly induced the mRNA expression of liver X receptors and their target genes (ABCA1, ABCG1, and ApoE) in RAW 246.7 murine macrophages, thus resulting in the removal of cholesterol from the cells [57]. These mechanisms could contribute to the protective role of SQ against atherosclerosis by stimulating reverse cholesterol transport.

Farnesol ($C_{15}H_{25}OH$), an acyclic sesquiterpene alcohol, is a constituent of essential oil derived from various plants such as citronella, lemon grass, tuberose, cyclamen, rose, neroli, balsam, and musk [58-62]. It is also an important intermediary metabolite in SQ biosynthesis. Therefore, in the course of the analysis of nutritional property of SQ, the interest in farnesol has been arisen. Farnesol has been reported to stimulate the immune system response and show anti-neoplastic effects in various human cancers

[63-72] through modulation of Ras protein and nuclear factor kappa-light-chain-enhancer of activated B cells activation which then down-regulate the expression of various inflammatory mediators such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNFα), and IL-6 [73-75]. Although some report has showed that farnesol exhibit beneficial effect on allergic asthma, diabetes, atherosclerosis, obesity, and hyperlipidemia [72; 76-78], the effect of farnesol on lipid metabolism and atherosclerosis are not clear yet.

In the present study, the effect of SQ on the lipid metabolism has been focused with special attention to cholesterol levels in the mouse models. In addition to SQ, the nutritional property of farnesol has also been analyzed together with that of sphingomyelin (SM).

It is widely recognized that cholesterol absorption influence fasting plasma lipids and risk for CVD [79]. Inflammation in adipose tissue [80] and liver [81; 82] in mouse models of obesity can be exacerbated by dietary cholesterol. Therefore, reduction of lipid absorption would be an important strategy to improve complications such as dyslipidemia, hepatic steatosis and low-grade inflammation. SM, an integral component of mammalian cell membranes and nerves has showed a dose-dependently reduce effect on the absorption of cholesterol, TAG and fatty acids in rodents [83-86]. Although SM has been shown repeatedly to inhibit lipid absorption, elevated levels of SM have also been identified as an independent risk factor for coronary heart disease. Lipoprotein lipase (LPL) is a major enzyme of lipoprotein metabolism. TAG on very low density lipoprotein (VLDL) and chylomicrons can be hydrolyzed by LPL [87; 88]. Reports have shown that SM inhibits the action of LPL [89; 90]. Another report showed that SM in lipoproteins delays remnant clearance by decreasing the binding of ApoE, an antiatherogenic protein, to cell membrane receptors [91]. What is more, the SM levels in plasma and in the aortic wall are also closely related to the development of atherosclerosis [92-96]. There is also a negative correlation between the SM content in HDL and lecithin:cholesterol acyltransferase (LCAT), an important enzyme

catalyzing cholesterol to its ester on HDL particle [97]. On the other hand, other reports indicate that high plasma SM level is not associated with an increased risk of coronary heart disease. Thus, further research is required to confirm the effect of SM on the lipids metabolism and CVD.

Under the background as described above, in **Chapter 1** and **Chapter 2**, we assessed the effect of SQ on lipid metabolism of animal models. For the animals, we used obese/diabetes KK-A^y mice and normal C57BL/6J mice. These mice have been widely used due its higher predisposition to atherosclerosis development.

In **Chapter 3**, we assessed the effect of farensol and SM on lipid metabolism of animal models. For the animals, we used obese/diabetes mice.

Chapter 1

Dietary Effect of Squalene on the Lipid Metabolism of Obese/diabetes KK-A^y Mice

1.1 Introduction

SQ, a naturally occurring triterpenic hydrocarbon, is widespread in nature, especially among shark liver oil and olive oil. Olive oil intake is known to show health beneficial effects [98-101] and these effects have been recognized to be, in part, derived from olive oil minor compounds, mainly phenolic compounds. In addition, due to the relatively high content in olive oil as compared with other vegetable oils, SQ has been also regarded as a contributing factor for the observations of reduced risk of diseases associated with olive oil intake. Up to date, SQ has been reported to show anticancer, anti-inflammatory, anti-oxidant, skin protection, liver protection, and neuroprotection activities [49; 55; 102; 103]. Especially, many studies have been conducted on the relationship between the reduced risk of cancer by olive oil intake and the role of SQ as vital dietary cancer chemopreventive agent [49; 55; 102-104].

Furthermore, the higher intake of olive oil in the Mediterranean countries than that in northern European countries has been related to the low incidence of CVD [55]. 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 [105]. Although the exact motive of atherosclerosis is still unknown, modification and deposition of lipids in the vascular wall can induce this event. Among the lipid deposition, LDL-C deposition, especially oxidized LDL, is regarded as a main cause. Thus, cholesterolemia is known as one of the major inducers 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 [55].

SQ is known as an important intermediate for the biosynthesis of phytosterol or cholesterol in plants/animals and humans [49] and its endogenous synthesis begins

with the conversion of acetyl coenzyme A to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), followed by reduction of HMG-CoA to mevalonate, mediated by HMG-CoA reductase. Thus, the involvement of SQ in the cholesterol synthesis is easily expected and the possibility has been demonstrated for the use of SQ as a biomarker to evaluate endogenous cholesterol biosynthesis. Indeed, numerous studies from this viewpoint have explored this possibility using several experimental approaches in both animals and humans [49].

There have been also many studies on the effect of dietary SQ on blood cholesterol levels. Several researchers have demonstrated that dietary SQ can inhibit the activity of HMG-CoA reductase, due to a negative feedback regulation of endogenous cholesterol synthesis [103; 104]. 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, controversial has been the effect of SQ on plasma lipids in humans and animals. Some authors have reported the decrease in plasma cholesterol levels in human studies by SQ intake, while other researchers reported the opposite effect [43; 55; 106; 107]. The different effect of SQ has been considered to be due to the difference in the experimental approaches and sexes [54; 55; 106].

The controversial effect of SQ on the blood cholesterol level has been also found in animal models. Several researchers have reported the reduction of blood lipid levels including cholesterol of rats after SQ intake [108-110], while other researchers have found the increase in the cholesterol levels in normal animals by SQ feeding [111; 112]. In addition, Chmelik et al. [53] found the increase in HDL-C levels of C57Bl/6J SPF mice fed SQ, while the TC and LDL-C levels were decrease by the SQ feeding. This specific increase in HDL-C by SQ intake has been also found in three different types of mouse models (wild-type, ApoA-1- and ApoE-deficient) [54].

Many animal and human studies have revealed SQ as a promising agent in CVD prevention [49; 55; 106]. The effect of SQ has been explained on the several different mechanisms including elimination of cholesterol as fecal bile acids [107], inhibition of HMG-CoA reductase by dietary SQ due to negative feed-back regulation [113], inhibition of oxidized LDL uptake by macrophages [56], stimulating reverse cholesterol transport [57], inhibition of isoprenaline-induced lipid peroxidation [108], and attenuation of homocysteine-induced endothelial dysfunction [49]. These mechanisms are basically dependent on the antioxidant activity of SQ and the involvement of SQ in cholesterol metabolism [43; 49; 55; 106].

However, SQ's role on plasma lipids is not clear yet, although hyperlipidemia, especially hypocholesterolemia, has been regarded as major risk factor for CVD. In the present study, the effect of SQ on plasma lipid level has been analyzed by using animal models. For the animals, we used C57BL/6J genetic background mice. These mice have been widely used due its higher predisposition to atherosclerosis development [114]. Furthermore, the comparison has been done on the effect of SQ on the obese/diabetes (**Chapter 1**) and normal (**Chapter 2**) C57BL/6J genetic background mice. Obesity and diabetes are a major risk factor for CVD [115; 116]. Therefore, the comparison may provide useful information to know the effect of SQ on the reduction of CVD risk.

1.2 Material and Methods

1.2.1 Material

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

analytical grade.

1.2.2 Animals and diets

Obese/diabetic KK-A^y mice (male, four weeks old) were obtained from Japan CREA Co., Tokyo, Japan. The mice were housed individually in an air-conditioned room (23±1℃ 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 5 groups of seven and were then fed experimental diets (**Table 1-1**) for four weeks. The body weight, diet and water intake of each mouse was recorded daily. Mice were sacrificed under diethyl ether anesthesia after 12 h fasting on day 28. Body weights after 12 h fasting on day 28 were determined.

The composition of fatty acid (FA) in the dietary lipids was shown in **Table 1-2**. After extraction of the lipids from each diet with chloroform/methanol (2:1, v/v), the fatty acyl groups in the lipids were converted to their methyl esters. And then, the FA composition was determined by gas chromatography (GC). The fatty acid methyl esters (FAME) were prepared according to the method of Prevot and Mordret [117]. 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 ℃ for 30 min. After the 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 x 0.32 mm i.d.); Supelco, Bellefonte, PA]. The injection port and flame ionization detector were set at 250 and 260 ℃, respectively, and the column temperature was held at 200 ℃. The carrier gas was helium at a flow rate of 50 kPa. FA content in the lipid samples was expressed as a weighted percentage of the total fatty acids. Each FA level of the liver tissue (1 g) was

calculated by comparing the peak ratio to that of the internal standard (17:0) and the total lipid content. And the composition of FA in the lipid samples was also determined which was expressed as percentage of total FA.

1.2.3 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 carried out under the approval by the Ethical Committee of Experimental Animal Care at Hokkaido University.

1.2.4 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 part 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, the Glutest Neo Sensor (Sanwa Kagaku Kenkyusyo Co. Ltd., Aichi, Japan). This sensor is an amperometric sensor with flavin adenine dinucleotide-dependent glucose dehydrogenase and $Fe(CN)_6^{3-}$. Liver, spleen, small 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 ℃ for lipid analysis. The parts of livers 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 (qRT-PCR) analysis.

1.2.5 Blood lipid analysis

The blood plasma analysis was performed by the Analytical Center of Hakodate

Medical Association (Hakodate, Japan). The analysis included the measurement of the following parameters: TC, HDL-C, non-HDL-C, TAG and phospholipids (PL).

1.2.6 Hepatic lipid analysis

Total lipids (TL) was extracted from the liver with chloroform/methanol (2:1, v/v) [118]. 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 PL 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. TC and TAG were measured using enzymatic kits (Cholesterol E-test and Triglyceride E-test) obtained from Wako Pure Chemical Industries, Osaka, Japan.

The FA composition of the TL was determined by GC after conversion of fatty acyl groups in the lipid to their methyl esters. The FAME was prepared as described as above.

In a preliminary experiment, we have found that SQ and DHA could not be separated clearly on the chromatogram when using the Omegawax-320 capillary column. Therefore, FAME sample prepared was submitted to thin layer chromatography (TLC) for the removal of 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) developing with hexane-diethyl ether-acetic acid (80:20:1, v/v/v).

1.2.7 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 then synthesized from total RNA using High-Capacity cDNA Reverse Transcription

Kits (Applied Biosystems Japan Ltd., Tokyo, Japan). Quantitative real-time PCR (qRT-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), and 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.

1.2.8 HMG-CoA reductase analysis

Activity of HMG-CoA reductase, a key enzyme of hepatic cholesterol synthesis, was measured according to the procedure as described by Rao and Ramakrlshnan (1975) [119]. This procedure is based on the formation of hydroxymate from the reaction of HMG-CoA and mevalonate with hydroxylamine. Resulted hydroxymate can be quantitatively measured by the colorimetric assay. The measurements of HMG-CoA and mevalonate concentration in the liver homogenate were done separately by

changing pH to avoid the 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 2.1 for mevalonate, respectively. HMG-CoA reductase activity was calculated from the ratio of mevalonate concentration to HMG-CoA concentration.

1.2.9 Statistic analysis

Results are expressed as the mean ± SEM. Statistical significances between groups were evaluated by one-way ANOVA with *post hoc* comparisons (Šidák correction). Differences with $P < 0.05$ were considered statistically significant.

1.3 Results

1.3.1 Initial weight, weight gain, final body weight on day 28 and water intake.

As shown in **Figure 1-1,** no difference was observed in initial body weights of mice. On the other hand, 2% SQ diet induced significant increase in the final body weight compared to linseed oil group (**Figure 1-2**). Although a same increasing tendency in the body weight was observed in 1% SQ group, no significant difference was detected. Significant increase in the weight gain was found in both 1% and 2% SQ group as compared with other groups (**Figure 1-3**). As shown in **Figure 1-4,** significant decrease in total water intake were observed in 1% SQ group as compared with soybean and fish oil groups.

1.3.2 Blood glucose levels and plasma lipid parameters.

The blood glucose was not significantly different between the groups (**Figure 1-5**). Although a tendency in the decrease in plasma non-HDL-C was found in KK-A^y mice

administrated with SQ (1% and 2%), other lipid parameters increased in SQ groups as compared with other groups (**Table 1-3**). The decrease in the plasma non-HDL-C was significantly different from that of mice fed soybean oil (**Table 1-3**). Administration of 2% SQ significantly increased plasma HDL-C as compared to soybean oil group, linseed oil group and fish oil group. Although a significant difference in plasma HDL-C was also observed among 1% SQ group and soybean oil and fish oil groups, no significance was observed between 1% SQ group and linseed oil group (**Table 1-3**). On the other hand, no significant difference was detected between the groups in other lipid parameters (such as TAG, PL and TC).

1.3.3 Tissue weights

The WAT weight and weight of major tissues were not significantly different between the different groups, except for a significant increase in the liver weight of KK-A^y mice fed SQ (1% and 2%) (**Table 1-4, Table 1-5, Table 1-6 and Table 1-7**).

1.3.4 Hepatic lipid parameters

SQ intake significantly increased TL, NL and TAG in the liver of KK-A^y mice (**Figure 1-6, Figure 1-7 and Figure 1-9**), while no significance was observed in the hepatic PL and TC levels (**Figure 1-8 and Figure 1-10**). When the FAME sample prepared from liver lipids was submitted to TLC for removal of SQ before GC analysis the hepatic SQ content was also determined. The content was 16.30 mg/g liver and 11.50 mg/g liver in the mice fed 1% SQ and 2% SQ, respectively (**Figure 1-11**).

1.3.5 Fatty acid levels of liver lipids

In this experiment, the composition ratio of FA in hepatic TL, the content of FA in hepatic TL, NL and PL of KK-A^y mice were determined. The composition ratio of FA in hepatic TL was shown in **Table 1-8**. SQ (1% and 2%) supplementation

significantly decreased the composition ratio of 18:0, 20:4n-6 and 22:6n-3 (DHA) in the liver of mice compared to the other 3 groups. The composition of saturated fatty acid (SFA) was also decreased in the liver of mice by SQ feeding, which probably as a result of the decrease of 18:0.

The content of fatty acids in hepatic TL, NL and PL were shown in **Table 1-9, Table 1-10 and Table 1-11.** SQ (1% and 2%) supplementation significantly increased each fatty acid contents in TL of the liver from KK-A^y mice except for C20:4n-6, resulting in the significant increase in the total fatty acid contents (**Table 1-9**). The increase in the total fatty acids presented in **Table 1-9** was consistent with the result in **Figure 1-6** showing the increasing effect of the SQ on liver TL.

The contents of SFA, monounsaturated fatty acid (MUFA) and PUFA in NL were significantly higher in SQ supplementation groups (1% and 2%) (**Table 1-10**), probably as a result of increment of liver TAG and NL. However, FA in PL was affected slightly or was not affected by SQ supplementation compared to the linseed oil group (**Table 1-11**).

1.3.6 Gene expressions 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 by qRT-PCR (**Figure 1-12**). Compared to linseed oil group, 1% SQ supplementation significantly reduced HMGCR mRNA expression. CYP7A1 gene encoding cholesterol 7 alpha-hydroxylase was also reduced in both SQ supplementation groups, and a significant difference was observed with fish oil group. However, the reduced CYP7A1 mRNA expression did not reach significance when comparing with linseed oil group. Administration of linseed oil increased CYP51 mRNA expression significantly comparing with soybean oil group and fish oil group.

There were no other differences observed between the groups in gene expressions related to cholesterol synthesis and metabolism (SREBP-2 and FDFT1). No significant difference was also detected between the groups in gene expressions related to TAG (LXR, ChREBP, LPIN1, DGAT1 and DGAT2) and FA (SCD1 and FASN) biosynthesis.

Furthermore, 2% SQ supplementation significantly decreased HMG-CoA reductase activity in the liver from KK-A^y mice (**Figure 1-13**).

Table 1-1 Composition of experimental diets (g kg^{-1})

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

Table 1-2 Fatty acid composition of dietary lipids (%)

	Soybean oil	Linseed oil	1% SQ	2% SQ	Fish oil
14:0	0.95±0.03	0.96±0.02	0.96±0.02	0.94±0.01	1.31±0.03
16:0	19.79±0.05	17.78±0.14	17.88±0.14	17.91±0.20	19.88±0.14
16:1	1.68±0.05	1.74±0.04	1.73±0.03	1.71±0.03	1.95±0.04
18:0	10.70±0.06	10.56±0.05	10.53±0.05	10.50±0.05	10.78±0.11
18:1n-9	35.02±0.16	35.09±0.19	35.21±0.18	35.33±0.19	33.45±0.28
18:2n-6	23.74±0.02	11.02±0.05	11.00±0.04	10.98±0.05	18.93±0.08
20:4n-6	ND	ND	ND	ND	0.29±0.30
18:3n-3	2.46±0.06	16.83±0.04	16.81±0.03	16.81±0.05	1.97±0.04
20:5n-3	ND	ND	ND	ND	1.45±0.03
22:6n-3	ND	ND	ND	ND	2.73±0.05
Total FA	95.46±0.09	95.09±0.10	95.19±0.13	95.24±0.16	94.56±0.18
SFA	31.44±0.05	29.30±0.10	29.37±0.09	29.36±0.13	31.97±0.11
MUFA	37.32±0.10	37.47±0.14	37.56±0.13	37.65±0.14	36.17±0.22
PUFA n-6	23.74±0.02	11.02±0.05	11.00±0.04	10.98±0.05	19.23±0.08
PUFA n-3	2.46±0.06	16.83±0.04	16.82±0.03	16.81±0.05	6.32±0.12
n-6/n-3	9.66±0.24	0.66±0.00	0.66±0.00	0.65±0.00	3.04±0.07

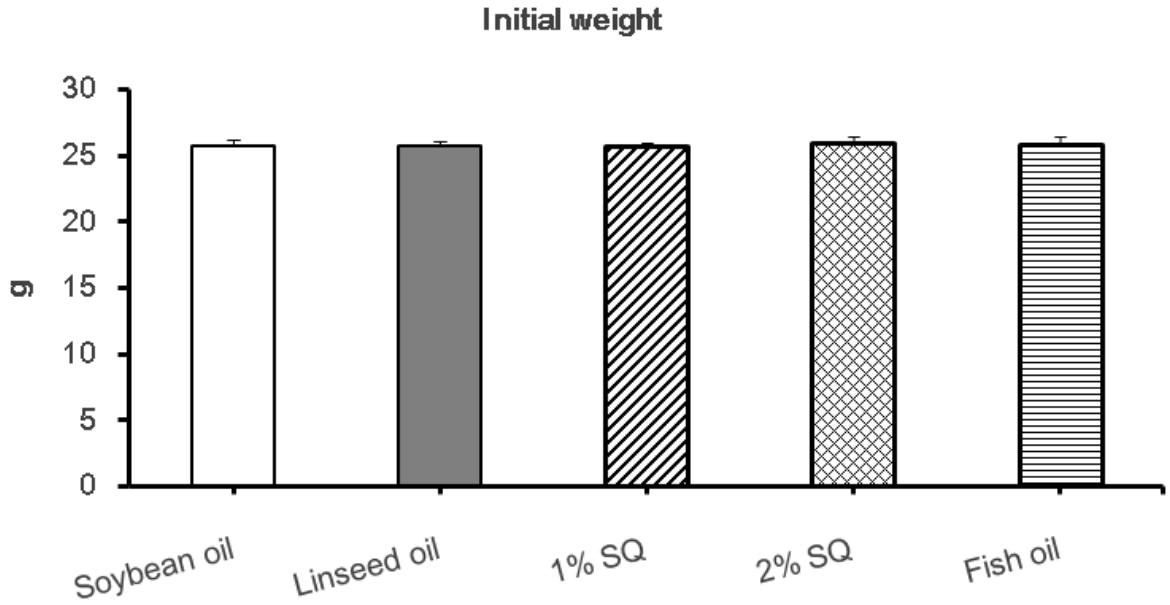

Figure 1-1 Initial weight of KK-A^y mice.

Initial weight of KK-A^y mice was determined after 12h fasting. Values are expressed as mean ± SEM (n=7).

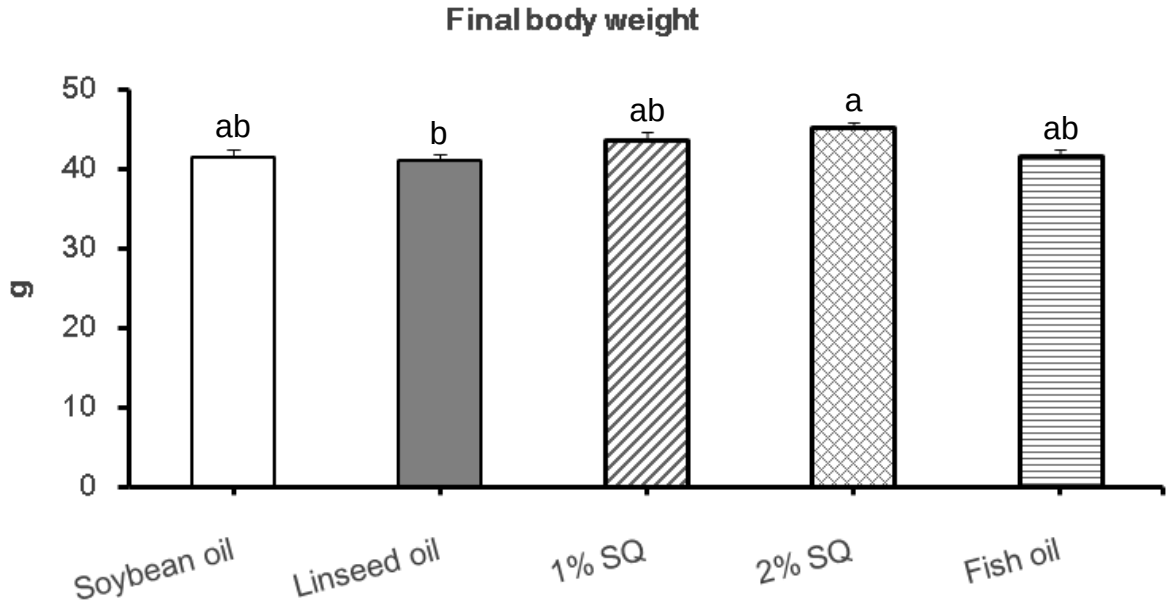

Figure 1-2 Final body weight of KK-A^y mice.

Final body weight of KK-A^y mice on day 28 was determined after 12h fasting. Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at $P < 0.05$ among each value.

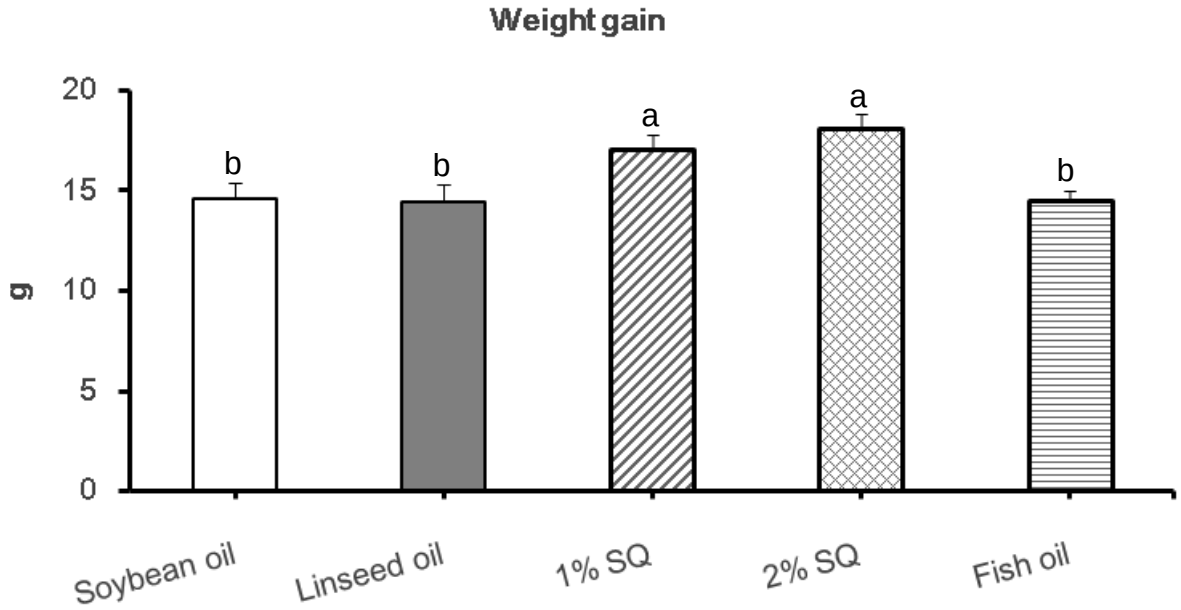

Figure 1-3 Weight gain of KK-A^y mice on day 28.

Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at P< 0.05 among each value.

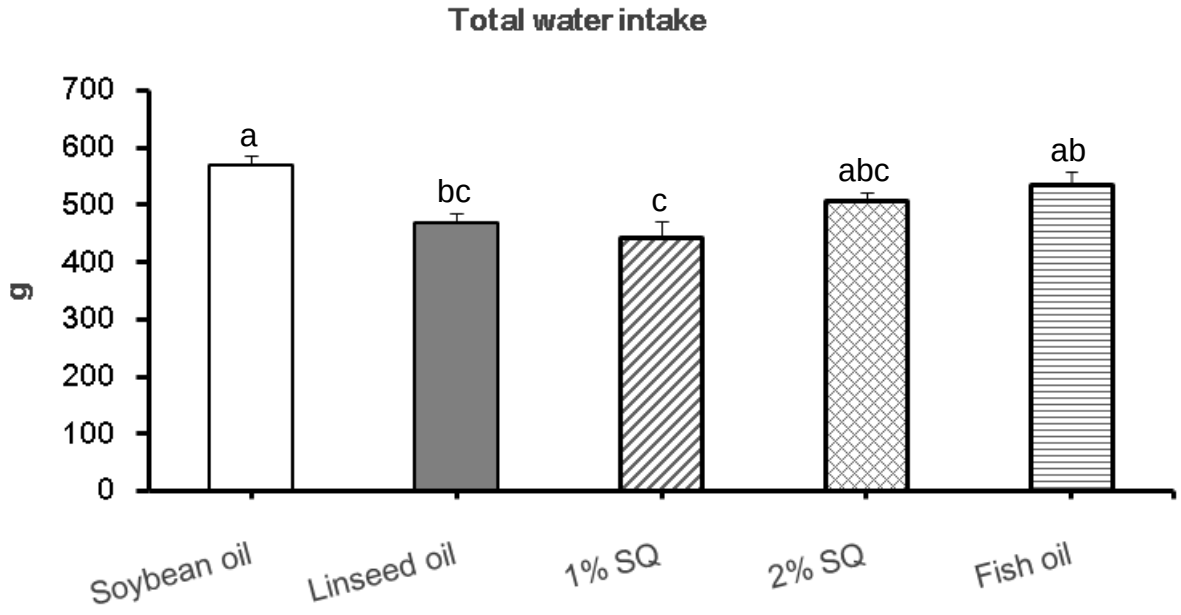

Figure 1-4 Total water intake of KK-A^y mice for 28 days feeding.

Water intake of each mouse was recorded daily. Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at P< 0.05 among each value.

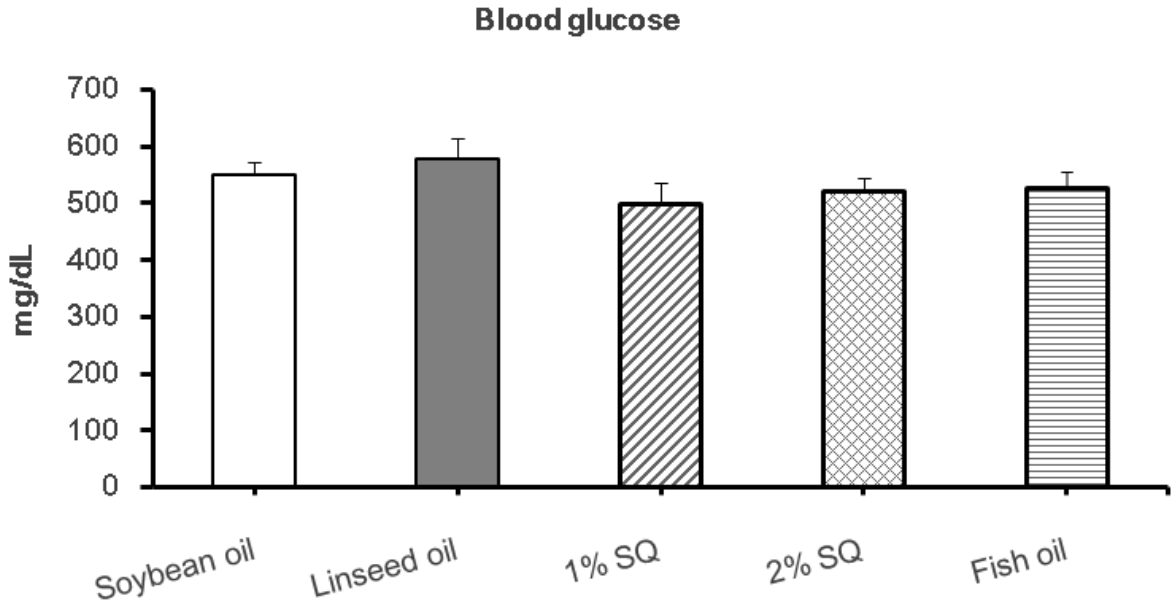

Figure 1-5 Effect of SQ on blood glucose level of KK-A^y mice.

Blood glucose was measured using a blood glucose monitor after 12 h fasting. Values are expressed as mean ± SEM (n=7).

Table 1-3 Effect of SQ on plasma lipid parameters of KK-A^y mice.

		TAG (mg/dL)	PL (mg/dL)	TC (mg/dL)	HDL-C (mg/dL)	NonHDL-C (mg/dL)
KK-A^y mice	Soybean oil	420.57±83.62	387.71±19.32	198.43±7.19	126.00±3.64[c]	72.43±4.74[a]
	Linseed oil	398.83±27.61	365.00±15.94	186.14±6.22	130.14±6.44[bc]	56.00±3.69[ab]
	1% SQ	471.14±54.78	377.29±9.52	197.57±4.79	152.86±6.19[ab]	44.71±4.51[b]
	2% SQ	375.43±87.51	376.00±15.33	200.71±9.20	155.43±5.23[a]	46.71±4.93[b]
	Fish oil	248.43±66.98	344.43±15.41	182.83±6.28	127.57±5.08[c]	56.71±2.51[ab]

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 1-4 Effect of SQ on absolute WAT weight of KK-A^y mice.

		WAT				
		Mesenteric	Epididymal	Perirenal	Inguinal	Total
		g				
KK-A^y mice	Soybean oil	1.03±0.05	1.84±0.08	0.78±0.03	1.49±0.10	5.15±0.23
	Linseed oil	1.01±0.03	1.78±0.09	0.74±0.06	1.33±0.05	4.86±0.19
	1% SQ	1.08±0.06	1.73±0.08	0.71±0.03	1.52±0.10	5.04±0.22
	2% SQ	1.18±0.03	1.86±0.07	0.84±0.04	1.67±0.13	5.55±0.14
	Fish oil	0.97±0.06	1.70±0.07	0.73±0.04	1.34±0.07	4.75±0.17

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at P< 0.05 among each value.

Table 1-5 Effect of SQ on absolute tissue weight of KK-A^y mice.

		Liver	Spleen	BAT	Small intestine	Pancreas	Kidney	Muscle	Heart	Brain
		g								
KK-A^y mice	Soybean oil	2.45±0.11^b	0.09±0.01	0.21±0.02	1.49±0.06	0.31±0.01	0.68±0.02	0.25±0.01	0.15±0.01	0.37±0.01
	Linseed oil	2.56±0.12^b	0.11±0.01	0.19±0.01	1.43±0.04	0.29±0.01	0.74±0.03	0.26±0.01	0.14±0.00	0.36±0.01
	1% SQ	3.97±0.15^a	0.10±0.01	0.19±0.01	1.41±0.06	0.28±0.02	0.72±0.03	0.25±0.01	0.15±0.00	0.36±0.01
	2% SQ	3.68±0.10^a	0.11±0.01	0.20±0.01	1.53±0.04	0.31±0.01	0.70±0.01	0.27±0.01	0.15±0.01	0.33±0.03
	Fish oil	2.60±0.10^b	0.12±0.01	0.21±0.01	1.52±0.05	0.30±0.01	0.72±0.04	0.26±0.01	0.15±0.00	0.38±0.02

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at P< 0.05 among each value.

Table 1-6 Effect of SQ on relative WAT weight of KK-A^y mice.

		WAT				
		Mesenteric	Epididymal	Perirenal	Inguinal	Total
		g per 100g body weight				
KK-A^y mice	Soybean oil	2.54±0.07	4.53±0.14	1.93±0.05	3.67±0.18	12.66±0.29
	Linseed oil	2.46±0.05	4.33±0.15	1.79±0.14	3.24±0.11	11.82±0.32
	1% SQ	2.46±0.10	3.96±0.16	1.62±0.07	3.46±0.20	11.50±0.38
	2% SQ	2.61±0.05	4.11±0.16	1.86±0.09	3.70±0.30	12.28±0.33
	Fish oil	2.33±0.10	4.09±0.16	1.75±0.08	3.21±0.13	11.38±.026

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at P< 0.05 among each value.

Table 1-7 Effect of SQ on relative tissue weight of KK-A^y mice.

		Liver	Spleen	BAT	Small intestine	Pancreas	Kidney	Muscle	Heart	Brain
		g per 100g body weight								
KK-A^y mice	Soybean oil	6.03±0.12^c	0.22±0.04	0.53±0.03	3.69±0.15	0.75±0.03	1.68±0.04	0.61±0.01	0.36±0.01	0.93±0.05
	Linseed oil	6.21±0.19^c	0.26±0.03	0.45±0.03	3.48±0.07	0.69±0.02	1.80±0.09	0.63±0.01	0.35±0.01	0.89±0.03
	1% SQ	9.07±0.21^a	0.22±0.02	0.43±0.02	3.23±0.12	0.64±0.02	1.64±0.08	0.57±0.02	0.33±0.01	0.81±0.03
	2% SQ	8.07±0.31^b	0.24±0.01	0.43±0.02	3.40±0.13	0.68±0.02	1.56±0.03	0.59±0.02	0.34±0.01	0.74±0.05
	Fish oil	6.23±0.18^c	0.28±0.02	0.51±0.02	3.66±0.10	0.71±0.02	1.72±0.11	0.63±0.02	0.35±0.03	0.91±0.04

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at P< 0.05 among each value.

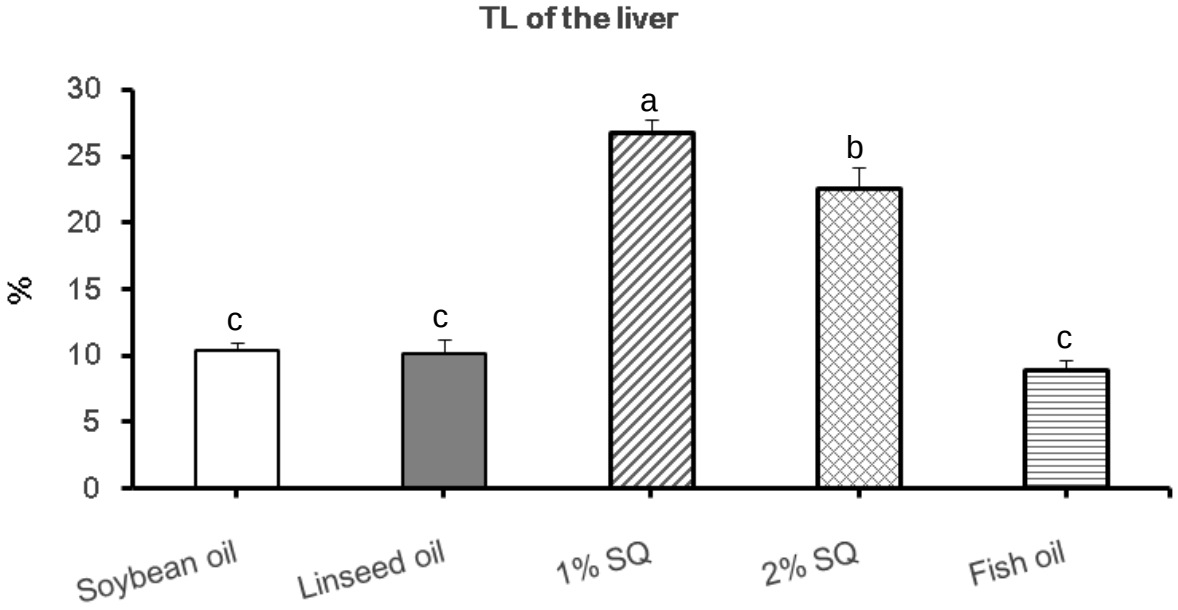

Figure 1-6 Effect of SQ on the hepatic TL content of KK-A^y mice.

TL was extracted from the liver with chloroform/methanol (2:1, v/v). Values are expressed as mean ± SE (n=7). Different letters indicate significant difference at P< 0.05 among each value.

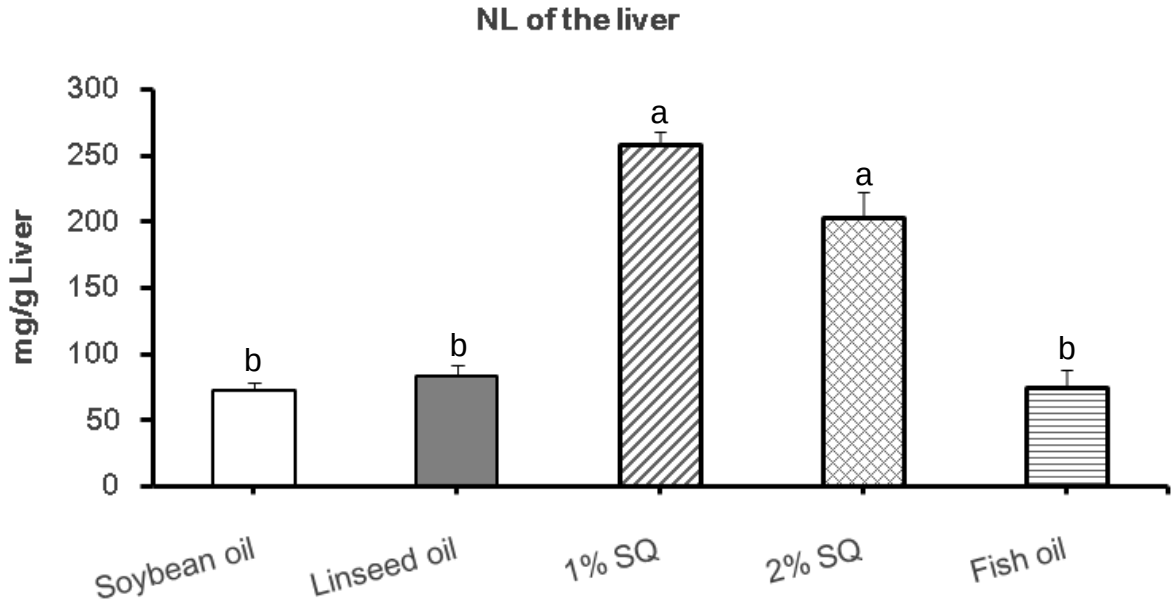

Figure 1-7 Effect of SQ on the hepatic NL content of KK-A^y mice.

The TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridge by elution with chloroform and methanol. The NL fraction was eluted with 70 mL chloroform. Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at $P < 0.05$ among each value.

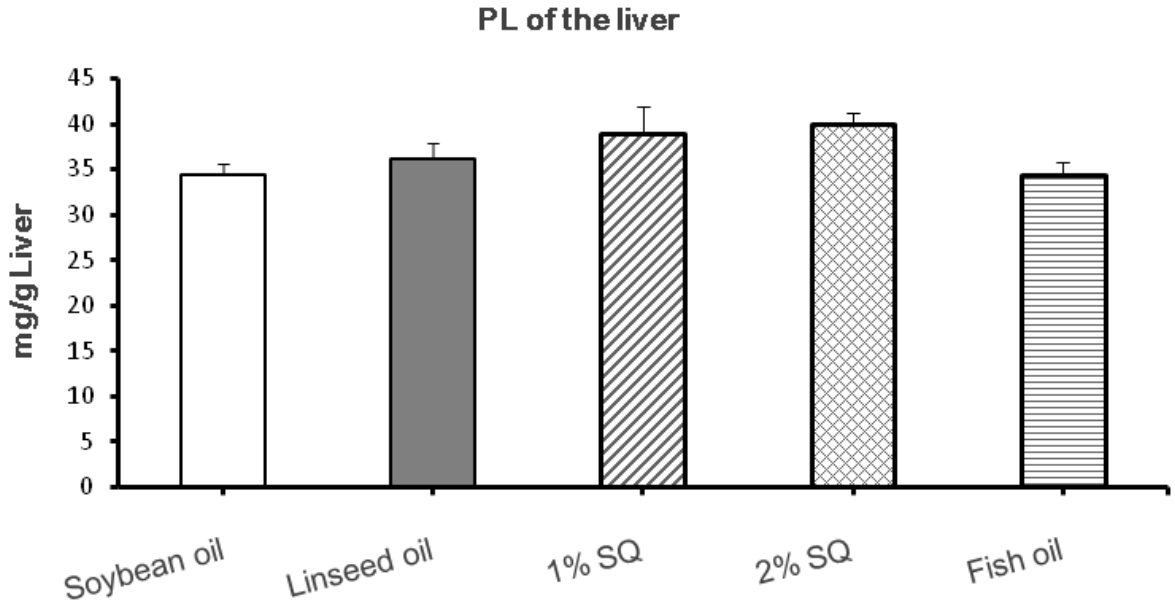

Figure 1-8 Effect of SQ on the hepatic PL content of KK-A^y mice.

The TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridgeby elution with chloroform and methanol. The PL fraction was eluted with 50 mL methanol. Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at P< 0.05 among each value.

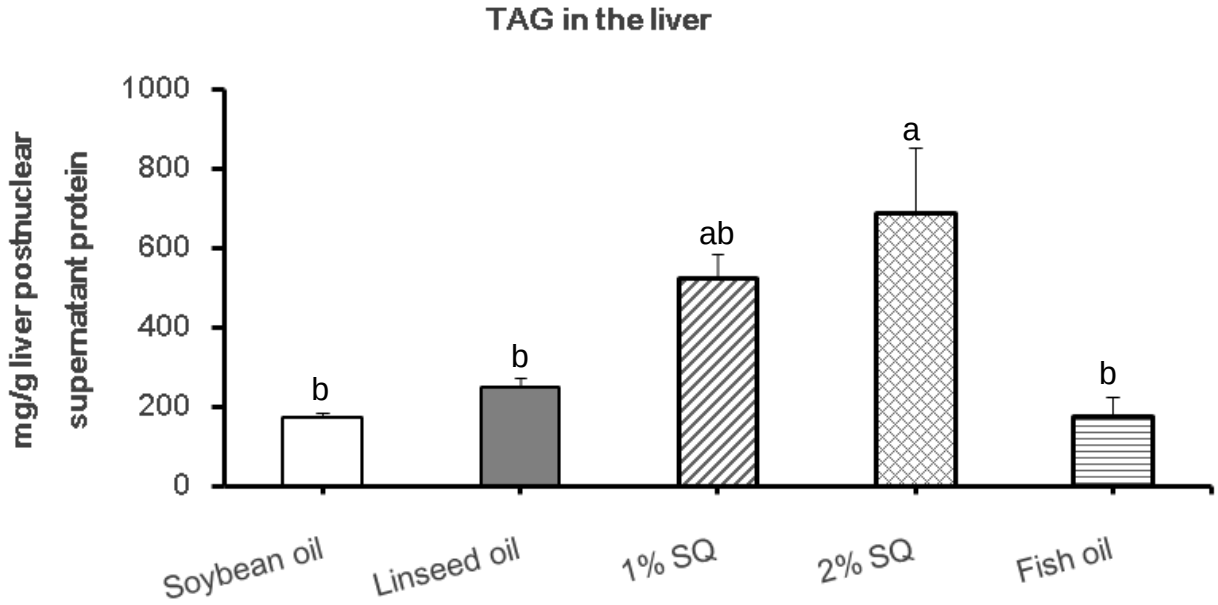

Figure 1-9 Effect of SQ on the hepatic TAG content of KK-A^y mice.

TAG was measured using enzymatic kits (Triglyceride E-test, Wako Pure Chemical Industries, Osaka, Japan). Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at $P < 0.05$ among each value.

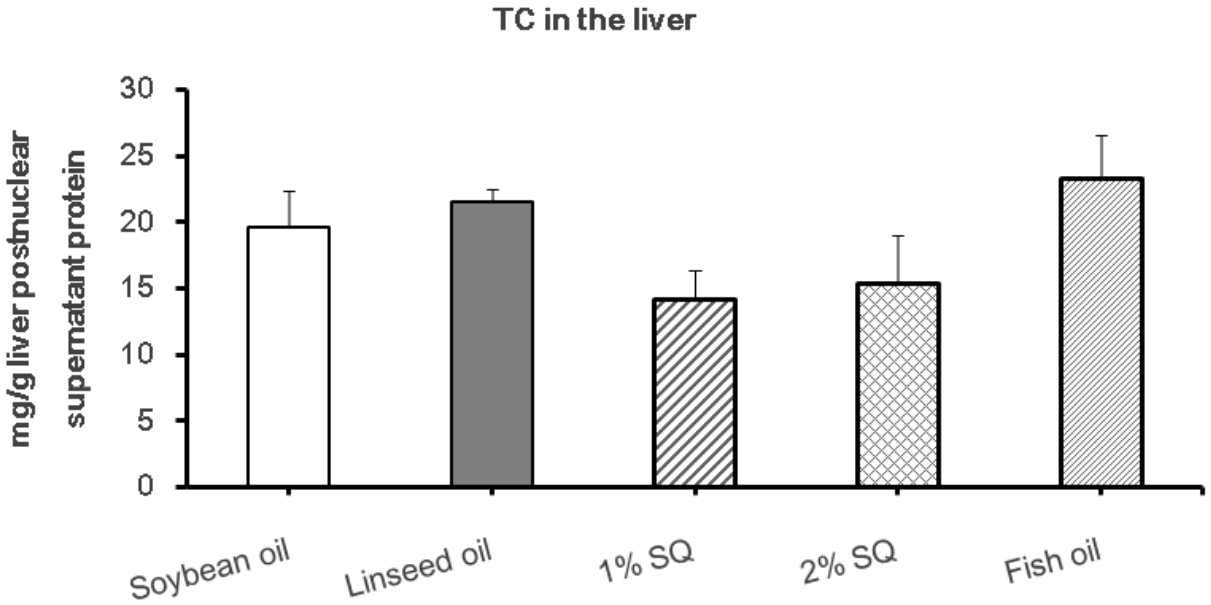

Figure 1-10 Effect of SQ on the hepatic TC content of KK-A^y mice.

TC was measured using enzymatic kits (Cholesterol E-test, Wako Pure Chemical Industries, Osaka, Japan). Values are expressed as mean ± SEM (n=7).

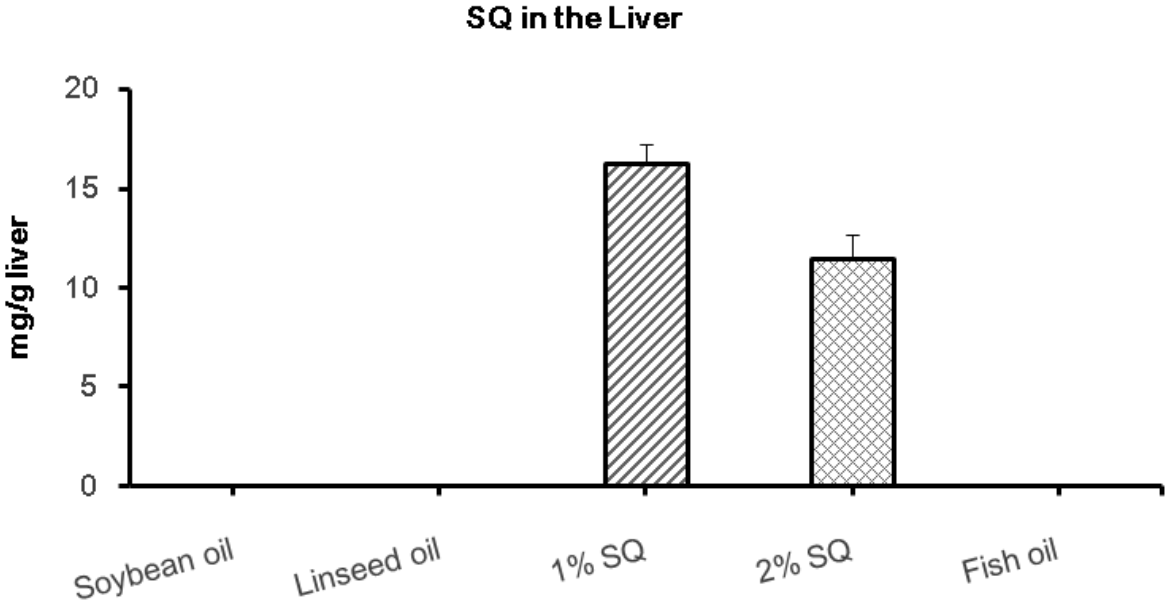

Figure 1-11 Content of SQ in the liver of KK-A^y mice.

Values are expressed as mean ± SEM (n=7).

Table 1-8 Effect of SQ on the fatty acids composition ratio in hepatic TL of KK-A^y mice (%).

	Soybean oil	Linseed oil	1% SQ	2% SQ	Fish oil
16:0	19.29±0.41	19.90±0.63	19.84±0.13	20.54±0.47	20.72±0.12
18:0	6.93±0.31 [a]	6.13±0.48 [a]	3.94±0.21 [b]	4.38±0.29 [b]	7.06±0.41 [a]
20:0	0.63±0.05	0.57±0.03	0.72±0.06	0.68±0.06	0.66±0.03
16:1	1.21±0.17 [b]	1.92±0.12 [a]	1.63±0.19 [ab]	1.71±0.12 [ab]	1.04±0.11 [b]
18:1n-9	32.71±0.62 [c]	36.95±0.82 [b]	42.06±1.35 [a]	40.74±1.27 [ab]	29.07±0.73 [c]
20:1n-9	1.22±0.03 [c]	1.19±0.06 [bc]	1.48±0.06 [a]	1.36±0.06 [ab]	1.01±0.03 [c]
18:2n-6	21.37±0.63	12.22±0.28 [b]	12.25±0.69 [b]	12.14±0.45 [b]	19.54±0.60 [a]
20:4n-6	4.79±0.16 [a]	2.32±0.19 [c]	0.98±0.06 [d]	1.25±0.09 [d]	3.42±0.30 [b]
18:3n-3	0.87±0.03 [c]	6.37±0.39 [b]	7.46±0.46 [a]	7.28±0.27 [ab]	0.89±0.04 [c]
20:5n-3	0.20±0.01 [c]	1.52±0.07 [a]	0.76±0.06 [b]	0.88±0.06 [b]	1.81±0.10 [a]
22:6n-3	2.82±0.08 [b]	2.53±0.18 [b]	1.40±0.13 [c]	1.60±0.11 [c]	7.56±0.26 [a]
Total FA	91.65±0.22	91.29±0.30	92.07±0.22	92.15±0.33	92.36±0.17
SFA	26.45±0.29 [ab]	26.28±0.96 [ab]	24.05±0.26 [c]	25.19±0.52 [bc]	28.02±0.46 [a]
MUFA	35.14±0.74 [bc]	40.05±0.88 [ab]	45.17±1.55 [a]	43.80±1.35 [a]	31.12±0.67 [c]
PUFA n-6	26.16±0.63 [a]	14.54±0.34 [c]	13.23±0.75 [c]	13.39±0.54 [c]	22.95±0.46 [b]
PUFA n-3	3.90±0.10 [b]	10.42±0.30 [a]	9.62±0.62 [a]	9.76±0.37 [a]	10.27±0.33 [a]
n-6/n-3	6.72±0.08 [a]	1.39±0.03 [c]	1.39±0.06 [c]	1.37±0.02 [c]	2.25±0.10 [b]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 1-9 Effect of SQ on the fatty acids content in hepatic TL of KK-A^y mice (mg/g liver).

	Soybean oil	Linseed oil	1% SQ	2% SQ	Fish oil
16:0	13.88±1.04 [c]	14.35±1.08 [c]	39.16±1.49 [a]	33.89±2.61 [b]	13.48±1.13 [c]
16:1	0.86±0.13 [b]	1.27±0.20 [b]	3.15±0.50 [a]	2.67±0.35 [a]	0.69±0.06 [b]
18:0	4.49±0.23 [c]	4.64±0.17 [c]	8.05±0.21 [a]	7.02±0.21 [b]	4.58±0.14 [c]
18:1n-9	23.01±1.93 [c]	23.99±2.68 [c]	79.37±5.74 [a]	64.62±7.67 [b]	18.91±1.90 [c]
20:1n-9	0.82±0.07 [c]	0.74±0.09 [c]	2.60±0.21 [a]	1.98±0.21 [b]	0.62±0.04 [c]
20:0	0.42±0.04 [c]	0.39±0.03 [c]	1.38±0.07 [a]	1.08±0.07 [b]	0.40±0.03 [c]
18:2n-6	15.44±1.19 [c]	8.95±0.94 [d]	24.66±0.57 [a]	19.12±1.39 [b]	12.53±1.22 [c]
20:4n-6	3.09±0.16 [a]	1.85±0.07 [b]	1.98±0.06 [b]	1.91±0.10 [b]	2.13±0.05 [b]
18:3n-3	0.65±0.06 [d]	4.70±0.51 [c]	16.30±0.88 [a]	11.93±0.64 [b]	0.59±0.06 [d]
20:5n-3	0.13±0.01 [d]	1.26±0.06 [c]	1.67±0.10 [a]	1.43±0.08 [b]	1.14±0.05 [c]
22:6n-3	1.80±0.18 [c]	2.22±0.16 [c]	3.32±0.25 [b]	3.82±0.19 [b]	4.76±0.22 [a]
Total FA	64.60±4.49 [c]	64.37±5.31 [c]	181.63±6.75 [a]	148.83±12.14 [b]	59.81±4.66 [c]
SFA	18.79±1.21 [c]	19.39±1.18 [c]	48.58±1.47 [a]	41.99±2.74 [b]	18.46±1.25 [c]
MUFA	24.69±2.06 [c]	26.00±2.96 [c]	85.12±6.42 [a]	69.27±8.15 [b]	20.21±1.98 [c]
PUFA n-6	18.54±1.30 [b]	10.80±0.77 [d]	26.63±0.61 [a]	21.03±1.48 [b]	14.66±1.23 [c]
PUFA n-3	2.58±0.24 [d]	8.19±0.59 [c]	21.29±1.11 [a]	16.55±0.67 [b]	6.49±0.32 [c]
n-6/n-3	7.40±0.66 [a]	1.32±0.02 [b]	1.26±0.05 [b]	1.27±0.09 [b]	2.26±0.13 [b]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 1-10 Effect of SQ on the fatty acids content in hepatic NL of KK-A^y mice (mg/g liver).

	Soybean oil	Linseed oil	1% SQ	2% SQ	Fish oil
16:0	11.97±0.83 [c]	10.95±0.84 [c]	41.73±0.78 [a]	32.76±2.37 [b]	11.03±1.82 [c]
16:1	0.84±0.06 [b]	1.14±0.16 [b]	3.62±0.45 [a]	3.13±0.46 [a]	0.73±0.06 [b]
18:0	1.58±0.14 [c]	1.13±0.05 [c]	5.02±0.16 [a]	3.79±0.26 [b]	1.20±0.28 [c]
18:1n-9	25.77±1.94 [c]	23.61±1.73 [c]	95.50±3.15 [a]	71.29±6.27 [b]	20.88±3.43 [c]
20:1n-9	0.96±0.07 [c]	0.73±0.07 [c]	3.10±0.14 [a]	2.46±0.22 [b]	0.72±0.13 [c]
20:0	0.46±0.04 [c]	0.34±0.03 [c]	1.55±0.19 [a]	1.27±0.05 [b]	0.42±0.11 [c]
18:2n-6	14.52±1.06 [bc]	5.99±0.40 [d]	26.01±2.09 [a]	18.83±0.75 [b]	10.68±2.40 [c]
20:4n-6	0.49±0.03 [a]	0.15±0.01 [a]	0.35±0.05 [b]	0.28±0.02 [b]	0.29±0.05 [b]
18:3n-3	0.72±0.06 [d]	4.35±0.41 [c]	20.23±1.09 [a]	13.76±0.49 [b]	0.66±0.13 [d]
20:5n-3	0.09±0.01 [d]	0.39±0.04 [c]	1.39±0.10 [a]	0.91±0.07 [b]	0.63±0.15 [c]
22:5n-3	0.14±0.01 [d]	0.36±0.05 [cd]	1.52±0.15 [a]	1.04±0.12 [b]	0.52±0.14 [c]
22:6n-3	0.46±0.05 [b]	0.46±0.07 [b]	2.13±0.13 [a]	1.17±0.08 [b]	2.45±0.64 [a]
Total FA	58.70±4.13 [c]	50.17±3.34 [c]	204.57±3.40 [a]	152.57±9.61 [b]	50.78±9.42 [c]
SFA	14.27±0.96 [c]	12.65±0.87 [c]	49.15±1.10 [a]	38.49±2.46 [b]	12.92±2.25 [c]
MUFA	27.71±2.08 [c]	25.60±1.95 [c]	102.67±3.62 [a]	77.26±6.96 [b]	22.47±3.63 [c]
PUFA n-6	15.28±1.09 [bc]	6.23±0.41 [d]	26.78±2.17 [a]	19.44±0.78 [b]	11.11±2.49 [c]
PUFA n-3	1.45±0.12 [d]	5.68±0.48 [c]	25.97±1.51 [a]	17.38±0.73 [b]	4.28±1.06 [c]
n-6/n-3	10.63±0.38 [a]	1.10±0.04 [c]	1.03±0.08 [c]	1.12±0.04 [c]	2.63±0.13 [b]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 1-11 Effect of SQ on the fatty acids content in hepatic PL of KK-A^y mice (mg/g liver).

	Soybean oil	Linseed oil	1% SQ	2% SQ	Fish oil
16:0	3.13±0.10^{b}	3.54±0.17^{ab}	3.34±0.07^{ab}	3.63±0.11^{a}	3.78±0.22^{a}
18:0	3.05±0.10^{d}	3.65±0.19^{bc}	4.03±0.10^{ab}	4.15±0.13^{a}	3.38±0.14^{cd}
18:1n-9	1.19±0.05^{b}	1.73±0.09^{a}	1.67±0.05^{a}	1.62±0.05^{a}	1.35±0.07^{b}
18:2n-6	2.77±0.11	2.97±0.20	3.17±0.16	3.25±0.13	3.01±0.13
20:4n-6	2.45±0.11^{a}	1.69±0.10^{b}	1.84±0.11^{b}	1.86±0.05^{b}	1.81±0.08^{b}
18:3n-3	0.02±0.00^{c}	0.21±0.02^{a}	0.16±0.01^{b}	0.17±0.01^{ab}	0.02±0.00^{c}
20:5n-3	0.05±0.00^{d}	0.89±0.06^{a}	0.63±0.03^{bc}	0.72±0.04^{b}	0.53±0.04^{c}
22:5n-3	0.06±0.00^{d}	0.23±0.02^{a}	0.19±0.01^{b}	0.20±0.01^{ab}	0.11±0.00^{c}
22:6n-3	1.23±0.05^{c}	1.64±0.17^{b}	1.61±0.10^{b}	1.62±0.07^{b}	2.22±0.10^{a}
Total FA	14.38±0.48	17.12±0.98	17.17±0.44	17.09±0.76	15.40±0.68
SFA	6.26±0.18^{b}	7.29±0.34^{a}	7.47±0.18^{a}	7.87±0.22^{a}	7.25±0.35^{a}
MUFA	1.52±0.05^{b}	2.14±0.11^{a}	2.03±0.07^{a}	1.98±0.07^{a}	1.66±0.08^{b}
PUFA n-6	5.23±0.21	4.69±0.29	5.04±0.25	5.15±0.18	4.84±0.21
PUFA n-3	1.37±0.05^{b}	3.01±0.27^{a}	2.63±0.11^{a}	2.75±0.13^{a}	2.74±0.03^{a}
n-6/n-3	3.81±0.06^{a}	1.57±0.07^{c}	1.92±0.09^{b}	1.88±0.06^{b}	1.70±0.06^{bc}

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

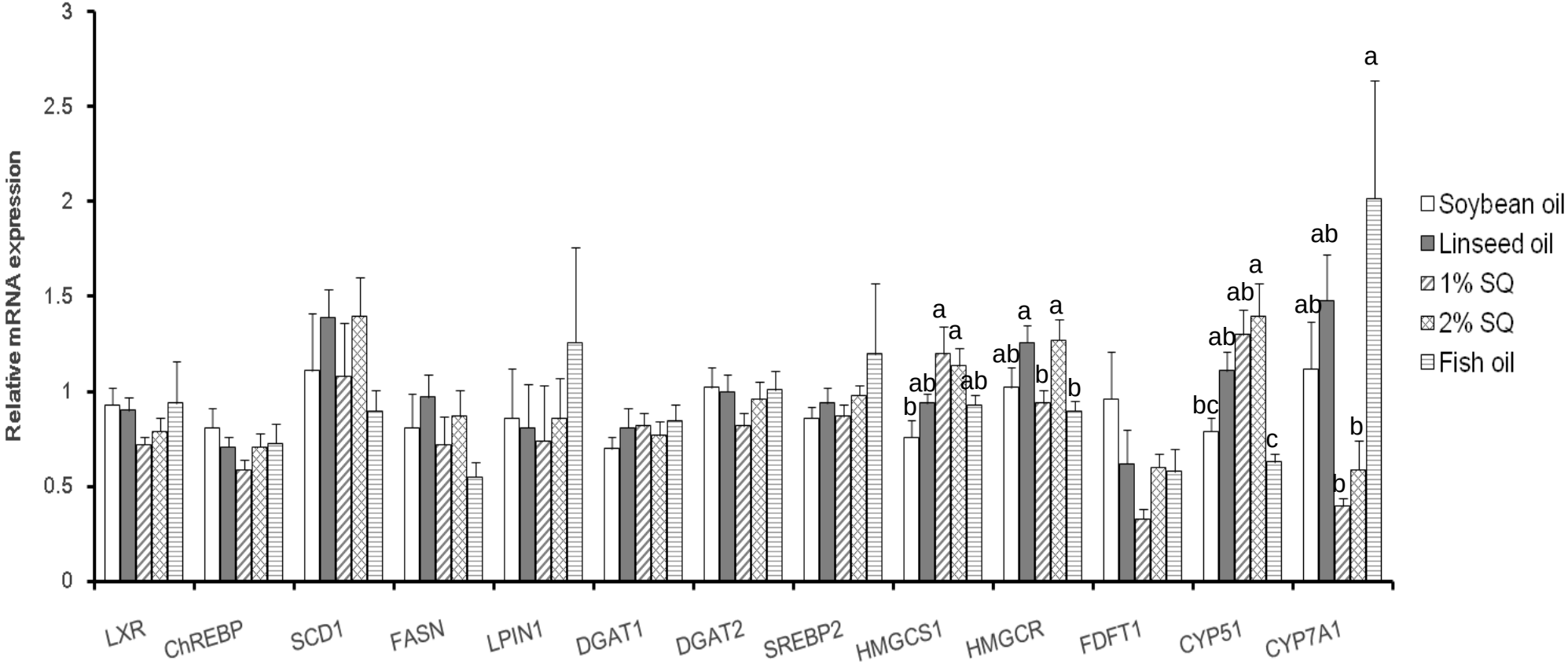

Figure 1-12 Effect of SQ on the hepatic mRNA expression of KK-A^y mice.

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. Values are expressed as mean ± SEM, different letters indicate significant difference at P< 0.05 among each value.

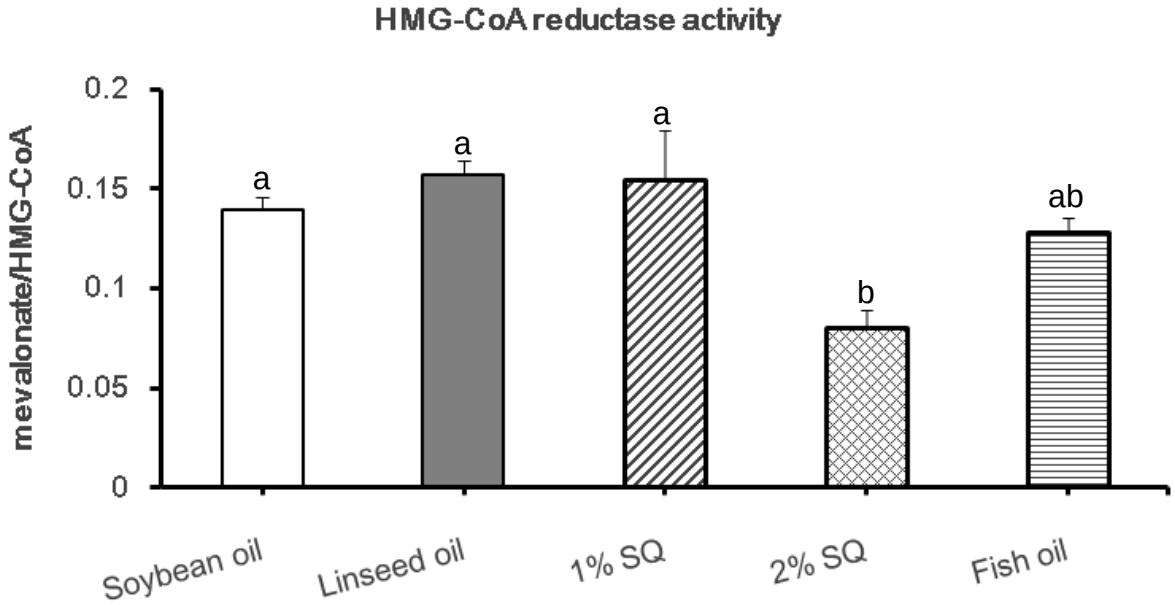

Figure 1-13 Effect of SQ on the HMG CoA reductase activity in the liver of KK-A^y mice.

HMG-CoA reductase activity was calculated from the ratio of mevalonate concentration to HMG-CoA concentration. Values are expressed as mean ± SEM. Different letters indicate significant difference at $P < 0.05$ among each value.

1.4 Discussion

Epidemiological studies have revealed an inverse correlation between HDL-C levels and the risk of CVD and of atherosclerosis. HDL-C promotes reverse cholesterol transport and has several atheroprotective functions such as anti-inflammation, anti-thrombosis, and anti-oxidation [120; 121]. 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 [122]. 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-C [123]. In the present study, we have found a significant increase in plasma HDL-C of obese/diabetic KK-A^y mice given SQ with no significant difference in the TC and non-HDL-C levels (**Table 1-3**). This result suggests the anti-atherosclerosis effect of SQ on obese/diabetic KK-A^y mice.

Various animal and human studies have shown that SQ could reduce the risk of atherosclerosis and this effect of SQ have been explained by the involvement of SQ in lipid metabolism and by its protective action on lipoproteins and artery endothelial cells against oxidative damage [49; 55; 106]. However, there has been a contradiction in the effect of SQ on the blood lipid levels. Several researchers have shown the significant decrease in blood cholesterol levels in animal and human studies by SQ intake, while other researchers have reported the opposite results [43; 55; 106; 107; 108; 109; 111; 112; 115]. Although there have been controversial results in the effect of SQ on plasma lipid levels, recent studies have demonstrated the effect of SQ on blood HDL-C level as important factor in the atherosclerosis protection [49]. Administration of SQ for seven weeks (2.1 g/kg) to C57Bl/6J SPF mice showed a 60% increase in HDL-C with no changes in total cholesterol [53]. Likewise, SQ administration for 11 weeks at a dose of 1 g/kg caused a specific increase in HDL-C levels in three male mouse models (wild-type, ApoA-1- and ApoE-deficient) having

C57BL/6J genetic background [54]. In rat model, the specific increase in HDL-C has been also reported [108]. These studies have demonstrated that high HDL level would be independent anti-atherosclerotic factor [35]. The present study confirmed that the increase in HDL-C level would be one of the major effects of SQ in atherosclerosis protection.

HDL-C biogenesis and its development are involved by different and complex metabolic networks such as up-regulation of ABCA1, ApoA-1 transcription and LXR [124]. Therefore, the increasing effect of SQ on the HDL-C would be related to these events; however, the mechanisms by which SQ elevate plasma HDL-C levels remains unclear. SQ is known to show a broad repertoire of biological actions based on its antioxidant activity [49]. This effect could be exerted in HDL that may prevent oxidative modifications of the ApoA-1, other HDL proteins, and HDL lipids. The prevention of HDL against oxidative stress can make it more fluid and thus more functional. Further study will be needed from this viewpoint.

In humans, orally administered SQ is well absorbed (60-85%). This and the intestinal de novo synthesized SQ are transported by chylomicrons into circulation, being rapidly taken up by the liver, where it is converted into cholesterol [54]. However, TC level decreased in the liver of obese/diabetic KK-A^y mice (**Figure 1-10**). Significant increase in NL by SQ intake was observed in KK-A^y mice, while no significant difference was observed in PL (**Figure 1-7 and Figure 1-8**). The increase in NL resulted in the higher TL levels in the liver. SQ administration to KK-A^y mice induced its accumulation in the liver. Moreover, SQ is eluted as NL fraction in the separation of TL with 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 **Figure 1-7** was mainly due to the increase in TAG. The TAG increase by SQ intake was strongly related to the higher level of total FA found in **Table 1-9** and **Table 1-10**.

It is shown in **Figure 1-9** and **Table 1-9** that that SQ administration to KK-A^y mice induces TAG accumulation in the liver. To know the effect of SQ, gene expressions related to FA (FASN and SCD1) and TAG (DGAT1 and DGAT2) syntheses were analyzed (**Figure 1-12**). 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 expressions and TAG content in the liver of KK-A^y mice. One possibility would be the involvement of TAG originated from other tissues.

HMGCR is gene related to cholesterol metabolism. Its expression was significantly decreased by SQ (1%) intake, but no significance by the administration of SQ (2%) (**Figure 1-12**), and significant decrease in HMG-CoA reductase activity was found in the liver of KK-A^y mice fed SQ (2%), but no significance in SQ (1%) (**Figure 1-13**). As one of the key factor in cholesterol synthesis, the down-regulation of HMG-CoA reductase activity and its gene expression might explain the decrease in cholesterol concentration in the liver of KK-A^y mice (**Figure 1-10**). CYP7A1 is known to catalyze cholesterol catabolic pathway. This enzyme expression was decreased by SQ intake (**Figure 1-12**) and this may also affect the cholesterol level of the liver.

In summary, the present study showed that SQ supplementation to KK-A^y mice increased plasma HDL-C level, important and independent anti-atherosclerotic factor. Although more research is needed to make clear the effect of SQ on the lipid metabolism and dynamics related to atherosclerosis, the present study suggests the anti-atherosclerotic effect of SQ on subjects with metabolic disorders.

Chapter 2

Dietary Effect of Squalene on the Lipid Metabolism of C57BL/6J Mice

2.1 Introduction

Triterpenes, consisting of six isoprene units, are one of the largest classes of chemical compounds. To date, more than 20,000 different triterpenes were reported in plants [125]. Although majority of diversity in natural triterpenes occurs in the plant kingdom, animal, fungi and bacteria can also create triterpenes.

In all of the triterpenes, SQ is arguably one of the most important examples as it forms the basis of almost all steroids [102]. Furthermore, SQ, as an important member of triterpene, is structurally similar to some physiologically active substance, such as β-carotene, coenzyme Q10, etc [126]. Due to its beneficial properties as antioxidant [127] and antitumor ability [102], drug carrier property [128] and advantages for the skin or adjuvants for vaccines as an emollient [102; 129], the biosynthesis of SQ in several organisms has been studied. In addition, SQ has also been interested as the characteristic phytochemicals in olive oil. Olive oil is known to be much more consumed in the Mediterranean countries than that in northern European countries. It has been believed that this higher consumption of olive oil has been related to the low incidence of CVD in these areas [55]. Therefore, much attention has been focused on the cardio-protective function of SQ.

In **Chapter 1**, we used KK-A^y mice as the experimental subject and showed that SQ supplementation to KK-A^y mice increased plasma HDL-C level, important and independent anti-atherosclerotic factor. SQ supplementation also affected body weight, hepatic lipid parameters, FA level of liver lipids, RNA expression related to lipid metabolism and activity of HMG-CoA reductase in KK-A^y mice.

In **Chapter 2**, C57BL/6J mice were used as the experimental subject and compared the effect of SQ on C57BL/6J normal mice with that on the obese/diabetes KK-A^y mice found in **Chapter 1**. The comparison may provide useful information to know

the effect of SQ on the reduction of CVD risk.

2.2 Material and Methods

2.2.1 Material

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

2.2.2 Animals and diets

C57BL/6J mice (male, four weeks old) were obtained from Japan CREA Co., Tokyo, Japan. The mice were housed individually in an air-conditioned room (23±1 ℃ 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 4 groups of seven and were then fed experimental diets (**Table 2-1**) for four weeks. The composition of fatty acid in the experimental diets (**Table 2-2**) was analyzed as described in 1.2.2 in **Chapter 1**. The body weight, diet and water intake of each mouse was recorded daily. Mice were sacrificed under diethyl ether anesthesia after 12 h fasting on day 28. Body weights after 12 h fasting on day 28 were determined.

2.2.3 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 carried out

under the approval by the Ethical Committee of Experimental Animal Care at Hokkaido University.

2.2.4 Sample collection

The sample collection was done following the method as described in section 1.2.4 in **Chapter 1**.

2.2.5 Blood lipid analysis

The blood plasma analysis was performed by the Analytical Center of Hakodate Medical Association (Hakodate, Japan) as described in section 1.2.5 in **Chapter 1**.

2.2.6 Hepatic lipid analysis

The hepatic lipid parameters were determined following the method as described in section 1.2.6 in **Chapter 1**.

2.2.7 Quantitative real-time PCR

The mRNA expression of the related genes in the liver of C57BL/6J mice were measured following the method as described in section 1.2.7 in **Chapter 1**.

The mRNA analyses were performed on genes associated with lipid metabolism, which included LXR, SREBP2, CYP7A1, ChREBP, FASN. The gene-specific primers were Hs00172885_m1 (LXR), Hs01081784_m1 (SREBP2), Hs00167982_m1 (CYP7A1), Hs00975714_m1 (ChREBP), Hs01005622_m1 (FASN), and Hs02786624_g1 (GAPDH; internal control), respectively.

2.2.8 HMG-CoA reductase analysis

The activity of HMG-CoA reductase was determined following the method as described in section 1.2.8 in **Chapter 1**.

2.2.9 Statistic analysis

Results are expressed as the mean ± SEM. Statistical significances between groups were evaluated by one-way ANOVA with *post hoc* comparisons (Šidák correction). Differences with $P < 0.05$ were considered statistically significant.

2.3 Results

2.3.1 Initial weight, weight gain, final body weight on day 28 and water intake

As shown in **Figure 2-1**, no difference was observed in initial body weights of C57BL/6J mice among the different groups. Administration of SQ did not affected weight gain, final body weight and total water intake of C57BL/6J mice (**Figure 2-2**, **Figure 2-3** and **Figure 2-4**).

2.3.2 Blood glucose levels and plasma lipid parameters

The blood glucose was not significantly different among the different groups (**Figure 2-5**). As shown in **Table 2-3**, all plasma cholesterols levels (TC, HDL-C and non-HDL-C) increased in C57BL/6J mice fed SQ when comparing with linseed oil and fish oil groups, especially significance was observed with HDL-C and non-HDL-C of fish oil group.

2.3.3 Tissue weights

Administration of SQ did not affect the absolute weight and relative weight of major tissues in C57BL/6J mice (**Table 2-4, Table 2-5, Table 2-6 and Table 2-7**).

2.3.4 Hepatic lipid parameters

SQ (1% and 2%) intake significantly increased TL in the liver of C57BL/6J mice when comparing with fish oil group (**Figure 2-6**). But no significant difference was observed between both SQ groups and linseed oil group. Although an increase tendency in the content of hepatic NL and TAG in the mice fed SQ was observed, no significant difference was found between both SQ groups and the other groups (**Figure 2-7** and **Figure 2-9**). No significance was also observed in the hepatic PL levels between the different groups (**Figure 2-8**). Compared to linseed oil group, TC level of the liver increased markedly in 2% SQ group (**Figure 2-10**).

2.3.5 FA levels of liver lipids

In this experiment, the content of FA in hepatic TL, NL and PL of C57BL/6J mice were determined. There was a little difference in the FA content in the hepatic TL of C57BL/6J mice (**Table 2-8**). As shown in **Table 2-9**, the content of 18:0 in NL was significantly lower in 1% and 2% SQ groups compared to linseed oil group. No significant difference was observed in the other FA contents in the hepatic NL of C57BL/6J mice. This was also expected from the result of TL and NL levels in the liver of C57BL/6J mice. In regard to PL (**Table 2-10**), 2% SQ supplementation significantly increased the contents of SFA, MUFA and PUFA as compared with linseed and fish oil groups. 1% SQ supplementation only significantly increased the content of 18:3n-3 compared to linseed oil group. However, no difference was detected in the other FA between 1% SQ group and linseed oil group.

2.3.6 Gene expressions 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 by real-time PCR (**Figure 2-11**). The analysis showed no significant effect of SQ on the gene expressions of C57BL/6J mice as compared with other dietary groups.

Furthermore, SQ supplementation did not affect HMG-CoA reductase activity in the liver from C57BL/6J mice (**Figure 2-12**).

Table 2-1 Composition of experimental diets (g kg^{-1})

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

Table 2-2 Fatty acid composition of dietary lipids (%)

	Linseed oil	1% SQ	2% SQ	Fish oil
14:0	0.96±0.02	0.96±0.02	0.94±0.01	1.31±0.03
16:0	17.78±0.14	17.88±0.14	17.91±0.20	19.88±0.14
16:1	1.74±0.04	1.73±0.03	1.71±0.03	1.95±0.04
18:0	10.56±0.05	10.53±0.05	10.50±0.05	10.78±0.11
18:1n-9	35.09±0.19	35.21±0.18	35.33±0.19	33.45±0.28
18:2n-6	11.02±0.05	11.00±0.04	10.98±0.05	18.93±0.08
20:4n-6	ND	ND	ND	0.30±
18:3n-3	16.83±0.04	16.81±0.03	16.81±0.05	1.97±0.04
20:5n-3	ND	ND	ND	1.56±
22:6n-3	ND	ND	ND	3.87±
Total FA	95.09±0.10	95.19±0.13	95.24±0.16	94.56±0.18
SFA	29.30±0.10	29.37±0.09	29.36±0.13	31.97±0.11
MUFA	37.47±0.14	37.56±0.13	37.65±0.14	36.17±0.22
PUFA n-6	11.02±0.05	11.00±0.04	10.98±0.05	19.23±0.08
PUFA n-3	16.83±0.04	16.82±0.03	16.81±0.05	6.32±0.12
n-6/n-3	0.66±0.00	0.66±0.00	0.65±0.00	3.04±0.07

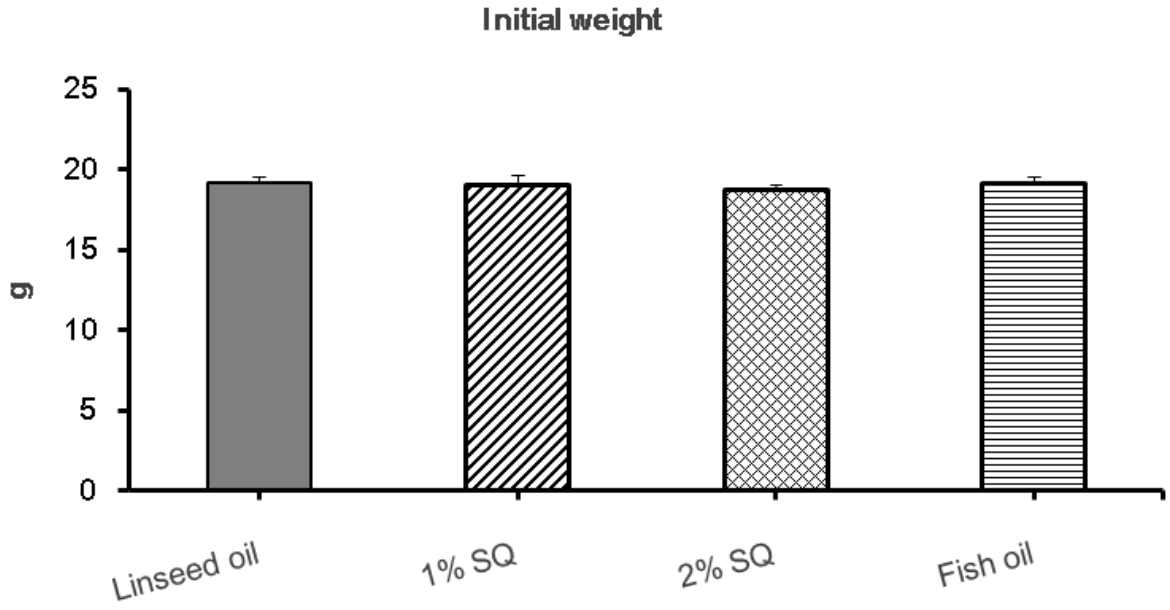

Figure 2-1 Initial weight of C57BL/6J mice.

Initial weight of C57BL/6J mice was determined after 12 h fasting. Values are expressed as mean ± SEM (n=7).

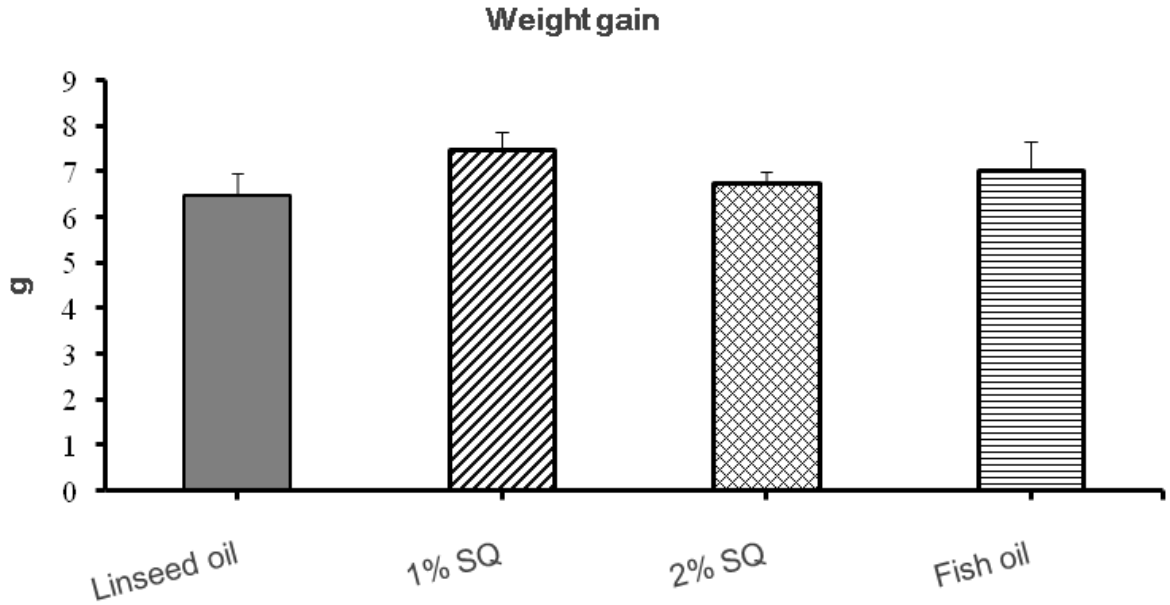

Figure 2-2 Weight gain of C57BL/6J mice.

Values are expressed as mean ± SEM (n=7).

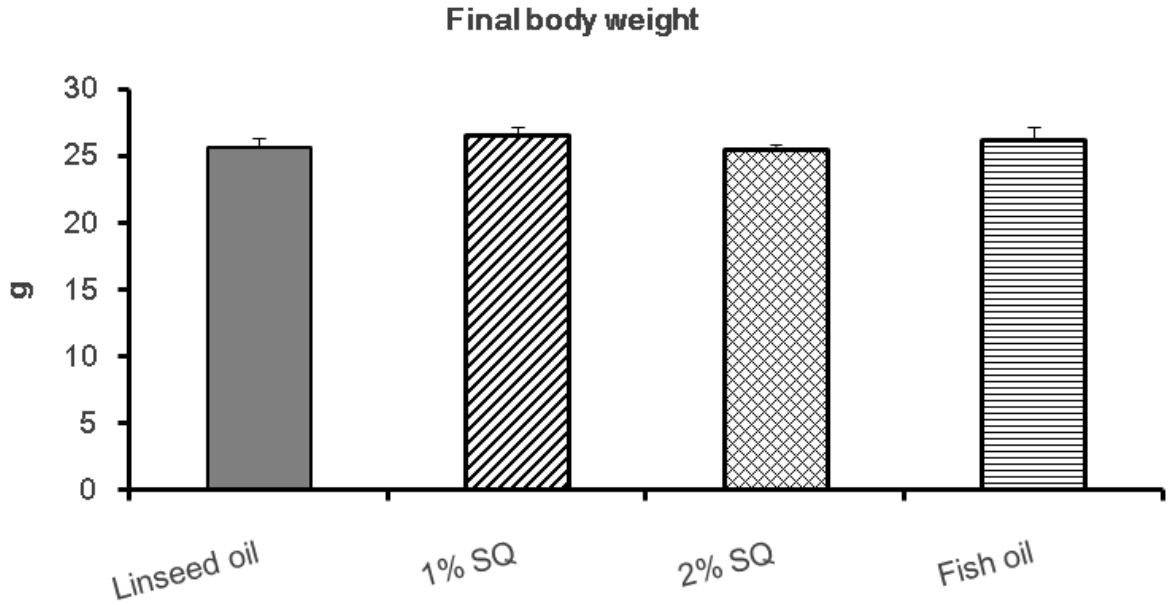

Figure 2-3 Final body weight of C57BL/6J mice on day 28.

Final body weight of C57BL/6J mice on day 28 was determined after 12h fasting. Values are expressed as mean ± SEM (n=7).

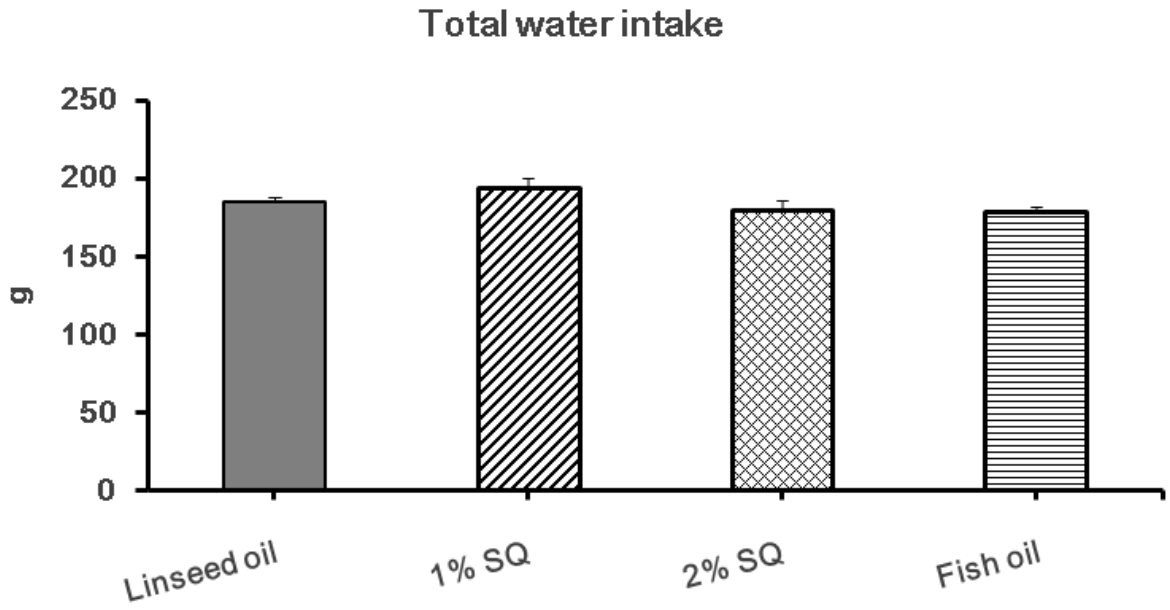

Figure 2-4 Total water intake of C57BL/6J mice during 28 days feeding.

Water intake of each mouse was recorded daily. Values are expressed as mean ± SEM (n=7).

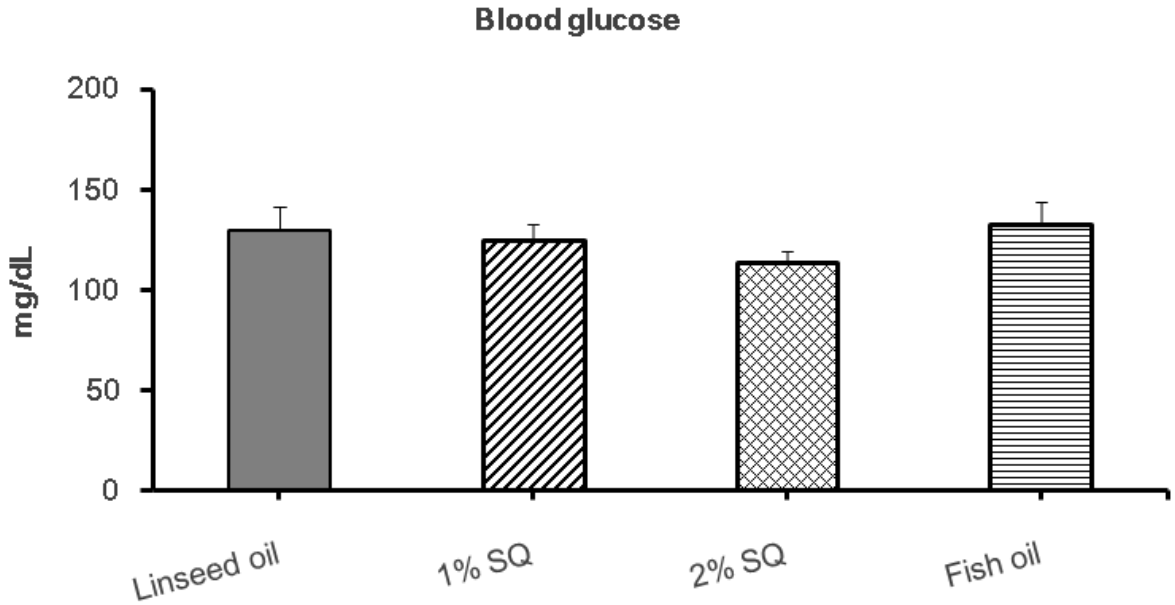

Figure 2-5 Effect of SQ on blood glucose of C57BL/6J mice on day 28.

Blood glucose was measured using a blood glucose monitor after 12 h fasting. Values are expressed as mean ± SEM (n=7).

Table 2-3 Effect of SQ on plasma lipid parameters of C57BL/6J mice.

		TAG (mg/dL)	PL (mg/dL)	TC (mg/dL)	HDL-C (mg/dL)	nonHDL-C (mg/dL)
C57BL/6J mice	Linseed oil	83.57±9.40	269.14±11.09	120.71±3.58	85.43±2.95[ab]	35.29±1.34
	1% SQ	87.17±15.67	282.00±5.38	134.50±3.21	92.50±3.25[a]	42.00±1.24
	2% SQ	86.00±10.26	284.00±9.48	134.71±5.79	93.29±3.28[a]	41.43±3.05
	Fish oil	92.86±16.70	266.86±10.52	114.71±3.91	77.14±1.44[b]	37.57±2.90

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 2-4 Effect of SQ on absolute WAT weight of C57BL/6J mice.

		WAT				
		Mesenteric	Epididymal	Perirenal	Inguinal	Total
		g				
C57BL/6J mice	Linseed oil	0.33±0.03	0.69±0.05	0.22±0.03	0.60±0.05	1.85±0.15
	1% SQ	0.36±0.03	0.84±0.06	0.27±0.03	0.63±0.05	2.10±0.15
	2% SQ	0.33±0.02	0.79±0.07	0.25±0.03	0.65±0.05	2.02±0.16
	Fish oil	0.31±0.04	0.69±0.07	0.24±0.04	0.62±0.06	1.86±0.20

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at P< 0.05 among each value.

Table 2-5 Effect of SQ on absolute tissue weight of C57BL/6J mice.

		Liver	Spleen	BAT	Small intestine	Pancreas	Kidney	Muscle	Heart	Brain
		g								
C57BL/6J mice	Linseed oil	1.46±0.06	0.08±0.01	0.11±0.01	0.82±0.03	0.24±0.01	0.32±0.01	0.30±0.01	0.12±0.00	0.40±0.05
	1% SQ	1.48±0.04	0.08±0.01	0.12±0.00	0.81±0.05	0.24±0.02	0.36±0.02	0.30±0.01	0.13±0.01	0.39±0.02
	2% SQ	1.47±0.02	0.08±0.01	0.12±0.01	0.78±0.03	0.28±0.03	0.33±0.01	0.28±0.01	0.12±0.00	0.40±0.02
	Fish oil	1.50±0.06	0.09±0.00	0.12±0.01	0.85±0.05	0.28±0.03	0.37±0.03	0.33±0.04	0.23±0.07	0.43±0.04

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at P< 0.05 among each value.

Table 2-6 Effect of SQ on relative WAT weight of C57BL/6J mice.

		WAT				
		Mesenteric	Epididymal	Perirenal	Inguinal	Total
		g per 100g body weight				
C57BL/6J mice	Linseed oil	2.68±0.15	1.29±0.09	0.85±0.09	2.34±0.18	7.16±0.50
	1% SQ	3.19±0.19	1.36±0.09	1.03±0.10	2.49±0.11	8.08±0.44
	2% SQ	3.08±0.28	1.27±0.08	0.96±0.14	2.52±0.19	7.83±0.62
	Fish oil	2.62±0.20	1.17±0.09	0.88±0.12	2.34±0.17	7.01±0.52

Values are expressed as mean ± SEM (n=7).

Table 2-7 Effect of SQ on relative tissue weight of C57BL/6J mice.

		Liver	Spleen	BAT	Small intestine	Pancreas	Kidney	Muscle	Heart	Brain
		g per 100g body weight								
C57BL/6J mice	Linseed oil	5.71±0.17	0.31±0.02	0.44±0.02	3.20±0.11	0.94±0.02	1.26±0.03	1.16±0.02	0.48±0.01	1.57±0.21
	1% SQ	5.80±0.13	0.31±0.01	0.46±0.02	3.16±0.17	0.88±0.04	1.30±0.02	1.12±0.02	0.47±0.01	1.42±0.81
	2% SQ	5.67±0.05	0.30±0.01	0.44±0.03	2.92±0.14	1.10±0.13	1.31±0.01	1.08±0.03	0.49±0.02	1.60±0.02
	Fish oil	5.73±0.15	0.32±0.01	0.45±0.02	3.24±0.12	1.07±0.12	1.41±0.14	1.27±0.15	0.48±0.01	1.52±0.16

Values are expressed as mean ± SEM (n=7).

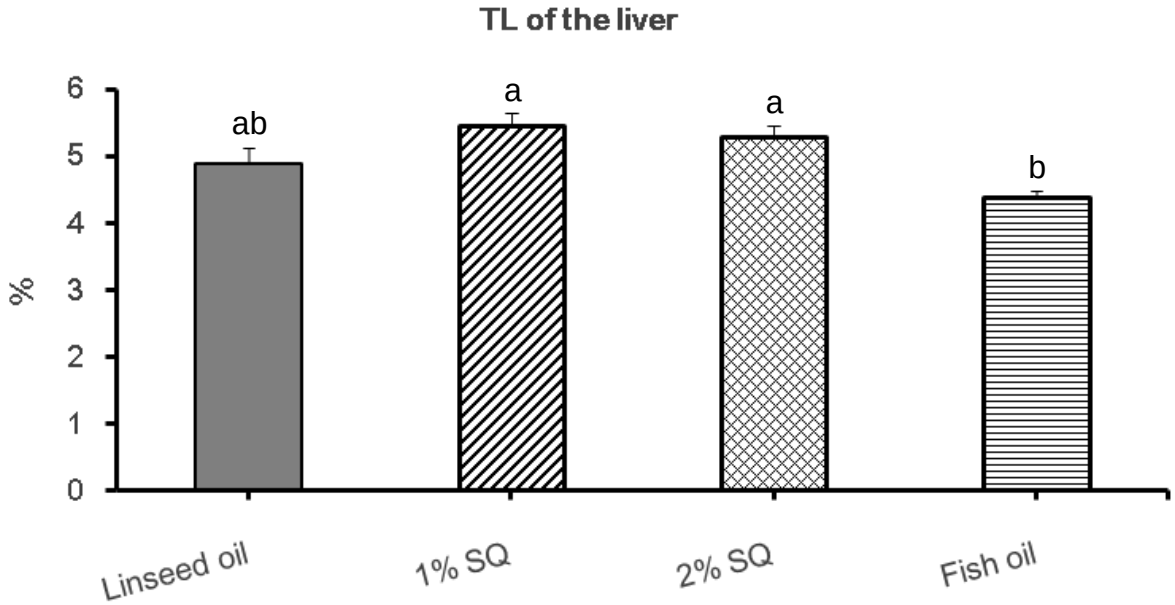

Figure 2-6 Effect of SQ on the hepatic TL content of C57BL/6J mice.

TL was extracted from liver with chloroform/methanol (2:1, v/v). Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at $P < 0.05$ among each value.

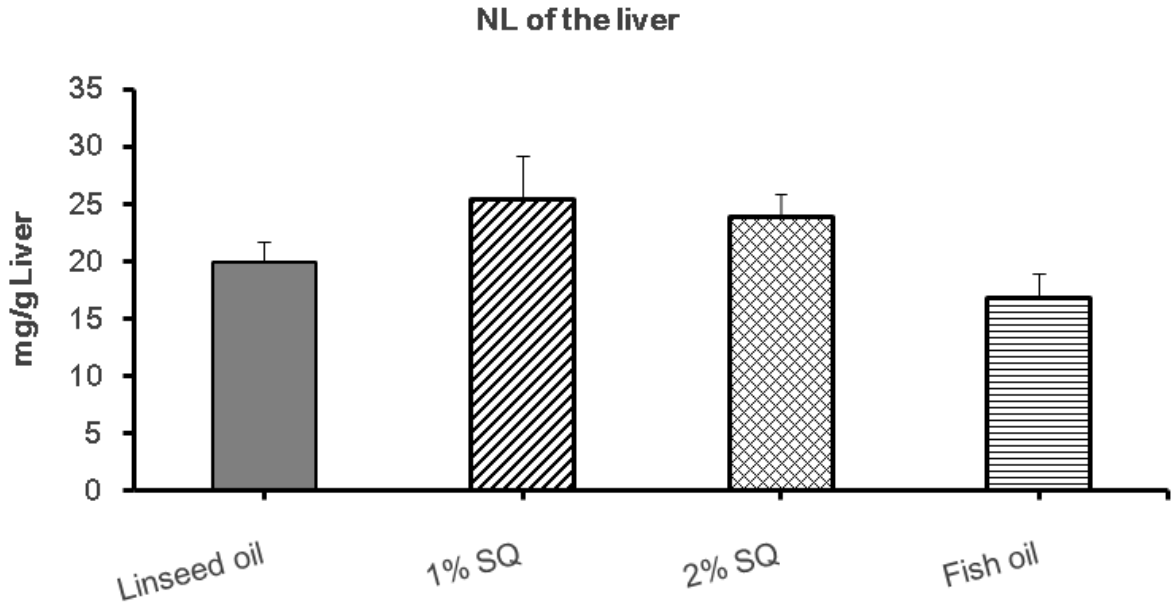

Figure 2-7 Effect of SQ on the hepatic NL content of C57BL/6J mice.

The TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridge by elution with chloroform and methanol. The NL fraction was eluted with 70 mL chloroform. Values are expressed as mean ± SEM (n=7).

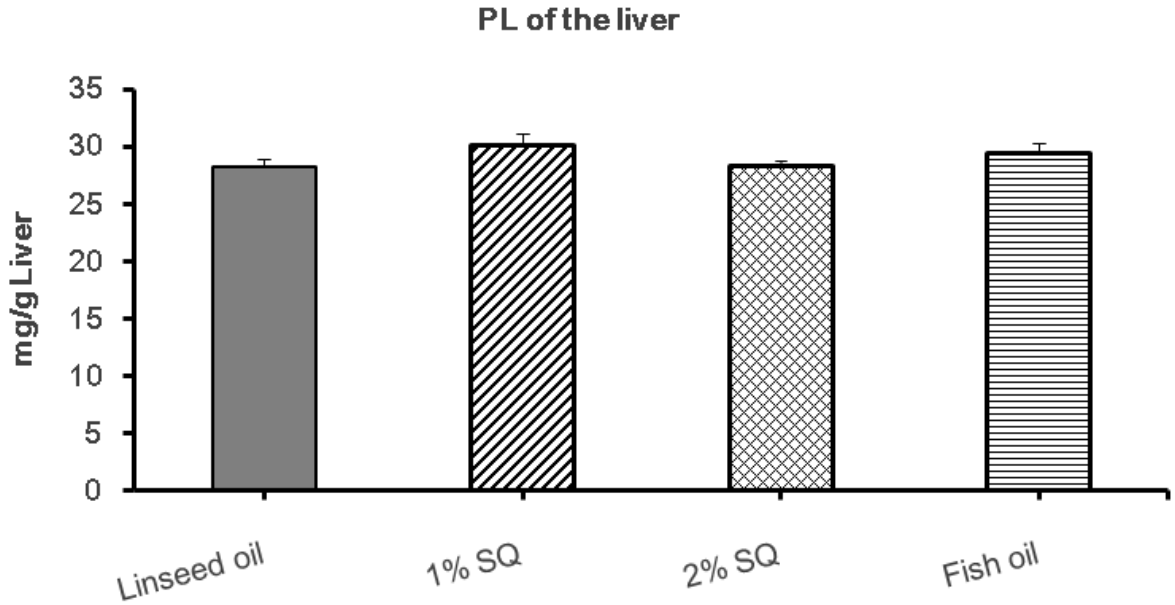

Figure 2-8 Effect of SQ on the hepatic PL content of C57BL/6J mice.

The TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridgeby elution with chloroform and methanol. The PL fraction was eluted with 50mL methanol. Values are expressed as mean ± SEM (n=7).

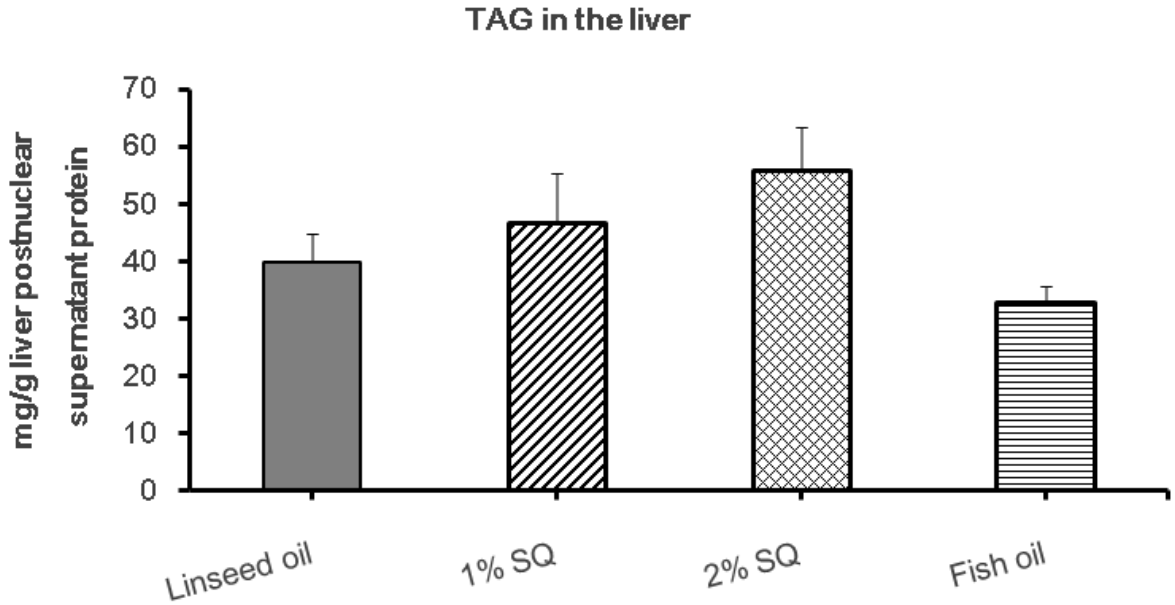

Figure 2-9 Effect of SQ on the hepatic TAG content of C57BL/6J mice.

TAG was measured using enzymatic kits (Triglyceride E-test, Wako Pure Chemical Industries, Osaka, Japan). Values are expressed as mean ± SEM (n=7).

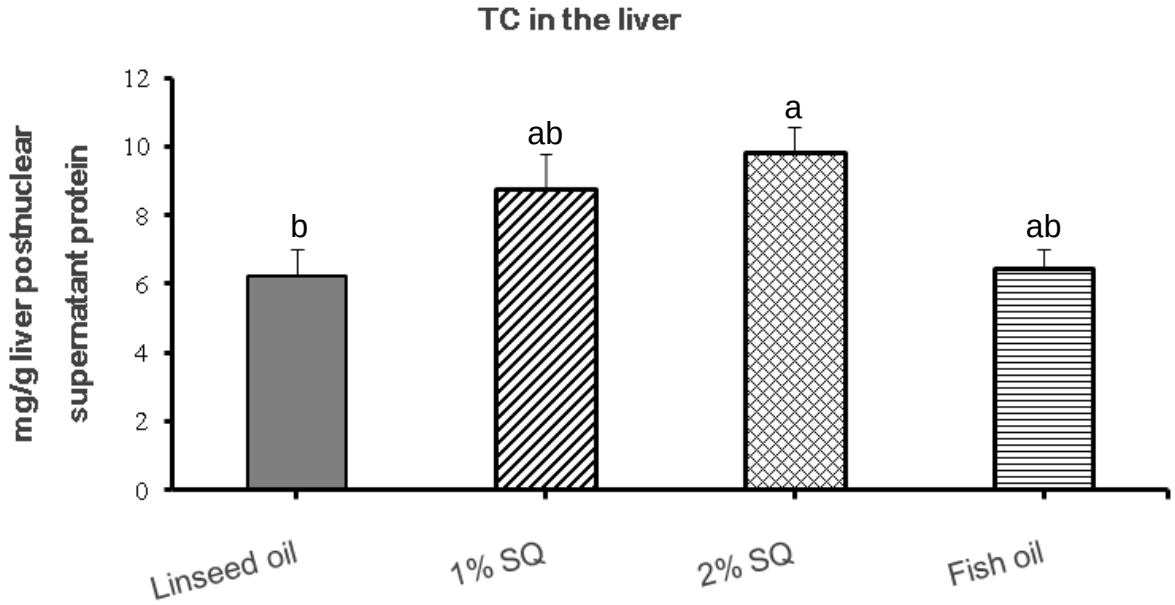

Figure 2-10 Effect of SQ on the hepatic TC content of C57BL/6J mice.

TC was measured using enzymatic kits (Cholesterol E-test, Wako Pure Chemical Industries, Osaka, Japan). Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at $P < 0.05$ among each value.

Table 2-8 Effect of SQ on the fatty acid content in hepatic TL of C57BL/6J mice (mg/g liver).

	Linseed oil	1% SQ	2% SQ	Fish oil
16:0	7.03±0.32	8.47±0.75	8.16±0.55	7.08±0.22
16:1	0.38±0.05 [ab]	0.65±0.11 [a]	0.70±0.11 [a]	0.31±0.03 [b]
18:0	3.25±0.10	3.49±0.11	3.35±0.05	3.31±0.08
20:0	0.14±0.01	0.16±0.01	0.14±0.00	0.14±0.01
18:1n-9	6.43±0.47 [ab]	8.63±0.93 [a]	8.48±0.81 [a]	4.86±0.15 [b]
20:1n-9	0.18±0.01 [bc]	0.25±0.02 [a]	0.23±0.02 [ab]	0.15±0.00 [c]
18:2n-6	3.95±0.13 [b]	4.86±0.20 [a]	4.48±0.18 [ab]	4.39±0.15 [ab]
20:4n-6	1.92±0.08 [ab]	1.99±0.05 [ab]	1.87±0.05 [b]	2.16±0.05 [a]
18:3n-3	1.20±0.06 [b]	1.76±0.15 [a]	1.72±0.20 [ab]	0.13±0.01 [c]
20:5n-3	0.99±0.05 [ab]	1.15±0.06 [a]	1.11±0.04 [a]	0.81±0.03 [b]
22:6n-3	2.51±0.13 [b]	2.56±0.11 [b]	2.40±0.10 [b]	3.73±0.17 [a]
Total FA	28.37±1.21 [ab]	34.42±2.31 [a]	33.07±1.75 [ab]	27.43±0.68 [b]
SFA	10.42±0.39	12.13±0.84	11.65±0.52	10.53±0.28
MUFA	7.00±0.53 [ab]	9.52±1.05 [a]	9.41±0.93 [a]	5.32±0.17 [b]
PUFA n-6	6.24±0.19 [b]	7.30±0.26 [a]	6.77±0.19 [ab]	6.92±0.18 [ab]
PUFA n-3	4.70±0.21	5.47±0.28	5.23±0.26	4.66±0.19
n-6/n-3	1.33±0.03 [b]	1.34±0.03 [b]	1.30±0.04 [b]	1.49±0.03 [a]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 2-9 Effect of SQ on the fatty acid content in hepatic NL of C57BL/6J mice (mg/g liver).

	Linseed oil	1% SQ	2% SQ	Fish oil
16:0	10.41±0.65	9.03±0.69	9.90±0.52	8.13±0.36
16:1	0.82±0.07 [ab]	0.89±0.09 [ab]	1.03±0.13 [a]	0.58±0.08 [b]
18:0	0.93±0.03 [a]	0.79±0.02 [b]	0.79±0.02 [b]	0.85±0.05 [ab]
18:1n-9	14.41±0.96 [a]	13.65±0.78 [a]	14.64±0.65 [a]	9.75±0.39 [b]
18:2n-6	4.09±0.28 [b]	4.40±0.27 [ab]	4.57±0.19 [ab]	5.42±0.29 [a]
20:4n-6	0.27±0.01 [b]	0.26±0.01 [b]	0.27±0.01 [b]	0.36±0.02 [a]
18:3n-3	2.41±0.13 [a]	2.94±0.24 [a]	2.96±0.24 [a]	0.31±0.02 [b]
20:5n-3	0.47±0.03	0.45±0.04	0.47±0.03	0.50±0.05
22:5n-3	0.69±0.04	0.65±0.04	0.64±0.03	0.65±0.04
22:6n-3	1.16±0.09 [b]	0.97±0.03 [b]	1.04±0.10 [b]	2.32±0.13 [a]
Total FA	36.64±2.10	35.04±2.14	37.33±1.29	29.76±0.93
SFA	11.83±0.69	10.30±0.72	11.17±0.51	9.45±0.39
MUFA	15.65±1.03 [a]	14.99±0.87 [a]	16.14±0.77 [a]	10.67±0.45 [b]
PUFA n-6	4.43±0.29 [b]	4.74±0.28 [ab]	4.91±0.19 [ab]	5.85±0.30 [a]
PUFA n-3	4.73±0.27 [ab]	5.01±0.34 [ab]	5.11±0.30 [a]	3.78±0.23 [b]
n-6/n-3	0.94±0.02 [b]	0.95±0.02 [b]	0.97±0.03 [b]	1.56±0.06 [a]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 2-10 Effect of SQ on the fatty acid content in hepatic PL of C57BL/6J mice (mg/g liver).

	Linseed oil	1% SQ	2% SQ	Fish oil
16:0	6.25±0.33^{b}	6.91±0.46^{ab}	7.80±0.27^{a}	6.42±0.21^{ab}
18:0	5.32±0.32^{b}	5.90±0.34^{ab}	6.55±0.25^{a}	5.15±0.15^{b}
18:1n-9	2.29±0.13^{bc}	2.60±0.13^{ab}	2.93±0.12^{a}	2.03±0.15^{c}
18:2n-6	3.73±0.18^{c}	4.60±0.27^{ab}	5.05±0.18^{a}	3.82±0.13^{bc}
20:4n-6	2.74±0.14^{b}	2.96±0.17^{ab}	3.37±0.13^{a}	2.79±0.09^{ab}
18:3n-3	0.24±0.01^{b}	0.33±0.01^{a}	0.35±0.01^{a}	0.04±0.00^{c}
20:5n-3	1.09±0.07^{ab}	1.29±0.06^{ab}	1.49±0.06^{a}	0.80±0.03^{b}
22:5n-3	0.29±0.02^{ab}	0.29±0.04^{ab}	0.35±0.03^{a}	0.21±0.01^{b}
22:6n-3	2.55±0.11^{c}	2.72±0.17^{bc}	3.15±0.11^{ab}	3.64±0.12^{a}
Total FA	25.40±1.27^{b}	28.68±1.59^{ab}	32.28±1.06^{a}	25.65±0.74^{b}
SFA	12.11±0.67^{b}	13.44±0.82^{ab}	15.05±0.51^{a}	12.03±0.36^{b}
MUFA	2.54±0.14^{c}	2.93±0.15^{ab}	3.32±0.15^{a}	2.26±0.16^{c}
PUFA n-6	6.52±0.32^{b}	7.62±0.44^{ab}	8.49±0.32^{a}	6.67±0.21^{b}
PUFA n-3	4.22±0.19^{b}	4.69±0.25^{ab}	5.41±0.18^{a}	4.69±0.15^{ab}
n-6/n-3	1.55±0.05^{ab}	1.63±0.05^{a}	1.57±0.04^{ab}	1.43±0.04^{b}

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

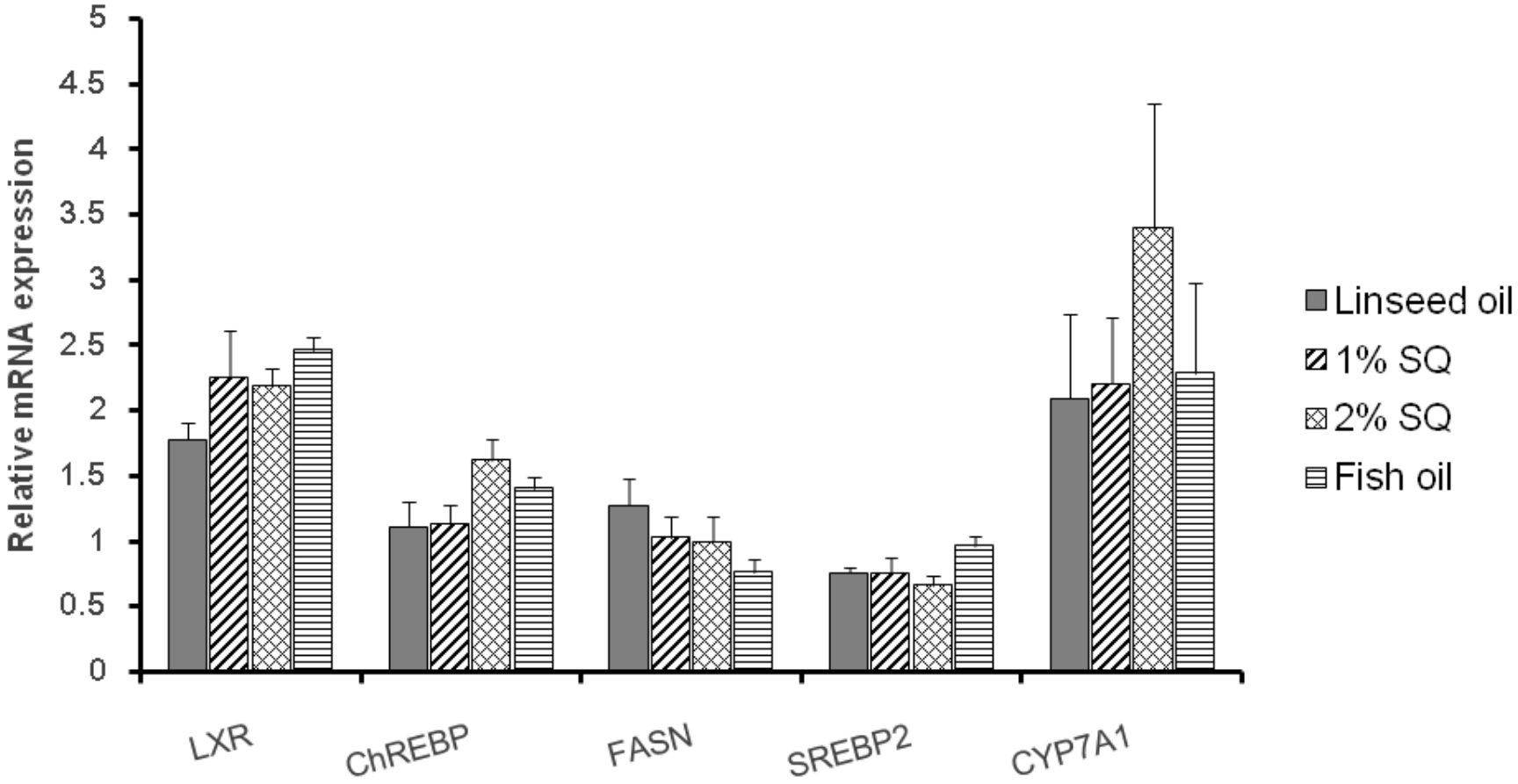

Figure 2-11 Effect of SQ on the hepatic mRNA expression of C57BL/6J mice.

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. Values are expressed as mean ± SEM.

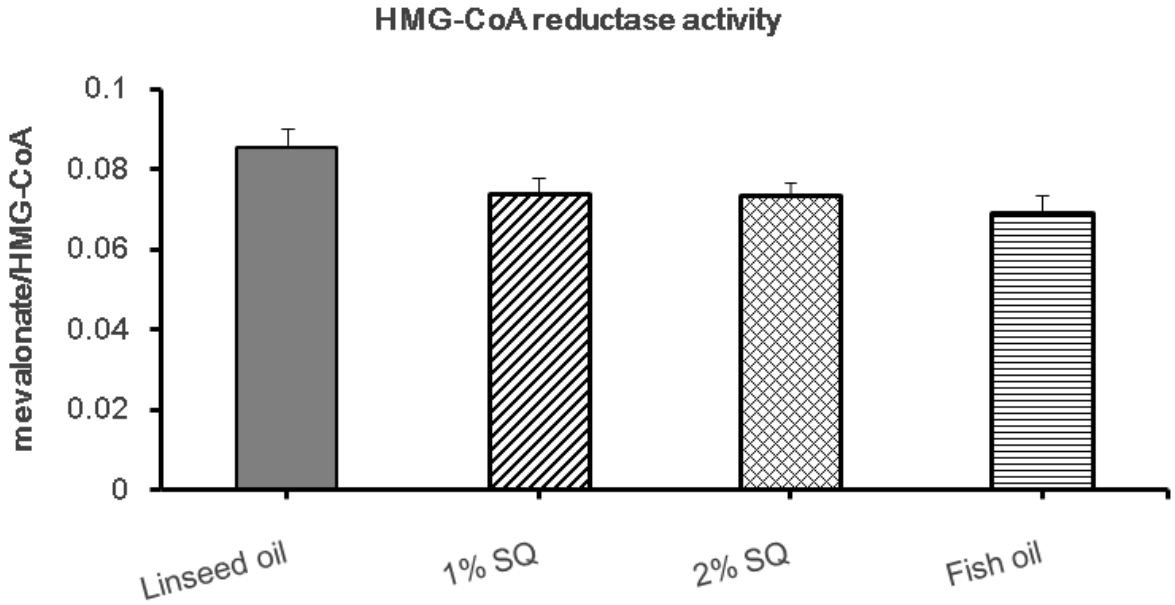

Figure 2-12 Effect of SQ on the HMG CoA reductase activity in the liver of C57BL/6J mice.

HMG-CoA reductase activity was calculated from the ratio of mevalonate concentration to HMG-CoA concentration. Values are expressed as mean ± SEM.

2.4 Discussion

The present study was designed to investigate the influence of SQ on the lipid metabolism of normal C57BL/6J mice. The major effects of SQ administration were increase in plasma HDL-C, nonHDL-C (**Table 2-3**) and hepatic TC (**Figure 2-10**); however, the significant difference was not observed between SQ groups and linseed oil group, except for the significant increase in hepatic TC of 2% SQ group. On the other hand, SQ significantly increased plasma HDL-C level in KK-A^y mice (**Table 1-3**). In addition, SQ decreased plasma non-HDL-C in the KK-A^y mice. These results indicate the higher anti-atherosclerotic effect of SQ on obese/diabetes KK-A^y mice than that on normal C57BL/6J mice.

HDL is a protective factor against coronary heart disease and the TC/HDL-C ratio has been reported to be a better predictor of ischemic heart disease than other conventional risk markers, such as TC, LDL-C or TAG [130-134]. Considering these studies and the results in the present study, SQ administration could show heart protection especially on the disease model animals such as KK-A^y mice, since it significantly raised plasma HDL-C level in KK-A^y mouse model (**Table 1-3**) than that seen in C57BL/6J (**Table 2-3**). These results are in agreement with the study on wild-type and ApoE-deficient mice on C57BL/6J genetic backgrounds which treated with 1 g SQ/kg body weight for 11 weeks [54].

Plasma TC level was increased by SQ supplementation without significant difference in C57BL/6J mice (**Table 2-3**). There have been controversial results on the effect of SQ on plasma cholesterol levels in animals and human. In animals, administration of SQ at a dose of 1g/kg for 4 weeks was reported to lower plasma TC levels [110]. In humans, no effect on serum cholesterol level was observed when treated with 900 mg SQ per day for 7 to 30 days [107]. Other study has reported the lowering effect of SQ on plasma TC and LDL-C in elderly patients with hypercholesterolemia [135]. These

results show that doses of SQ may be related to the different effect of SQ on plasma cholesterol levels in animals and humans.

Hepatic steatosis has been proposed to be associated with liver disease, type 2 diabetes mellitus, arterial hypertension and metabolic syndrome [136; 137]. In this way, large amount of TAG accumulated in liver via the process of steatosis could contribute to metabolic syndrome and its clinical complications. In a previous study, hepatic fat content showed a significant sex difference in ApoE-knockout mice consuming chow diet with SQ. Females showed a trend to increase hepatic fat. In contrast, hepatic fat content appeared to be decreased significantly in males [109]. In the present study, we used male KK-A^y mice and male C57BL/6J mice. SQ administration significantly increased TL (**Figure 1-6**), NL (**Figure 1-7**), and TAG (**Figure 1-9**) of the liver from KK-A^y mice. On another hand, there was no significant increase in these hepatic lipid levels by SQ administration to normal C57BL/6J mice, although an increase tendency of TAG by SQ feeding was found in male C57BL/6J mice (**Figure 2-6**, **Figure 2-7** and **Figure 2-9**). These results may show the increasing risk of hepatic steatosis by SQ administration especially in obese/diabetes condition than in normal condition.

In the present study, an increase tendency in hepatic TC was also found in C57BL/6J mice fed SQ (**Figure 2-10**), especially the significant increase was observed by 2% SQ administration. On the other hand, hepatic TC level was decreased by SQ administration in KK-A^y mice (**Figure 1-10**). There would be several possible factors related to the increase in hepatic TC in C57BL/6J mice fed SQ. For example, SQ monooxygenase catalyzes the first oxygenation step in cholesterol synthesis, and the degradation of this enzyme has been reported to be a control point in cholesterol synthesis beyond HMG-CoA reductase [137]. Therefore, significant increase in hepatic TC found in C57BL/6J mice fed SQ may be reflected by the conversion of SQ to cholesterol through the promotion of SQ monooxygenase in the liver. However, when mRNA expression related to lipid synthesis in the liver was analyzed (**Figure**

2-12), there was no significant difference in these mRNA expressions. Further studies will be needed to make clear the different effect of SQ on the hepatic lipid levels in obese/diabetes KK-A^y mice and normal C57BL/6J mice.

In summary, the present study showed that SQ supplementation to C57BL/6J mice increased plasma HDL cholesterol level, important and independent anti-atherosclerotic factor. But, the effectiveness of SQ on normal C57BL/6J mice was lower than that on obese/diabetes KK-A^y mice. On the other hand, hepatic TAG and TC had also been increased by administration of SQ to C57BL/6J mice. Although the present study suggests the anti-atherosclerotic effect of SQ on normal C57BL/6J mice, more research is needed to make clear the effect of SQ on the lipid metabolism of normal C57BL/6J mice.

Chapter 3

Dietary Effect of Farnesol and Sphingomyelin on the Lipid Metabolism of Obese/diabetes KK-A^y Mice

3.1 Introduction

Fatty acids (FA), a carboxylic acid with a long aliphatic chain, are important dietary sources of fuel for animals and important structural components for cells. It was reported that differences in dietary FA structure can induce marked differences in plasma lipid and lipoprotein concentrations. For example, lauric, myristic, and palmitic acids were reported to increase both plasma LDL-C and HDL-C. Oleic and linoleic acids were reported to increase plasma HDL-C [138]. Additionally, several kinds of FA have also been reported to exhibit protective effects against many diseases. n-3 PUFA such as EPA and DHA have been reported to exhibit protection on CVD [139; 140], diabetes [141; 142], cancer [143; 144], Alzheimer's disease [145], depression [146; 147], visual and neurological development [148; 149]. Positive association of red blood cell membranes oleic acid with breast cancer risk was also reported [150]. Therefore, the composition of FA in animal is closely related with health.

From above viewpoint, it may be interesting to analyze the effect of farnesol and sphingomyelin (SM). Because farnesol and SM have been reported to up-regulated the bioconversion of α-linolenic acid (C18:3n-3) to EPA and DHA [151; 152]. By this effect of farnesol and SM, both food components may show the cardio-protective activity.

Faronesol is a typical isoprenol present in plant products including fruits and berries [153]. A number of studies have shown that farnesol exhibit protective effects against many human cancers, such as colon [154], pancreas [155] and skin cancer [65]. Farnesol was also reported to prevent renel cell oxidative stress which was mediated by Fe-nitrilotriacetic acid [156]. Furthermore, it has been demonstrated that farnesol ameliorates inflammation in allergic asthmatic mice by regulating pro-inflammatory cytokines [72] and the lung inflammation induced by cigarette smoke extract [67]. In

addition, the effect of farnesol on lipids metabolism has also been demonstrated. Farnesol lowers serum cholesterol [157] and serum TAG [158] in rats. De novo synthesis of TAG in rat hepatocytes has also been inhibited by farnesol [159]. Furthermore, farnesol, as a substrate of SQ, may also affect the plasma HDL-C level.

SM, an integral component of mammalian cell membranes and nerves, has showed a dose-dependently reduce effect on the absorption of cholesterol, TAG and FA in rodents [83-86]. Although SM has been shown repeatedly to inhibit lipid absorption, elevated levels of SM have also been identified as an independent risk factor for coronary heart disease.

In this chapter, the effect of farnesol and SM on lipid metabolism was analyzed using KK-A^y mice.

3.2 Material and Methods

3.2.1 Material

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

SM was extracted from dried whey which was kindly donated from Snow Brand Milk Products Co., Saitama, Japan. 300 g whey and 10 volumes (v/w) of chloroform/methanol (2:1, v/v) were mixed and leaved it for standing overnight. Then the solution was filtered, and the filtrates were concentrated under vacuum using a rotary evaporator to obtain TL. The TL of the whey contained sphingolipids (SL), PL, and NL. Then, polar lipids such as SL and PL were separated from the TL based on

the insolubility of polar lipids in acetone and diethyl ether. 10 volumes of acetone (v/w) were added to the TL and allowed to stand overnight. The precipitate was recovered by centrifugation at 1260 g for 5 min and dissolved again in 10 volumes (v/w) of diethyl ether. After leaving the solution overnight, the crude polar lipids were precipitated by centrifugation at 1680 g for 10 min. Next step is to remove the glycerol-o-esters, such as PL. The glycerol-o-acyl esters could be saponified under the weak alkaline, while N-acyl esters such as SL were resistant to the saponification. Then the crude polar lipids were saponified with 200 volumes of 0.2 N NaOH in methanol at 37 ℃ for 20 min to remove these glycerol-o-esters. After neutralization with 2.6 N HCl in methanol, the unsaponifiable fraction was dissolved in chloroform/methanol/water (10:5:3, v/v/v). Then the solution was placed into a separatory funnel and was shaken vigorously. After allowing the funnel to stand overnight, the lower layer, mainly SL, was evaporated under reduced pressure in a rotary evaporator.

SM was refined form the SL. The SL were passed through a column packed with a chloroform/methanol/water (65:25:4, v/v/v) slurry mixture of Silica gel BW-80S (Fuji Silysia Chem. Ltd, Kasugai, Aichi, Japan) (700 g) by eluting the same solvent. The effluent was fractionated and then analyzed using TLC and then the purity of SM was detected by HPLC-ELSD. The pure SM was collected and used as experiment sample. TLC was performed on a 0.25 mm silica gel plate (Silica gel 60G; Merck) developed with chloroform/methanol/water (65:25:4, v/v/v). The lipid spot was detected with the Dittmer reagent. The procedure of SM determination using HPLC-ELSD is based on the method as described below.

The methodology was that:

Column: normal-phase Microsorb silica column (250-4.6)

Column temperature: 25℃

Detector: ELSD-LT Ⅱ

Detector temperature: 40-42℃

Gas: N_2

Nitrogen flow pressure : 170-220 kpa，gain: 7

Flow rate: 1 mL/min

Pump: LC-20A

The procedure was that:

0-5 min: chloroform/methanol/30% ammonium hydroxide (80:19:1)

5-20 min: chloroform/methanol/30% ammonium hydroxide (60:39:1)

20-45 min: chloroform/methanol/water/30% ammonium hydroxide (60:34:5:1)

45-60 min: chloroform/methanol/30% ammonium hydroxide (80:19:1)

Each sample (ca. 1 mg) was dissolved in chloroform/methanol (2:1, v/v) and the 20 μL was injected onto the HPLC. Standard SM from milk (Nagara Science Co., Ltd, Oritate, Gifu, Japan) was used for the identification.

3.2.2 Animals and diets

Obese/diabetic KK-A^y mice (male, four weeks old) were obtained from Japan CREA Co., Tokyo, Japan. The mice were housed individually in an air-conditioned room (23±1 ℃ 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 6 groups of seven and were then fed experimental diets (**Table 3-1**) for four weeks. The composition of FA in the experimental diets **(Table 3-2)** was analyzed as described in 1.2.2 in **Chapter 1**. The body weight, diet and water intake of each mouse was recorded daily. Mice were sacrificed under diethyl ether anesthesia after 12 h fasting on day 28. Body weights after 12 h fasting on day 28 were determined.

3.2.3 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 carried out under the approval by the Ethical Committee of Experimental Animal Care at Hokkaido University.

3.2.4 Sample collection

The sample collection was done following the method as described in section 1.2.4 in **Chapter 1**.

3.2.5 Blood lipid analysis

The blood plasma analysis was performed by the Analytical Center of Hakodate Medical Association (Hakodate, Japan) as described in section 1.2.5 in **Chapter 1**.

Plasma TL was extracted and the FA composition was determined. 0.1 mL of plasma was added to the test tube containing 0.25 mL chloroform, 0.5 mL of methanol and 0.1ml double distilled water (chloroform/methanol/water, 1:2:0.8, v/v/v). After 1 min mixing by using the mixer, the test tube was allowed standing for 1h. Then the mixture was diluted with 0.25 mL chloroform and 0.25 mL double distilled water (chloroform/methanol/water, 1:1:0.9, v/v/v). After centrifugation at 3000 rpm for 15 min, the lower layer was collected to another test tube and used as plasma TL.

FA composition of plasma TL was determined following the method in section 1.2.6 in **Chapter 1**

3.2.6 Hepatic lipid analysis

The hepatic lipid parameters were determined following the method as described in section 1.2.6 in **Chapter 1**.

3.2.7 Brain lipid analysis

The extraction of brain TL, lipids fraction elution, and determination of FA composition was done following the method as described in section 1.2.6 in **Chapter 1**

3.2.8 Kidney lipid analysis

The extraction of kidney TL and determination of FA composition was done following the method as described in section 1.2.6 in **Chapter 1**

3.2.9 Feces lipid analysis

The extraction of faeces TL and determination of FA composition was done following the method as described in section 1.2.6 in **Chapter 1**

3.2.10 Quantitative real-time PCR

The mRNA expression of the related genes in the liver of Obese/diabetic KK-A^y mice were measured following the method as described in section 1.2.7 in **Chapter 1**.

The mRNA analyses were performed on genes associated with lipid metabolism, which included sterol regulatory element-binding protein 1 (SREBP1), stearoyl-CoA desaturase-1 (SCD1), nuclear receptor subfamily 1 group h member 3 (NR1H3),

nuclear receptor subfamily 1 group h member 2 (NR1H2), interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6). The gene-specific primers were Mm00550338_m1 (SREBP1), Mm00772290_m1 (SCD-1), Hs00172885_m1 (NR1H3), Hs01027215_g1 (NR1H2), Mm00434228_m1 (IL-1β), Dr03126850_m1 (TNFα), Hs00174131_m1 (IL-6) and Hs02786624_g1 (GAPDH; internal control), respectively.

3.2.11 Endogenous antioxidant status

The superoxide dismutase (SOD) activity was measured by using the SOD Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan). The analysis is based on the formation of water-soluble formazan dye produced by the reduction with superoxide anion (O_2^-). The dye formation can be recorded spectrophotometrically at 450 nm. The activity was expressed as units/g liver (one unit of SOD is defined as the amount of the enzyme in 20 μL of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%).

The glutathione (GSH) and oxidized glutathione (GSSG) were quantified respectively by using GSSG/GSH Quantification Kit (Dojindo Laboratories, Kumamoto, Japan). The GSH can be converted into its oxidized form (GSSG) by stimulation such as oxidative stress, so the ratio of GSH and GSSG was determined as index of oxidative stress.

3.2.12 Statistic analysis

Results are expressed as the mean ± SEM. Statistical significances between groups were evaluated by one-way ANOVA with *post hoc* comparisons (Šidák correction). Differences with $P < 0.05$ were considered statistically significant.

3.3 Results

3.3.1 Initial weight, weight gain, final body weight on day 28 and water intake

As shown in **Figure 3-1**, no difference was observed in initial body weights of KK-A^y mice between the different groups. Administration of 0.1% farnesol and 0.5% SM did not affect weight gain, final body weight, and total water intake of KK-A^y mice (**Figure 3-2**, **Figure 3-3** and **Figure 3-4**).

3.3.2 Plasma lipid parameters

As shown in **Table 3-3**, administration of 0.1% farnesol increased all plasma cholesterols levels (TC, HDL-C and non-HDL-C) in KK-A^y mice. On the other hand, these cholesterol levels decreased in KK-A^y mice fed 0.5% SM. As a result, a significant difference in these levels was observed between 0.1% farnesol group and 0.5% SM group. Administration of lard significantly increased plasma nonHDL-C level when comparing with the other groups (linseed oil group, 0.5% SM group, 0.1% farnesol group, 0.5% SM+0.1% farnesol group and fish oil group) (**Table 3-3**). The content of plasma PL was also increased in 0.1% farnesol group than in linseed oil group and 0.5% SM group (**Table 3-3**).

3.3.3 FA levels of plasma TL

To determine whether farnesol and SM can affect the FA metabolism, FA composition of plasma TL was measured (**Table 3-4**). As described in the introduction, the conversion of α-linolenic acid (18:3n-3) to EPA and DHA was mainly focused in the present study; however, there was no increase in EPA and DHA by farnesol and SM supplementation. On the other hand, there was a little effect of farnesol on other kinds of FA composition. The composition ratio of 18:0 in plasma TL was significantly

lower in 0.1% farnesol group than in other groups. In contrast, administration of 0.1% farnesol tended to increase the composition ratio of 18:1n-9 as compared with other groups except for lard group, but this difference was not significant. The increase in 18:1n-9 in 0.1% farnesol group increased total MUFA (**Table 3-4**). This finding may indicate that farnesol treatment induce the conversion to MUFA from SFA, especially from 18:0 to 18:1n-9. The composition of SFA and MUFA was significantly high in lard group than those of other groups. But, the composition ratio of PUFA n-3 was significantly low in this group (**Table 3-4**).

3.3.4 Tissue weights

The absolute weight and relative weight of major tissues were not significantly different, except for a significant decrease in the kidney weight of KK-A^y mice fed 0.5% SM than in linseed oil group and 0.5% SM+0.1% farnesol group (**Table 3-5, Table 3-6, Table 3-7 and Table 3-8**).

3.3.5 Hepatic lipid parameters

Although there was little difference in hepatic PL (**Figure 3-7**), some differences were found in hepatic TL (**Figure 3-5**), NL (**Figure 3-6**), and TC (**Figure 3-8**). As shown in **Figure 3-5** and **Figure 3-6**, 0.1% farnesol or 0.5% SM intake increased the content of hepatic TL and NL, but this increase was not significantly different with linseed oil group. The content of hepatic TC significantly decreased in 0.5% SM group and in 0.5% SM+0.1% farnesol group as compared with lard group (**Figure 3-8**). SM feeding also decreased plasma TC and non-HDL-C (**Table 3-3**). These results may indicate the cholesterol lowering effect of SM.

3.3.6 FA levels of liver lipids

Plasma MUFA composition ratio was increased by farnesol treatment (**Table 3-4**). To determine whether this effect was due to the increase in the conversion from SFA to MUFA in the liver, FA composition of hepatic TL was determined (**Table 3-9**). In 0.1% farnesol group, hepatic FA composition ratio of 18:1n-9 together with total MUFA was significantly higher than that in linseed oil group. On the contrary, 0.1% farnesol treatment decreased the composition ratio of 18:0 as compared with linseed oil group, although no significant difference was observed. The treatment of 0.1% farnesol dramatically decreased hepatic composition ratio of 18:2n-6 and total n-6 PUFA as compared with those in linseed oil group.

On the other hand, administration of 0.5% SM did not show any effect on hepatic FA composition of KK-A^y mice (**Table 3-9**).

To confirm the increase effect of farnesol on hepatic MUFA, hepatic TL was separated into NL and PL on a Sep-Pak Silica cartridge, and the composition of fatty acids in NL and PL was determined (**Table 3-10** and **Table 3-11**). In 0.1% farnesol group, the composition ratio of 18:1n-9 and total MUFA in NL were higher than those in linseed oil group (**Table 3-10**). Farnesol treatment also increased the composition ratio of total MUFA and 18:1n-9 in PL as compared with linseed oil group (**Table 3-11**). On the other hand, administration of 0.5% SM or 0.5% SM+0.1% farnesol significantly decreased the composition ratio of 18:1n-9 and total MUFA in hepatic NL comparing with linseed oil group and 0.1% farnesol group, respectively (**Table 3-10**).

3.3.7 Brain lipid parameters

As shown in **Figure 3-9**, **Figure 3-10** and **Figure 3-11**, there was little effect of 0.1% farnesol and 0.5% SM on the brain TL, NL and PL of KK-A^y mice.

3.3.8 FA levels of brain lipids

There was a little difference in the FA composition ratio in the brain of KK-A^y mice between different groups (**Table 3-12**, **Table 3-13** and **Table 3-14**).

3.3.9 Kidney lipid parameters

As shown in **Figure 3-12**, administration of 0.1% farnesol and 0.5% SM had no effect on kidney TL of KK-A^y mice.

3.3.10 FA levels of kidney TL

0.1% Farnesol supplementation significantly increased SFA composition ratio of the kidney lipids as comparing with linseed oil group (**Table 3-15**). Although significant difference was not observed, administration of 0.5% SM+0.1% farnesol also increased SFA composition ratio of the kidney lipids (**Table 3-15**). No significance was observed between 0.5% SM group and other groups.

3.3.11 Feces lipid parameters

As shown in **Figure 3-13**, administration of 0.1% farnesol and 0.5% SPH did not show any effect on the feces TL of KK-A^y mice.

3.3.12 FA levels of feces TL

Administration of 0.5% SM increased SFA composition ratio and decreased MUFA composition ratio in fecal lipids as comparing with other groups (**Table 3-16**), although there was no significant difference. This tendency was also observed in 0.5% SM+0.1% farnesol group, On the other hand, administration of 0.1% farnesol did not show any effect on the composition ratio of FA in feces lipids.

3.3.13 Gene expressions related to lipid metabolism

Farnesol affected the FA composition of hepatic lipids, especially, MUFA ratio such as C18:1n-9 (**Table 3-9**). To determine whether farnesol acts as an agonist of genes participating in the formation of MUFA, hepatic mRNA expression levels of SCD-1, SREBP-1c, NR1H3 and NR1H2 were measured. As shown in **Figure 3-14**, the mRNA expression level of SCD-1 significantly increased in the liver of 0.1% farnesol treated mice as compared with that of linseed oil treated mice. 0.1% Farnesol treatment did not change the mRNA levels of SREBP-1c, NR1H3 and NR1H2 gene in the liver of KK-A^y male mice. This finding indicates that farnesol may serve as a SCD-1 activator in liver of KK-A^y male mice. On the other hand, the mRNA expression level of SCD-1 in SM group was lower than that of linseed oil group.

Hepatic mRNA expression levels of cytokine involved in systemic inflammation (IL-1β，TNFα and IL-6) were also measured (**Figure 3-15**). As shown in **Figure 3-15**, administration of 0.1% farnesol and 0.5% SM did not show any effects on these mRNA expression levels.

3.3.14 Endogenous antioxidant status

Oxidative stress is thought to be involved in the development of many diseases, such as cancer, Alzheimer's disease and atherosclerosis, etc. It has been previously reported [160] that farnesol ameliorated LPS-induced oxidative stress by enhancing the antioxidant defense system as evident from the increased levels of SOD, CAT, GSH and GST in the cortex and hippocampus. In the present study, 0.1% farnesol and fish oil treatment increased hepatic level of SOD when comparing with linseed oil group (**Figure 3-16**). But, significant difference was not observed. On the other hand, no significant difference was observed in the level of GSH/GSSG (**Figure 3-17**).

Table 3-1 Composition of experimental diets (g kg^{-1})

Ingredient %	Lard	Linseed oil	0.5% SM	0.1% Farnasol	0.5% SM + 0.1% Farnesol	Fish oil
β-corn starch	224.586	224.586	224.586	224.586	224.586	224.586
α-corn starch	84.9	84.9	84.9	84.9	84.9	84.9
Milk casein	260	260	260	260	260	260
Sucrose	130	130	130	130	130	130
Cellulose	50	50	50	50	50	50
L-cystine	3	3	3	3	3	3
AIN-93G mineral mix	35	35	35	35	35	35
AIN-93G vitamin mix	10	10	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Ter-butyl hydroquinone	0.014	0.014	0.014	0.014	0.014	0.014
Lard	200	130	125	130	125	130
Linseed oil	0	70	70	70	70	0
Soybean oil	0	0	0	0	0	50
Fish oil	0	0	0	0	0	20
Sphingomyelin	0	0	5	0	5	0
Farnesol	0	0	0	1	1	0
Total	1000	1000	1000	1001	1001	1000

Table 3-2 Fatty acid composition of dietary lipids (%)

	Lard	Linseed oil	0.5% SM	0.1% Farnesol	0.5% SM + 0.1% Farnesol	Fish oil
14:0	1.57±0.01	1.02±0.05	1.01±0.05	1.00±0.04	1.32±0.05	1.16±0.05
16:0	25.95±0.29	19.13±0.42	18.93±0.36	19.21±0.36	18.91±0.36	20.94±0.41
16:1	2.42±0.04	1.50±0.05	1.45±0.04	1.52±0.04	1.45±0.03	1.75±0.04
18:0	15.98±0.10	12.42±0.09	12.48±0.08	12.49±0.17	12.53±0.15	12.46±0.13
18:1n-9	41.40±0.35	32.54±0.36	32.28±0.46	32.53±0.33	32.31±0.50	32.52±0.56
18:2n-6	8.65±0.04	11.27±0.06	11.35±0.05	11.23±0.03	11.33±0.05	19.37±0.15
18:3n-3	0.60±0.01	19.17±0.25	19.59±0.18	18.94±0.10	19.46±0.24	1.88±0.00
20:0	0.22±0.01	0.19±0.01	0.20±0.01	0.19±0.01	0.18±0.00	0.25±0.01
20:1n-9	0.74±0.02	0.51±0.01	0.55±0.01	0.56±0.02	0.51±0.02	0.76±0.02
20:2n-6	0.33±0.01	0.24±0.01	0.24±0.01	0.24±0.01	0.24±0.01	0.26±0.01
20:4n-6	0.13±0.01	0.10±0.00	0.10±0.00	0.11±0.00	0.10±0.00	0.32±0.01
20:5n-3	-	-	-	-	-	1.53±0.06
22:6n-3	-	-	-	-	-	2.71±0.19

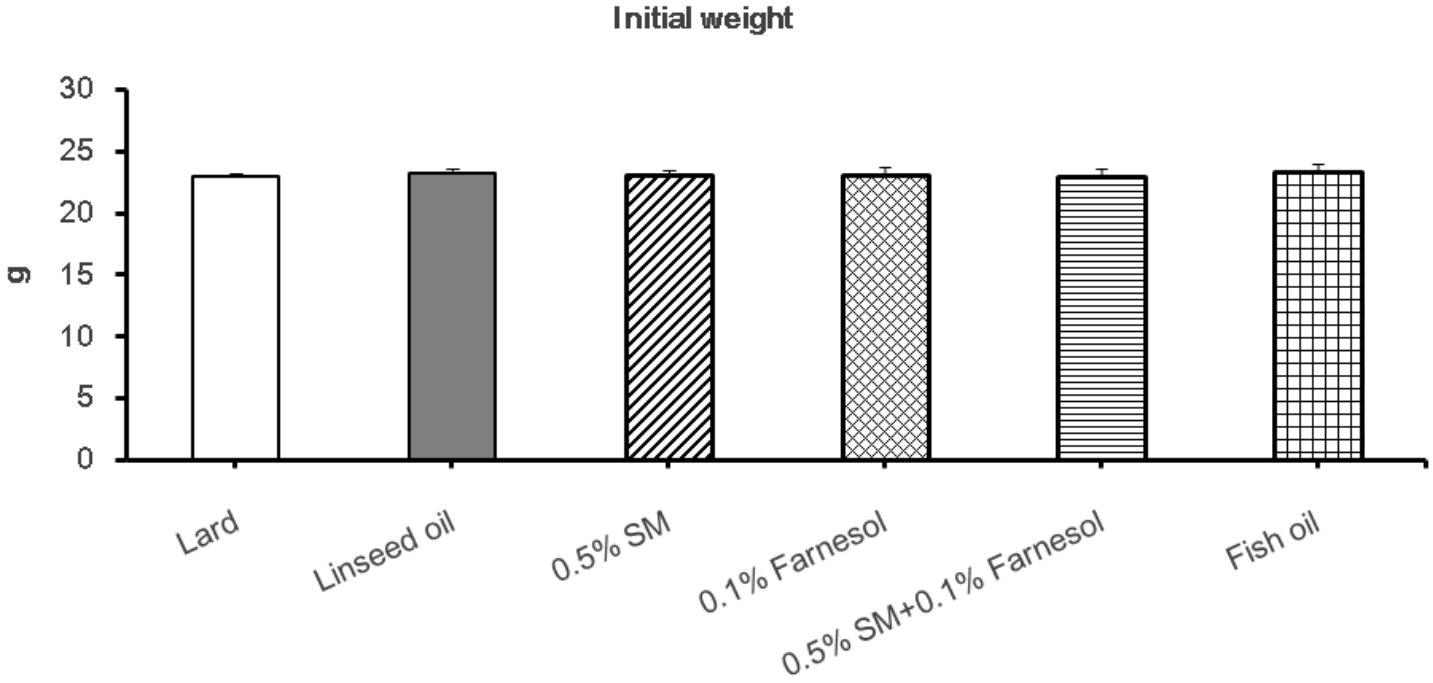

Figure 3-1 Initial weight of KK-A^y mice.

Initial weight of KK-A^y mice was determined after 12h fasting. Values are expressed as mean±SEM (n=7).

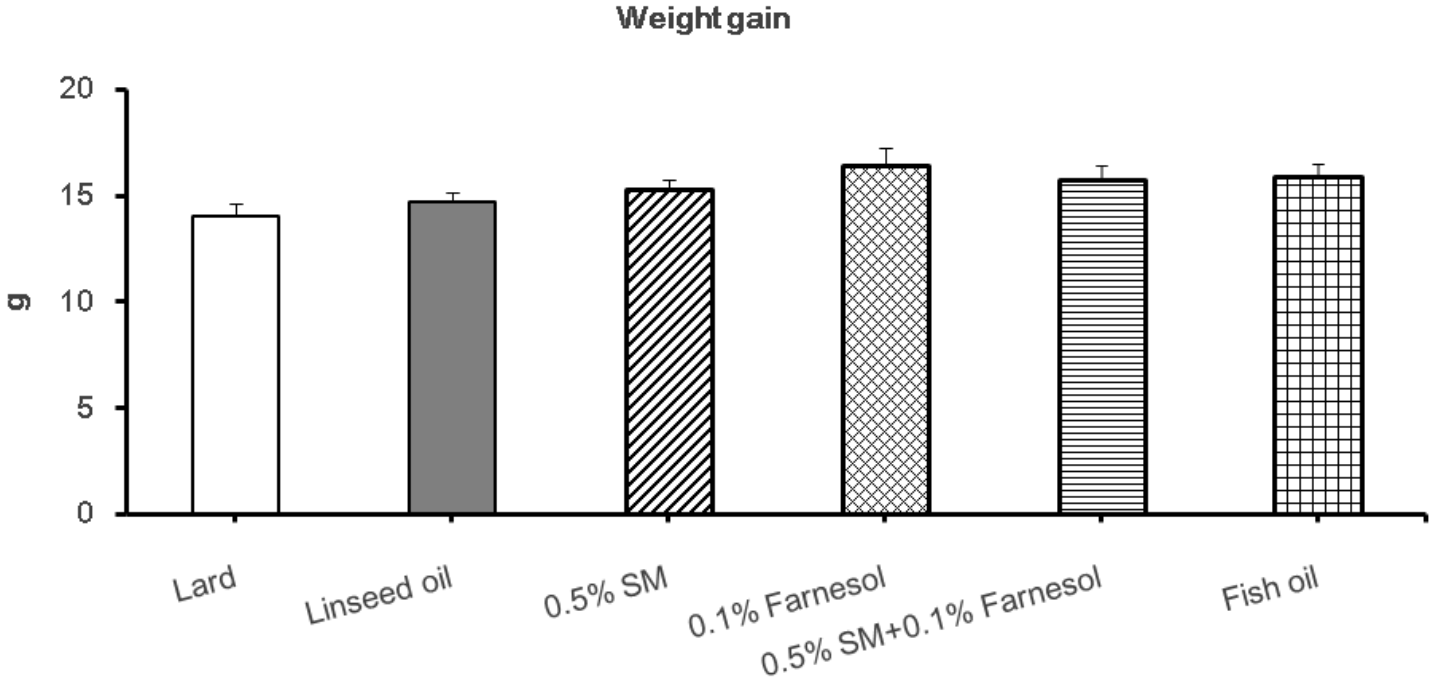

Figure 3-2 Weight gain of KK-A^y mice during experimental feeding.

Values are expressed as mean ± SEM (n=7).

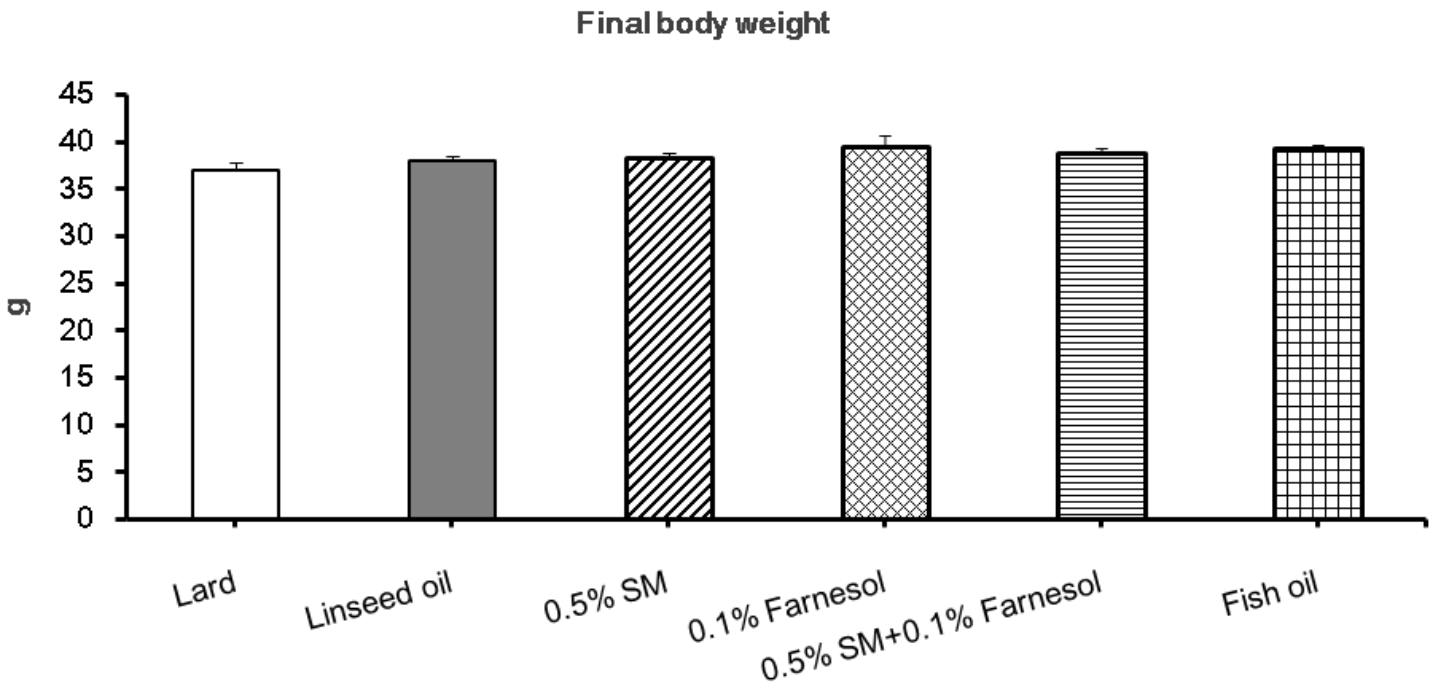

Figure 3-3 Final body weight of KK-A^y mice on day 28.

Weight of KK-A^y mice on day 28 was determined after 12h fasting. Values are expressed as mean ± SEM (n=7).

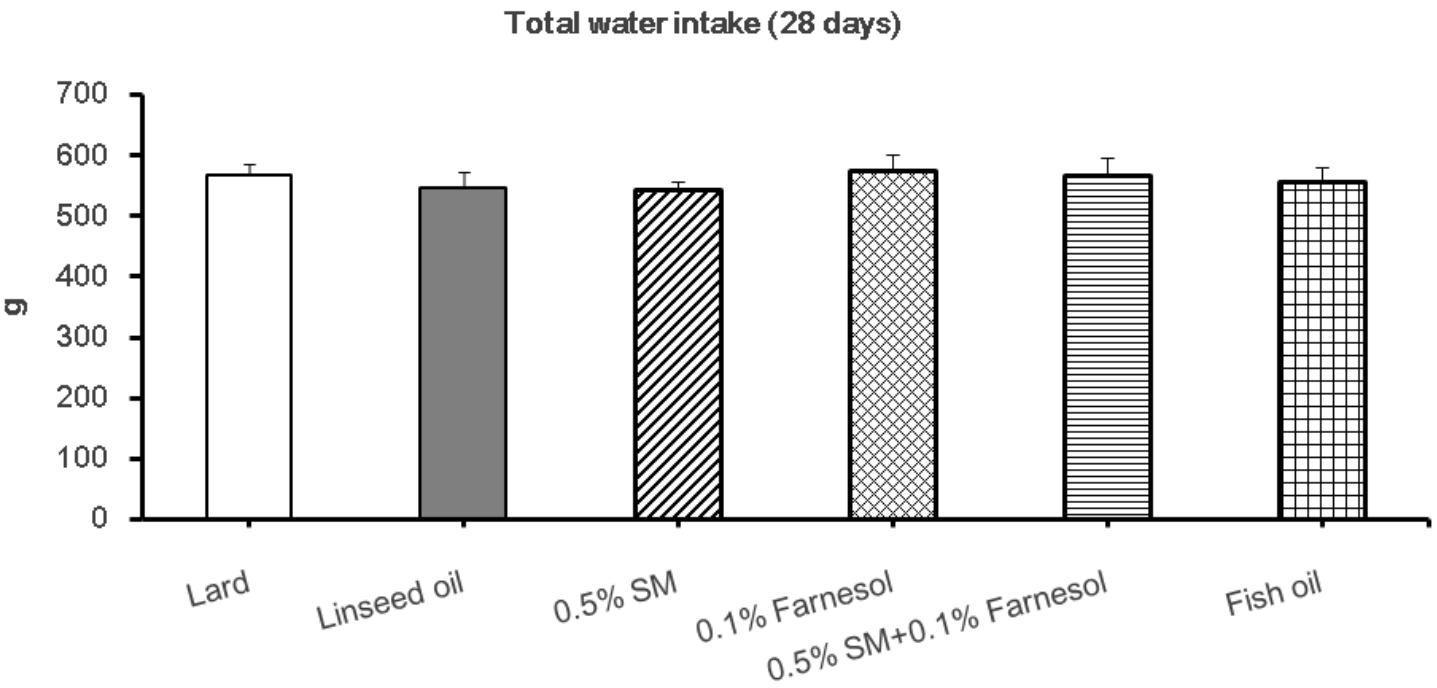

Figure 3-4 Total water intake of KK-A^y mice during 28 days feeding.

Water intake of each mouse was recorded daily. Values are expressed as mean ± SEM (n=7).

Table 3-3 Effect of farnesol and SM on plasma lipid parameter of KK-A^y mice.

		TAG (mg/dL)	PL (mg/dL)	TC (mg/dL)	HDL-C (mg/dL)	nonHDL-C (mg/dL)
KK-A^y mice	Lard	576.29±72.64	405.14±18.17^{ab}	189.43±8.92^{a}	102.86±5.84^{ab}	86.57±4.44^{a}
	Linseed oil	451.71±57.95	341.57±10.80^{b}	151.43±3.87^{b}	101.43±4.13^{ab}	50.00±2.37^{bc}
	0.5% SM	569.33±135.53	340.33±10.27^{b}	143.00±3.51^{b}	98.00±3.29^{b}	45.00±2.76^{c}
	0.1% Farnesol	751.71±131.83	418.14±26.06^{a}	189.57±13.72^{a}	124.71±9.46^{a}	64.86±6.63^{b}
	0.5% SM+0.1% Farnesol	706.14±132.32	370.29±13.02^{ab}	158.00±3.87^{ab}	108.57±4.04^{ab}	49.43±2.48^{bc}
	Fish oil	510.00±74.48	366.00±6.98^{ab}	171.00±4.21^{ab}	110.00±2.81^{ab}	61.00±3.12^{bc}

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 3-4 Effect of farnesol and SM on the fatty acid composition ratio of plasma TL of KK-A^y mice (%).

	Lard	Linseed oil	0.5% SM	0.1% Farnesol	0.5% SM + 0.1% Farnesol	Fish oil
16:0	25.11±0.49 [a]	21.56±0.27 [b]	22.23±0.24 [b]	21.83±0.35 [b]	21.64±0.37 [b]	23.02±0.21 [b]
18:0	11.35±0.14 [a]	12.19±0.31 [a]	11.09±0.33 [a]	10.79±0.25 [b]	11.03±0.38 [a]	11.62±0.27 [a]
18:1n-9	28.07±0.66 [a]	21.78±0.81 [bc]	22.19±0.70 [bc]	24.33±0.72 [b]	23.82±1.33 [bc]	20.25±0.59 [c]
20:1n-9	0.67±0.05	0.55±0.03	0.63±0.05	0.69±0.04	0.64±0.09	0.59±0.05
18:2n-6	16.67±0.49 [c]	19.83±0.68 [b]	19.25±0.79 [bc]	18.13±0.42 [bc]	18.14±1.05 [bc]	24.54±0.42 [a]
20:4n-6	8.12±0.41 [a]	3.55±0.42 [b]	3.03±0.34 [b]	3.07±0.19 [b]	2.67±0.29 [b]	4.08±0.16 [b]
18:3n-3	0.27±0.02 [b]	9.57±0.60 [a]	10.49±0.58 [a]	10.02±0.52 [a]	11.71±0.75 [a]	0.93±0.03 [b]
20:5n-3	0.17±0.01 [c]	2.21±0.21 [b]	2.07±0.21 [b]	2.02±0.14 [b]	1.63±0.16 [b]	2.97±0.08 [a]
22:5n-3	0.05±0.03 [c]	0.68±0.05 [a]	0.82±0.02 [a]	0.72±0.04 [a]	0.72±0.07 [a]	0.55±0.03 [b]
22:6n-3	2.16±0.21 [b]	2.44±0.26 [b]	2.25±0.15 [b]	2.25±0.17 [b]	2.04±0.20 [b]	6.06±0.33 [a]
SFA	38.22±0.46 [a]	35.28±0.33 [bc]	34.90±0.24 [bc]	34.15±0.49 [c]	34.27±0.47 [c]	36.20±0.30 [b]
MUFA	30.47±0.58 [a]	23.25±0.82 [bc]	23.97±0.87 [bc]	26.19±0.71 [b]	25.63±1.27 [b]	21.78±0.74 [c]
PUFA n-6	25.17±0.43 [b]	23.71±0.91 [bc]	22.63±1.11 [bc]	21.51±0.57 [bc]	21.15±1.30 [c]	28.97±0.59 [a]
PUFA n-3	2.65±0.23 [c]	14.90±0.21 [a]	15.63±0.28 [a]	15.00±0.32 [a]	16.11±0.40 [a]	10.52±0.38 [b]
n-6/n-3	9.84±0.65 [a]	1.60±0.08 [bc]	1.46±0.09 [c]	1.44±0.06 [c]	1.33±0.11 [c]	2.77±0.08 [b]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 3-5 Effect of farnesol and SM on absolute WAT weight of KK-A^y mice.

		WAT				
		Mesenteric	Epididymal	Perirenal	Inguinal	Total
		g				
KK-A^y mice	Lard	0.93±0.06	1.75±0.13	0.50±0.06	1.37±0.09	4.44±0.30
	Linseed oil	0.99±0.07	1.56±0.10	0.45±0.06	1.36±0.05	4.38±0.20
	0.5% SM	1.01±0.05	1.58±0.05	0.47±0.03	1.45±0.09	4.52±0.19
	0.1% Farnesol	1.02±0.03	1.74±0.04	0.58±0.08	1.57±0.07	4.92±0.15
	0.5% SM+0.1% Farnesol	0.91±0.13	1.61±0.09	0.60±0.07	1.54±0.03	4.66±0.17
	Fish oil	1.01±0.04	1.73±0.10	0.61±0.06	1.48±0.03	4.83±0.15

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at P< 0.05 among each value.

Table 3-6 Effect of farnesol and SM on absolute tissue weight of KK-A^y mice.

		Liver	Spleen	BAT	Small intestine	Pancreas	Kidney	Muscle	Heart	Brain
		g								
KK-A^y mice	Lard	2.01±0.08	0.08±0.00	0.24±0.01	1.50±0.09	0.17±0.02	0.63±0.01^{ab}	0.26±0.01	0.15±0.00	0.43±0.03
	Linseed oil	2.13±0.03	0.09±0.01	0.25±0.01	1.55±0.03	0.16±0.02	0.69±0.01^{a}	0.28±0.04	0.16±0.01	0.41±0.00
	0.5% SM	2.14±0.09	0.09±0.00	0.24±0.01	1.53±0.03	0.17±0.02	0.60±0.01^{b}	0.24±0.01	0.20±0.03	0.40±0.00
	0.1% Farnesol	2.32±0.09	0.09±0.01	0.25±0.01	1.62±0.05	0.16±0.01	0.65±0.02^{ab}	0.27±0.02	0.15±0.01	0.38±0.02
	0.5% SM+0.1% Farnesol	2.23±0.06	0.09±0.00	0.23±0.01	1.72±0.06	0.15±0.01	0.68±0.02^{a}	0.26±0.01	0.17±0.00	0.40±0.01
	Fish oil	2.21±0.06	0.09±0.00	0.25±0.01	1.55±0.06	0.16±0.01	0.67±0.02^{ab}	0.25±0.01	0.18±0.03	0.44±0.03

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at P< 0.05 among each value.

Table 3-7 Effect of farnesol and SM on relative WAT weight of KK-A^y mice.

		WAT				
		Mesenteric	Epididymal	Perirenal	Inguinal	Total
		g per 100g body weight				
KK-A^y mice	Lard	2.52±0.12	4.70±0.29	1.35±0.13	3.69±0.18	11.89±0.50
	Linseed oil	2.61±0.18	4.11±0.25	1.20±0.16	3.60±0.15	11.53±0.54
	0.5% SM	2.64±0.12	4.12±0.11	1.22±0.08	3.78±0.21	11.76±0.39
	0.1% Farnesol	2.59±0.08	4.42±0.15	1.48±0.20	3.97±0.15	12.46±0.38
	0.5% SM+0.1% Farnesol	2.35±0.34	4.15±0.23	1.54±0.18	3.97±0.09	12.02±0.48
	Fish oil	2.56±0.09	4.39±0.23	1.55±0.13	3.76±0.08	12.27±0.32

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at P< 0.05 among each value.

Table 3-8 Effect of farnesol and SM on relative tissue weight of KK-A^y mice.

		Liver	Spleen	BAT	Small intestine	Pancreas	Kidney	Muscle	Heart	Brain
		g per 100g body weight								
KK-A^y mice	Lard	5.42±0.15	0.21±0.01	0.66±0.03	1.69±0.02^{ab}	4.07±0.17	0.46±0.07^{abc}	0.70±0.04	0.42±0.01	1.16±0.11
	Linseed oil	5.60±0.09	0.25±0.02	0.66±0.03	1.81±0.02^{ab}	4.10±0.08	0.41±0.04^{a}	0.76±0.11	0.42±0.02	1.07±0.02
	0.5% SM	5.56±0.19	0.23±0.01	0.62±0.03	1.56±0.03^{ab}	4.00±0.04	0.44±0.06^{c}	0.63±0.02	0.52±0.09	1.05±0.01
	0.1% Farnesol	5.85±0.12	0.23±0.01	0.64±0.03	1.63±0.03^{ab}	4.10±0.10	0.41±0.02^{bc}	0.67±0.04	0.38±0.01	0.96±0.05
	0.5% SM+0.1% Farnesol	5.75±0.11	0.22±0.01	0.60±0.02	1.75±0.05^{a}	4.43±0.11	0.38±0.03^{ab}	0.66±0.02	0.43±0.01	1.02±0.01
	Fish oil	5.62±0.14	0.22±0.01	0.65±0.03	1.69±0.05^{b}	3.93±0.13	0.41±0.03^{abc}	0.63±0.02	047±0.09	1.11±0.08

Values are expressed as mean ± SEM (n=7). Different letters in the same row indicate significant difference at P< 0.05 among each value.

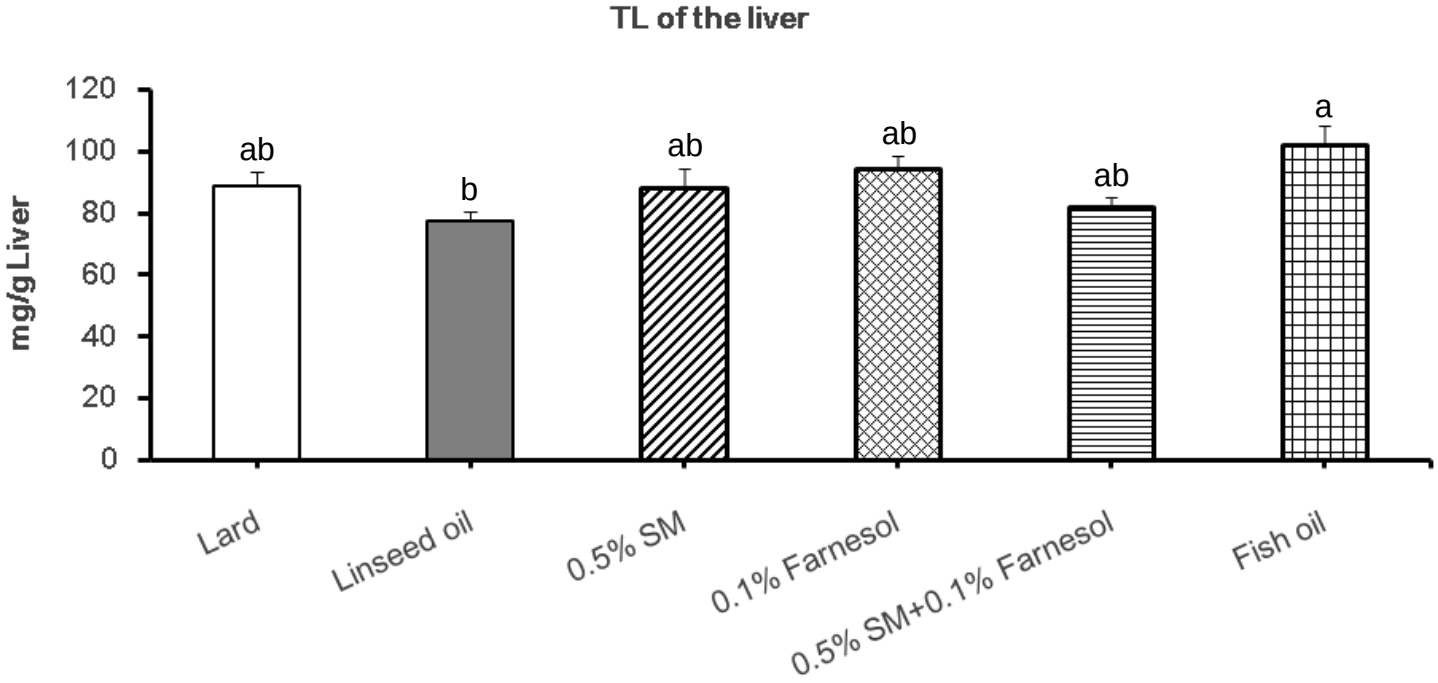

Figure 3-5 Effect of farnesol and SM on the hepatic TL content of KK-A^y mice.

TL was extracted from liver with chloroform/methanol (2:1, v/v). Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at P< 0.05 among each value.

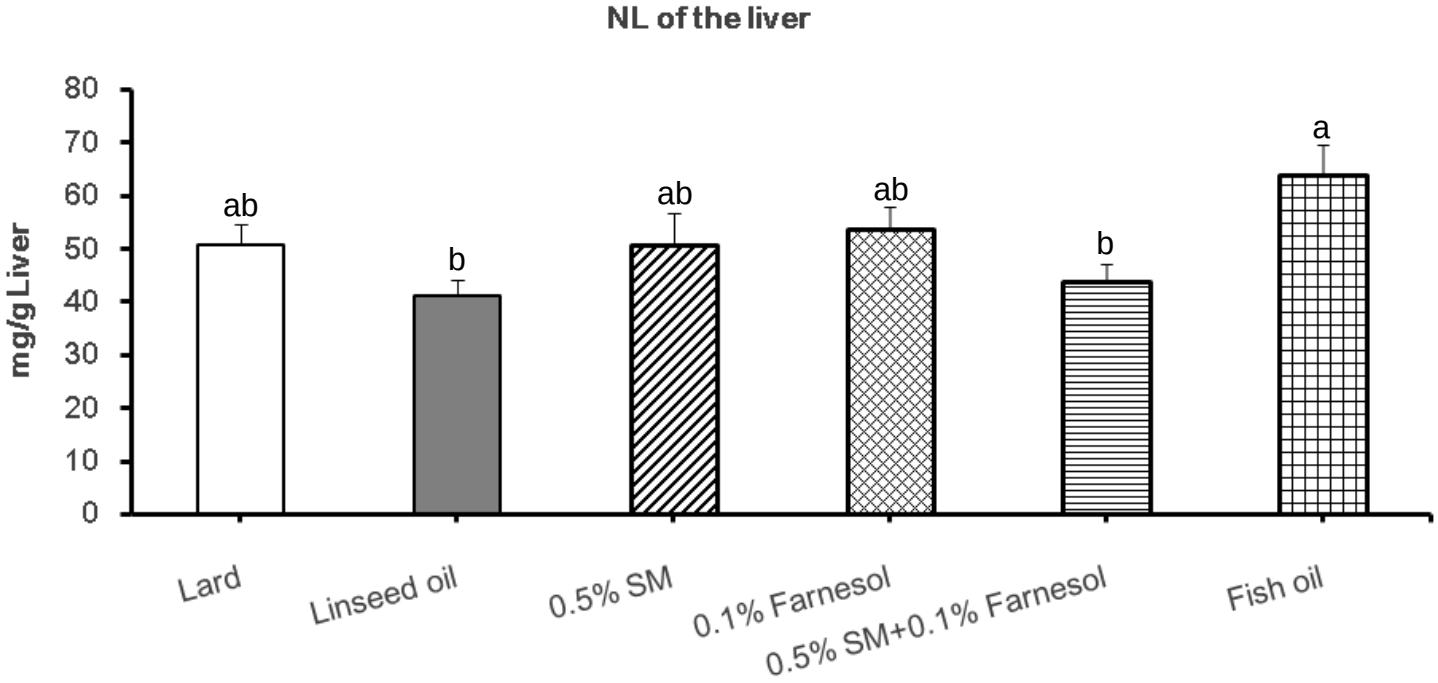

Figure 3-6 Effect of farnesol and SM on the hepatic NL content of KK-A^y mice.

The TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridge by elution with chloroform and methanol. The NL fraction was eluted with 70 mL chloroform. Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at $P < 0.05$ among each value.

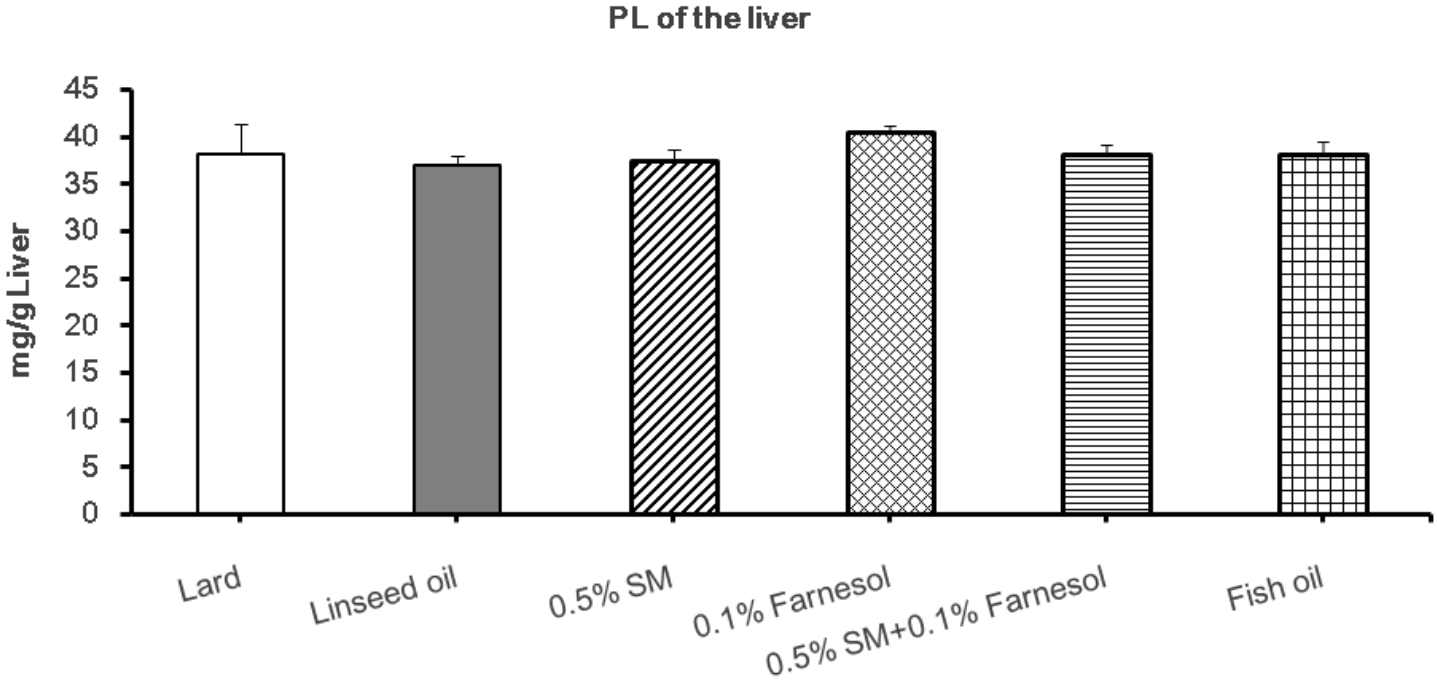

Figure 3-7 Effect of farnesol and SM on the hepatic PL content of KK-A^y mice.

The TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridgeby elution with chloroform and methanol. The PL fraction was eluted with 50mL methanol. Values are expressed as mean ± SEM (n=7).

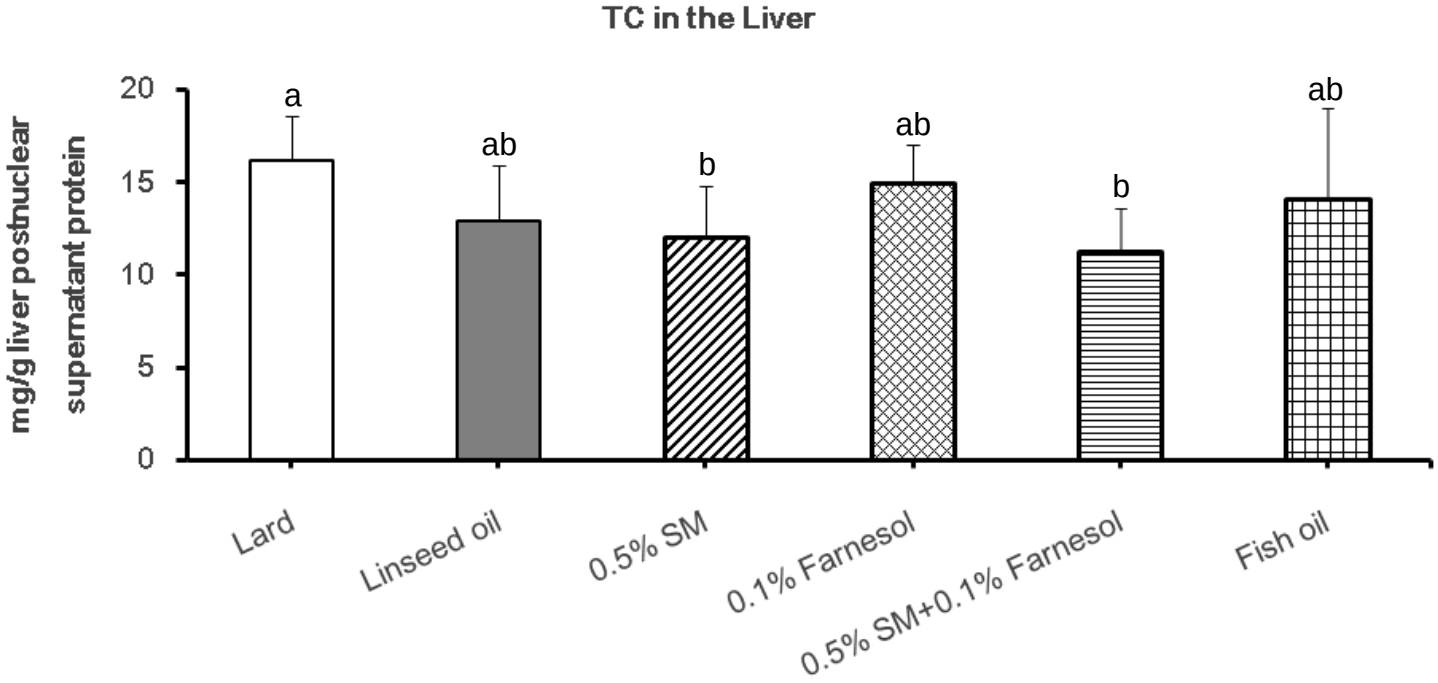

Figure 3-8 Effect of farnesol and SM on the hepatic TC content of KK-A^y mice.

TC was measured using enzymatic kits (Cholesterol E-test, Wako Pure Chemical Industries, Osaka, Japan). Values are expressed as mean ± SEM (n=7). Different letters indicate significant difference at P< 0.05 among each value.

Table 3-9 Effect of farnesol and SM on the fatty acid composition ratio in hepatic TL of KK-A^y mice (%).

	Lard	Linseed oil	0.5% SM	0.1% Farnesol	0.5% SM + 0.1% Farnesol	Fish oil
16:0	25.12±0.49 ^{a}	21.19±0.44 ^{c}	22.17±0.47 ^{bc}	22.09±0.41 ^{bc}	22.56±0.31 ^{bc}	23.44±0.25 ^{ab}
16:1	1.38±0.08 ^{a}	0.99±0.05 ^{bc}	0.86±0.04 ^{c}	1.13±0.07 ^{b}	0.82±0.04 ^{c}	0.82±0.04 ^{c}
18:0	7.90±0.31 ^{ab}	9.45±0.47 ^{a}	8.67±0.44 ^{ab}	8.10±0.39 ^{ab}	8.97±0.41 ^{ab}	7.63±0.36 ^{b}
18:1n-9	37.74±0.83 ^{a}	25.32±0.71 ^{c}	24.98±0.64 ^{c}	29.25±0.64 ^{b}	24.57±0.65 ^{c}	24.23±0.66 ^{c}
20:1n-9	1.09±0.01 ^{a}	0.80±0.02 ^{bc}	0.73±0.02 ^{c}	0.90±0.04 ^{b}	0.70±0.02 ^{c}	0.72±0.02 ^{c}
18:2n-6	10.88±0.27 ^{d}	14.32±0.36 ^{b}	14.73±0.21 ^{b}	13.01±0.34 ^{c}	14.56±0.16 ^{b}	19.51±0.24 ^{a}
20:4n-6	6.26±0.40 ^{a}	4.54±0.23 ^{b}	4.22±0.36 ^{b}	3.76±0.21 ^{b}	4.35±0.20 ^{b}	3.61±0.24 ^{b}
18:3n-3	-	7.14±0.21 ^{a}	8.13±0.28 ^{a}	7.25±0.55 ^{a}	7.51±0.17 ^{a}	0.94±0.04 ^{b}
20:5n-3	0.11±0.01 ^{c}	2.36±0.13 ^{a}	2.26±0.12 ^{a}	2.04±0.12 ^{ab}	2.21±0.10 ^{ab}	1.82±0.06 ^{b}
22:5n-3	-	1.77±0.05 ^{b}	2.11±0.07 ^{a}	1.59±0.07 ^{b}	2.20±0.05 ^{a}	1.34±0.06 ^{c}
22:6n-3	3.13±0.12 ^{c}	5.37±0.20 ^{b}	5.23±0.30 ^{b}	4.60±0.15 ^{b}	5.52±0.20 ^{b}	10.05±0.33 ^{a}
SFA	33.02±0.38 ^{a}	30.64±0.44 ^{b}	30.83±0.37 ^{b}	30.19±0.60 ^{b}	31.53±0.28 ^{ab}	31.08±0.52 ^{ab}
MUFA	40.22±0.85 ^{a}	27.11±0.74 ^{c}	26.56±0.67 ^{c}	31.28±0.72 ^{b}	26.09±0.68 ^{c}	25.77±0.69 ^{c}
PUFA n-6	17.14±0.53 ^{bc}	18.86±0.52 ^{b}	18.96±0.55 ^{b}	16.78±0.32 ^{c}	18.92±0.30 ^{b}	23.11±0.17 ^{a}
PUFA n-3	3.25±0.13 ^{d}	16.64±0.35 ^{ab}	17.73±0.22 ^{a}	15.49±0.49 ^{bc}	17.44±0.32 ^{a}	14.15±0.42 ^{c}
n-6/n-3	5.29±0.10 ^{a}	1.13±0.02 ^{c}	1.07±0.03 ^{c}	1.09±0.03 ^{c}	1.09±0.02 ^{c}	1.64±0.04 ^{b}

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 3-10 Effect of farnesol and SM on the fatty acid composition ratio in hepatic NL of KK-A^y mice (%).

	Lard	Linseed oil	0.5% SM	0.1% Farnesol	0.5% SM + 0.1% Farnesol	Fish oil
16:0	25.47±0.66 [a]	21.89±0.61 [b]	22.72±0.38 [b]	22.35±0.62 [b]	23.51±0.44 [ab]	23.17±0.37 [ab]
16:1	1.71±0.08 [a]	1.29±0.06 [bc]	1.08±0.04 [cd]	1.39±0.10 [b]	1.09±0.05 [cd]	0.94±0.04 [d]
18:0	3.45±0.11 [ab]	3.49±0.17 [a]	3.47±0.03 [a]	2.98±0.17 [b]	3.2±0.06 [ab]	3.17±0.07 [ab]
18:1n-9	47.51±0.56 [a]	35.77±0.66 [bc]	32.78±0.25 [d]	38.16±0.66 [b]	33.19±0.49 [cd]	30.79±0.78 [d]
20:1n-9	1.49±0.05 [a]	1.13±0.03 [b]	0.97±0.01 [c]	1.13±0.03 [b]	0.94±0.03 [c]	0.92±0.02 [c]
18:2n-6	10.50±0.31 [d]	14.10±0.39 [bc]	14.66±0.22 [b]	12.77±0.39 [c]	14.56±0.27 [b]	20.62±0.27 [a]
20:4n-6	1.24±0.07 [a]	0.72±0.04 [bc]	0.79±0.04 [b]	0.60±0.02 [c]	0.79±0.02 [b]	0.88±0.05 [b]
18:3n-3	0.26±0.01 [c]	9.72±0.19 [ab]	10.94±0.16 [a]	9.63±0.59 [b]	10.49±0.26 [ab]	1.19±0.03 [c]
20:5n-3	0.07±0.00 [d]	1.21±0.03 [bc]	1.37±0.03 [ab]	1.11±0.07 [c]	1.31±0.04 [ab]	1.46±0.06 [a]
22:5n-3	0.17±0.03 [d]	1.68±0.06 [bc]	2.03±0.10 [a]	1.44±0.09 [c]	1.93±0.05 [ab]	1.50±0.07 [c]
22:6n-3	0.88±0.07 [c]	2.17±0.12 [b]	2.23±0.13 [b]	1.80±0.12 [b]	2.12±0.08 [b]	7.67±0.41 [a]
SFA	31.43±0.66 [a]	27.62±0.49 [b]	28.60±0.39 [b]	27.08±0.49 [b]	28.91±0.49 [b]	28.45±0.35 [b]
MUFA	51.19±0.60 [a]	38.56±0.66 [b]	35.19±0.27 [c]	41.06±0.77 [b]	35.57±0.47 [c]	33.00±0.83 [c]
PUFA n-6	12.25±0.37 [d]	15.19±0.41 [bc]	15.84±0.26 [b]	13.70±0.41 [cd]	15.73±0.27 [b]	22.11±0.30 [a]
PUFA n-3	1.38±0.12 [d]	14.79±0.31 [ab]	16.57±0.23 [a]	13.98±0.85 [b]	15.85±0.30 [ab]	11.82±0.54 [c]
n-6/n-3	9.13±0.59 [a]	1.03±0.02 [b]	0.96±0.02 [b]	0.99±0.04 [b]	0.99±0.03 [b]	1.89±0.07 [b]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 3-11 Effect of farnesol and SM on the fatty acid composition ratio in hepatic PL of KK-A^y mice (%).

	Lard	Linseed oil	0.5% SM	0.1% Farnesol	0.5% SM + 0.1% Farnesol	Fish oil
16:0	19.93±0.45	19.34±0.55	19.93±0.56	18.16±0.15	19.37±0.41	19.49±0.52
16:1	2.64±0.09 [a]	1.58±0.34 [b]	2.15±0.21 [ab]	2.35±0.04 [ab]	2.39±0.05 [ab]	2.29±0.07 [ab]
18:0	18.96±0.42 [b]	21.10±0.39 [a]	21.14±0.25 [a]	20.36±0.18 [a]	20.28±0.14 [ab]	20.21±0.24 [ab]
18:1n-9	9.88±0.23 [a]	7.74±0.14 [b]	6.97±0.13 [c]	7.83±0.15 [b]	6.98±0.07 [c]	5.93±0.34 [d]
18:1n-7	3.28±0.03	2.21±0.41	2.73±0.26	3.14±0.04	2.96±0.05	2.92±0.05
18:2n-6	9.49±0.42 [c]	14.16±0.46 [ab]	13.50±0.26 [ab]	12.72±0.34 [b]	13.45±0.21 [ab]	14.65±0.51 [a]
18:3n-6	1.15±0.05	1.05±0.28	1.44±0.15	1.53±0.04	1.60±0.04	1.68±0.05
20:4n-6	18.19±0.59 [a]	10.92±0.19 [b]	10.32±0.24 [b]	10.78±0.22 [b]	10.39±0.33 [b]	10.27±0.23 [b]
18:3n-3	-	1.11±0.06 [a]	1.11±0.07 [a]	0.94±0.06 [a]	1.05±0.06 [a]	0.10±0.01 [b]
20:5n-3	0.17±0.01 [c]	3.89±0.20 [a]	3.44±0.09 [a]	3.85±0.18 [a]	3.28±0.12 [a]	2.37±0.10 [b]
22:5n-3	0.39±0.03 [c]	1.54±0.09 [b]	1.74±0.05 [ab]	1.54±0.06 [b]	1.96±0.06 [a]	0.66±0.01 [c]
22:6n-3	8.59±0.09 [c]	9.76±0.31 [bc]	9.71±0.33 [bc]	9.94±0.24 [b]	10.05±0.16 [b]	13.49±0.30 [a]
SFA	40.32±0.65	41.90±0.91	42.50±0.69	40.13±0.16	41.08±0.35	41.07±0.35
MUFA	16.01±0.27 [a]	11.72±0.69 [c]	12.02±0.44 [bc]	13.53±0.12 [b]	12.48±0.05 [bc]	11.31±0.37 [c]
PUFA n-6	29.23±0.36 [a]	26.29±0.45 [b]	25.41±0.31 [b]	25.19±0.46 [b]	25.58±0.25 [b]	26.83±0.54 [b]
PUFA n-3	9.15±0.11 [b]	16.29±0.57 [a]	16.01±0.28 [a]	16.26±0.35 [a]	16.34±0.22 [a]	16.62±0.20 [a]
n-6/n-3	3.20±0.06 [a]	1.63±0.07 [b]	1.59±0.03 [b]	1.56±0.06 [b]	1.57±0.02 [b]	1.61±0.02 [b]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

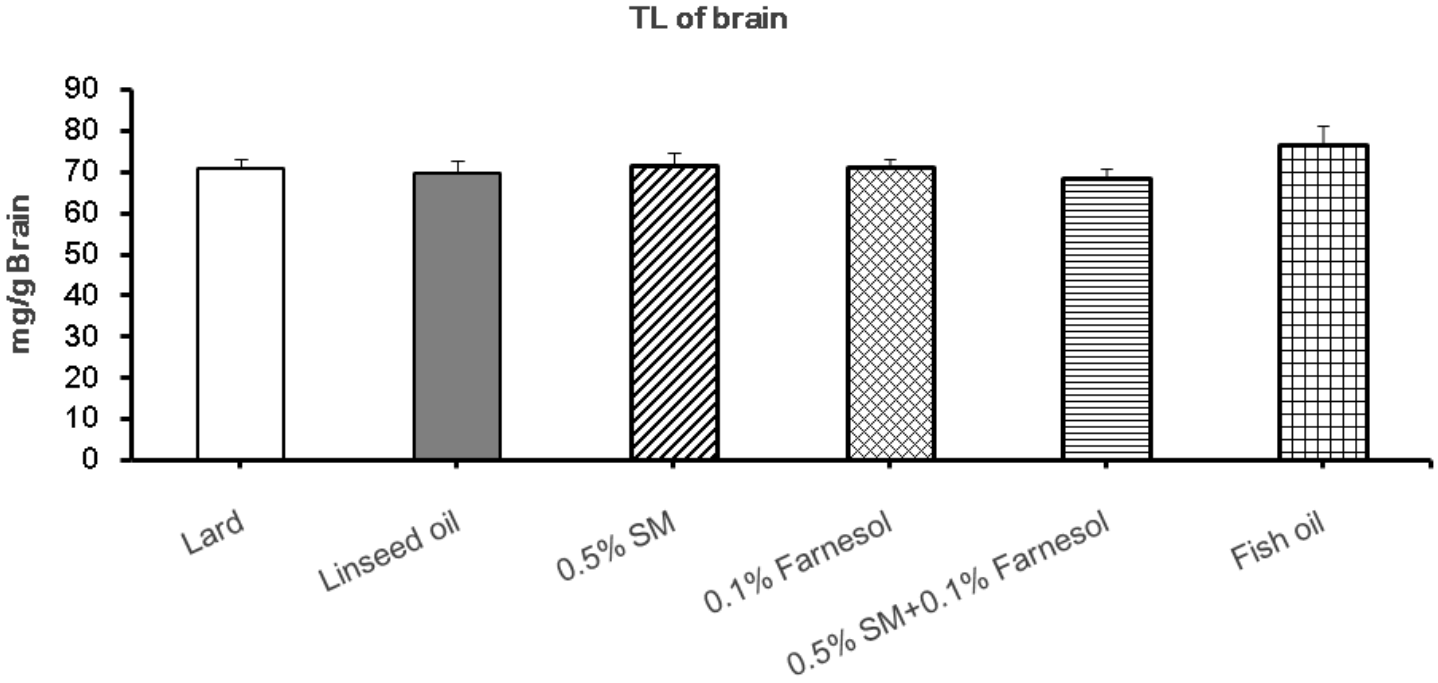

Figure 3-9 Effect of farnesol and SM on the brain TL content of KK-A^y mice.

TL was extracted from brain with chloroform/methanol (2:1, v/v). Values are expressed as mean ± SEM (n=7).

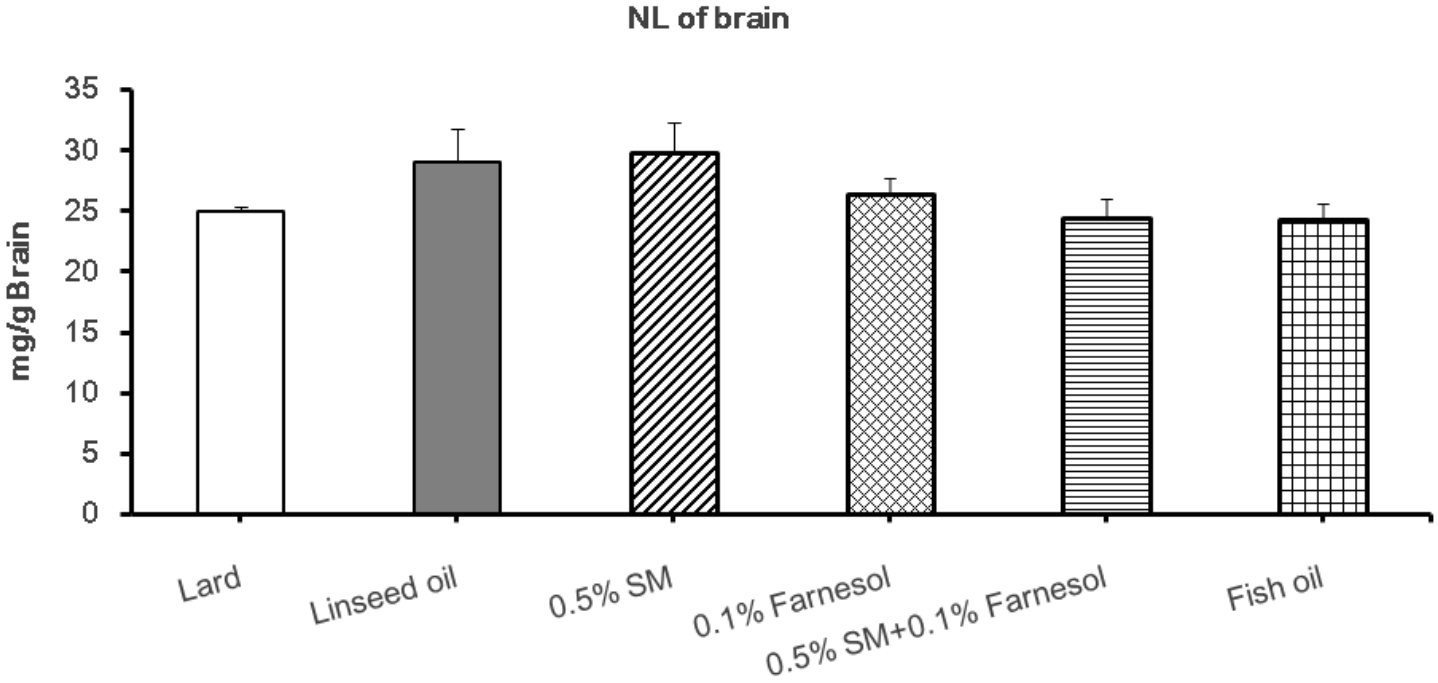

Figure 3-10 Effect of farnesol and SM on the brain NL content of KK-A^y mice.

The TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridge by elution with chloroform and methanol. The NL fraction was eluted with 70 mL chloroform. Values are expressed as mean ± SEM (n=7).

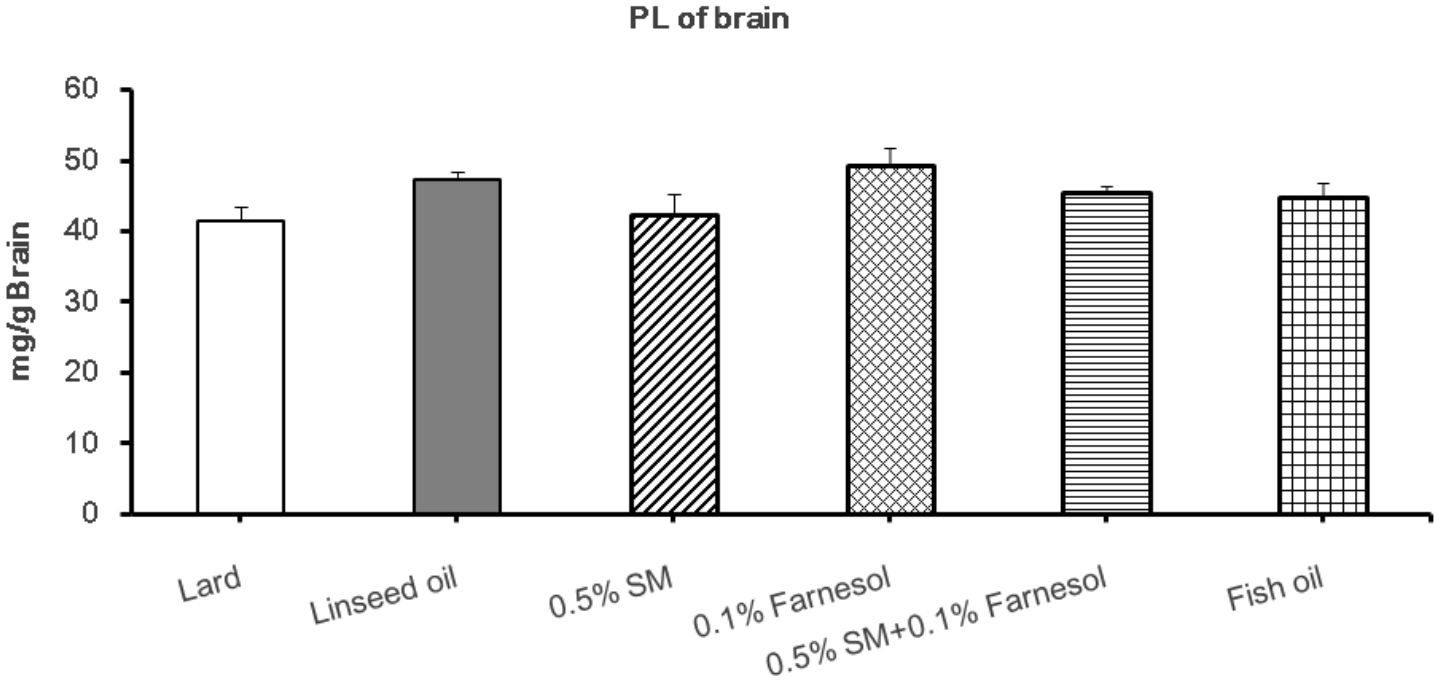

Figure 3-11 Effect of farnesol and SM on the brain PL content of KK-A^y mice.

The TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridge by elution with chloroform and methanol. The PL fraction was eluted with 50 mL methanol. Values are expressed as mean ± SEM (n=7).

Table 3-12 Effect of farnesol and SM on the fatty acid composition ratio in brain TL of KK-A^y mice (%).

	Lard	Linseed oil	0.5% SM	0.1% Farnesol	0.5% SM + 0.1% Farnesol	Fish oil
16:0	23.28±0.62	23.60±0.39	22.44±0.71	23.10±0.47	22.22±0.71	21.03±0.91
18:0	20.13±0.54	21.18±0.17	20.64±0.31	20.85±0.34	21.19±0.22	20.88±0.50
18:1n-9	19.62±0.81	17.41±0.35	18.38±0.68	18.02±0.63	17.37±0.42	18.37±1.11
18:1n-7	4.40±0.19 [a]	3.44±0.06 [b]	3.61±0.15 [b]	3.55±0.10 [b]	3.57±0.20 [b]	3.57±0.17 [b]
20:1n-9	2.30±0.30	1.73±0.16	2.17±0.30	1.77±0.08	1.86±0.19	2.45±0.50
20:4n-6	8.83±0.38	8.92±0.17	8.19±0.37	9.00±0.23	9.14±0.18	8.22±0.50
22:6n-3	13.13±0.74	15.53±0.32	14.51±0.61	14.76±1.14	15.82±0.65	16.48±0.88
SFA	43.41±0.84	44.78±0.52	43.08±0.94	43.95±0.37	43.41±0.84	41.91±1.39
MUFA	26.32±1.00	22.58±0.54	24.15±1.11	23.34±0.77	22.79±0.76	24.39±1.77
PUFA n-6	8.83±0.38	8.92±1.67	8.19±0.37	9.00±0.23	9.14±0.18	8.22±0.50
PUFA n-3	13.13±0.74	15.53±0.32	14.52±0.61	14.76±1.14	15.82±0.65	16.48±0.88
n-6/n-3	0.68±0.03 [a]	0.58±0.01 [ab]	0.57±0.02 [ab]	0.63±0.05 [a]	0.58±0.02 [ab]	0.50±0.01 [b]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 3-13 Effect of farnesol and SM on the fatty acid composition ratio in brain NL of KK-A^y mice (%).

	Lard	Linseed oil	0.5% SM	0.1% Farnesol	0.5% SM + 0.1% Farnesol	Fish oil
16:0	14.01±1.60	11.99±0.62	14.50±0.45	12.35±0.42	12.68±0.80	12.76±0.18
16:1	2.79±0.05	2.40±0.36	3.60±1.18	2.83±0.10	2.77±0.12	2.51±0.19
18:0	9.62±0.78	12.46±1.30	9.06±1.65	10.73±0.34	10.40±0.32	11.60±0.86
20:0	0.85±0.21	1.01±0.05	1.02±0.39	0.86±0.12	0.89±0.12	1.18±0.15
21:0	1.10±0.03	1.32±0.11	1.14±0.28	1.29±0.06	1.35±0.08	1.59±0.16
18:1n-9	24.83±0.24	23.45±1.65	27.61±2.35	25.82±0.47	24.79±0.68	24.41±0.71
18:1n-7	4.76±0.31	4.51±0.30	4.52±1.02	4.04±0.28	4.38±0.40	4.95±0.41
18:2n-6	4.75±0.31	5.01±0.67	7.63±1.61	6.38±0.32	6.10±0.30	6.81±0.62
20:4n-6	11.01±1.18	10.44±0.21	7.57±1.16	11.00±0.23	10.83±0.83	11.44±0.72
18:3n-3	0.77±0.36	0.80±0.04	3.33±1.34	2.54±0.51	2.13±0.54	1.03±0.38
20:5n-3	-	0.06±0.06	0.04±0.04	0.12±0.02	0.17±0.04	0.10±0.05
22:6n-3	9.53±0.54	13.42±1.98	8.18±1.67	11.24±0.54	11.19±0.37	8.89±1.55
SFA	29.07±3.31	30.37±0.77	27.18±2.83	26.76±0.64	26.48±1.24	27.09±0.88
MUFA	33.85±0.79	31.56±1.61	37.05±2.54	34.91±0.05	34.10±0.42	32.20±0.75
PUFA n-6	16.13±1.26	15.16±0.79	15.38±0.82	17.46±0.06	17.52±1.04	17.14±0.39
PUFA n-3	10.58±0.20	14.47±1.85	12.06±0.50	13.72±0.50	13.52±1.14	13.23±0.87
n-6/n-3	1.52±0.09	1.09±0.17	1.28±0.08	1.28±0.05	1.31±0.11	1.31±0.07

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

Table 3-14 Effect of farnesol and SM on the fatty acid composition ratio in brain PL of KK-A^y mice (%).

	Lard	Linseed oil	0.5% SM	0.1% Farnesol	0.5% SM + 0.1% Farnesol	Fish oil
16:0	22.09±0.22	22.36±0.64	21.87±0.98	23.55±0.31	22.55±0.63	22.70±0.67
16:1	0.43±0.06	0.46±0.05	0.51±0.01	0.49±0.06	0.48±0.06	0.51±0.01
18:0	20.86±0.29	20.83±0.13	20.48±0.44	21.41±0.20	20.92±0.32	21.09±0.09
20:0	0.45±0.05	0.49±0.04	0.50±0.11	0.36±0.03	0.41±0.05	0.43±0.04
21:0	0.44±0.02	0.55±0.01	0.54±0.05	0.49±0.01	0.53±0.05	0.57±0.02
18:1n-9	16.99±0.95	18.45±0.40	18.17±1.36	16.50±0.63	17.30±0.77	17.21±0.55
18:1n-7	3.56±0.12	3.55±0.09	3.54±0.25	3.32±0.04	3.31±0.13	3.48±0.10
20:1n-9	2.42±0.36	2.60±0.18	2.73±0.66	1.87±0.23	2.21±0.30	2.29±0.22
18:2n-6	0.44±0.02[b]	0.66±0.04[a]	0.62±0.03[a]	0.60±0.02[a]	0.61±0.02[a]	0.71±0.02[a]
20:4n-6	8.86±0.39	7.69±0.30	7.72±0.69	8.51±0.30	8.31±0.32	7.72±0.21
22:6n-3	15.31±1.18	15.03±0.78	15.87±1.11	16.04±0.84	16.34±0.78	16.78±0.77
SFA	44.47±0.06	44.87±0.63	44.06±1.18	46.35±0.24	45.00±0.83	45.38±0.70
MUFA	23.61±1.47	25.30±0.55	25.19±2.29	22.36±0.89	23.48±1.25	23.71±0.86
PUFA n-6	9.49±0.34	8.58±0.27	8.58±0.61	9.28±0.27	9.11±0.30	8.65±0.17
PUFA n-3	15.41±1.16	15.75±0.82	16.62±1.11	16.70±0.86	17.11±0.81	17.21±0.76
n-6/n-3	0.62±0.03[a]	0.55±0.02[ab]	0.52±0.02[ab]	0.56±0.01[ab]	0.53±0.02[ab]	0.50±0.02[b]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

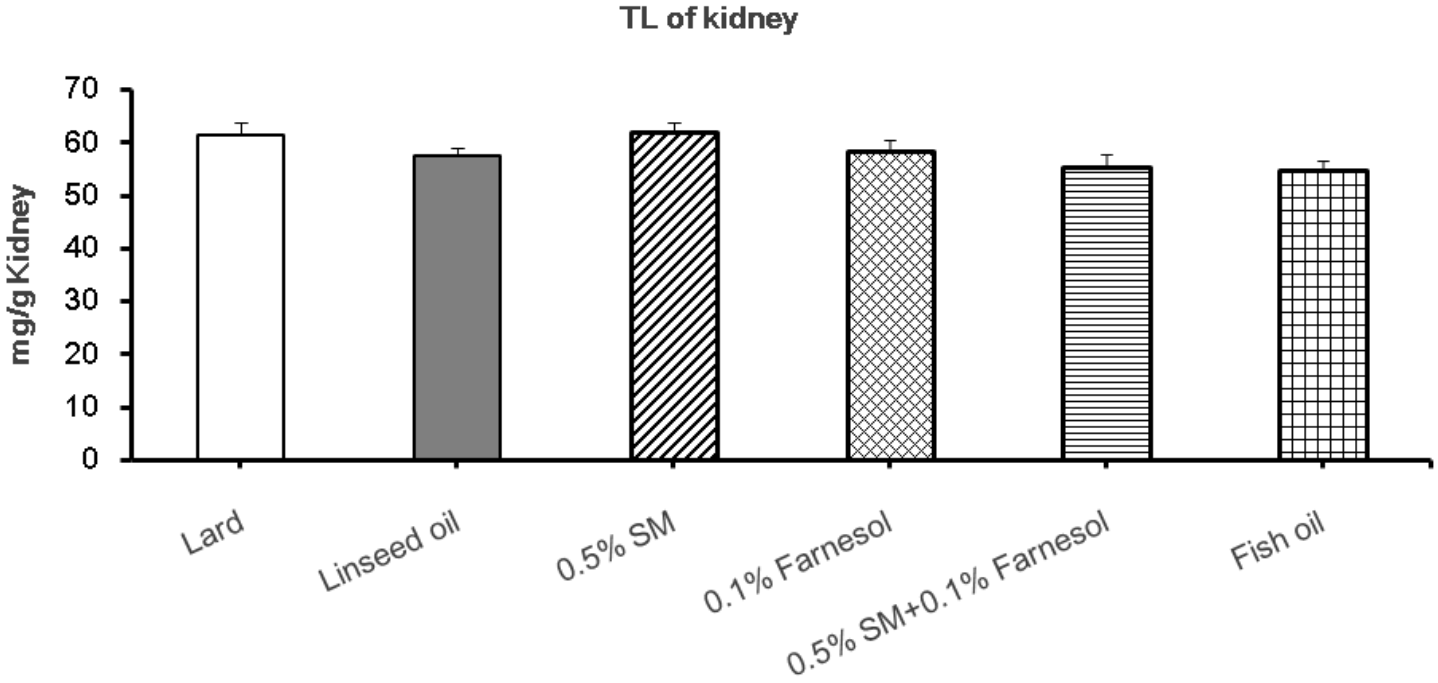

Figure 3-12 Effect of farnesol and SM on the kidney TL content of KK-A^y mice.

TL was extracted from brain with chloroform/methanol (2:1, v/v). Values are expressed as mean ± SEM (n=7).

Table 3-15 Effect of farnesol and SM on the fatty acid composition ratio in kidney TL of KK-A^y mice (%).

	Lard	Linseed oil	0.5% SM	0.1% Farnesol	0.5% SM + 0.1% Farnesol	Fish oil
16:0	17.52±0.35 [b]	16.47±0.48 [ab]	16.74±0.49 [ab]	17.83±0.51 [a]	17.19±0.50 [ab]	18.14±0.50 [a]
18:0	14.38±0.38	15.40±0.27	15.38±0.43	16.48±0.61	16.11±0.50	16.51±0.23
18:1n-9	14.82±1.64	14.89±1.29	14.43±1.61	13.59±1.41	13.46±1.98	10.87±1.14
20:1n-9	0.41±0.04 [a]	0.39±0.03 [a]	0.37±0.04 [a]	0.32±0.03 [a]	0.32±0.04 [a]	0.26±0.02 [b]
18:2n-6	8.65±0.23 [b]	11.24±0.33 [a]	11.63±0.29 [a]	11.31±0.25 [a]	11.88±0.27 [a]	11.89±0.35 [a]
20:4n-6	15.98±0.75 [a]	10.84±0.64 [b]	11.01±0.69 [b]	11.83±0.78 [b]	11.99±0.93 [b]	11.81±0.50 [b]
18:3n-3	0.11±0.01 [b]	3.14±0.26 [a]	3.34±0.42 [a]	3.09±0.34 [a]	3.11±0.44 [a]	0.27±0.03 [b]
20:5n-3	0.17±0.01 [b]	1.85±0.15 [a]	1.93±0.11 [a]	1.91±0.16 [a]	1.97±0.17 [a]	2.23±0.09 [a]
22:5n-3	0.91±0.06 [b]	1.95±0.16 [a]	2.15±0.16 [a]	1.68±0.10 [a]	2.15±0.19 [a]	0.78±0.04 [b]
22:6n-3	19.47±1.30 [ab]	18.06±1.06 [ab]	17.03±1.49 [ab]	15.73±1.20 [b]	16.01±1.47 [ab]	21.72±1.23 [a]
SFA	33.08±0.53 [b]	32.95±0.49 [b]	33.21±0.57 [ab]	35.53±0.71 [a]	34.49±0.40 [ab]	35.56±0.44 [a]
MUFA	17.89±1.65	17.22±1.33	16.72±1.63	15.88±1.44	15.72±2.00	13.15±1.15
PUFA n-6	25.31±0.68 [a]	22.94±0.36 [b]	22.95±0.54 [ab]	23.45±0.64 [ab]	24.18±0.77 [ab]	24.06±0.24 [ab]
PUFA n-3	20.72±1.35	25.30±1.11	24.77±1.38	22.73±1.17	23.58±1.40	25.06±1.33
n-6/n-3	1.24±0.07 [a]	0.89±0.03 [b]	0.94±0.04 [b]	1.04±0.05 [ab]	1.04±0.04 [ab]	0.98±0.05 [b]

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

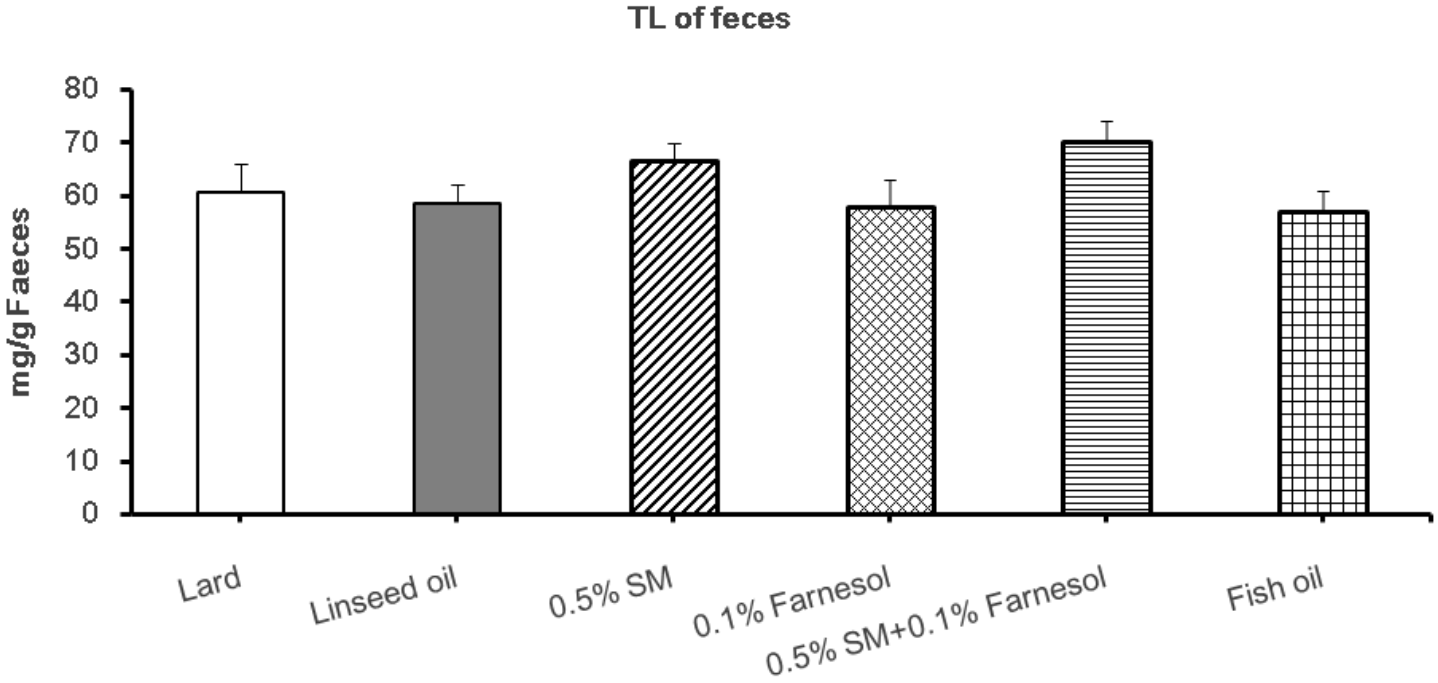

Figure 3-13 Effect of farnesol and SM on the feces TL content of KK-A^y mice.

TL was extracted from feces with chloroform/methanol (2:1, v/v). Values are expressed as mean ± SEM (n=7).

Table 3-16 Effect of farnesol and SM on the fatty acid composition ratio in faeces TL of KK-A^y mice (%).

	Lard	Linseed oil	0.5% SM	0.1% Farnesol	0.5% SM + 0.1% Farnesol	Fish oil
14:0	2.64±0.19	2.73±0.13	2.30±0.07	2.44±0.09	2.27±0.09	2.65±0.14
16:0	29.43±1.14	28.99±1.21	31.67±0.48	28.40±1.46	28.51±0.49	28.47±0.86
18:0	18.33±1.18 ^{b}	21.76±2.54 ^{ab}	30.24±0.64 ^{a}	23.06±2.90 ^{ab}	25.31±1.83 ^{ab}	21.54±2.06 ^{ab}
20:0	1.21±0.06 ^{d}	1.67±0.24 ^{bcd}	2.15±0.12 ^{ab}	1.36±0.11 ^{cd}	2.70±0.16 ^{a}	1.88±0.08 ^{bc}
18:1n-9	20.79±1.99 ^{a}	14.14±1.58 ^{ab}	11.31±0.42 ^{b}	15.04±2.26 ^{ab}	14.24±0.80 ^{ab}	14.74±1.52 ^{ab}
18:1n-7	3.17±0.07 ^{a}	3.06±0.20 ^{a}	2.01±0.02 ^{c}	2.98±0.14 ^{ab}	2.36±0.12 ^{bc}	3.06±0.27 ^{a}
20:1n-9	1.33±0.17	1.25±0.18	0.96±0.03	1.20±0.22	1.35±0.09	1.31±0.09
18:2n-6	4.11±0.24 ^{ab}	3.77±0.45 ^{ab}	3.02±0.15 ^{b}	4.00±0.41 ^{ab}	3.78±0.32 ^{ab}	5.43±0.61 ^{a}
20:4n-6	0.72±0.07 ^{a}	0.29±0.02 ^{bc}	0.17±0.01 ^{c}	0.32±0.01 ^{bc}	0.18±0.01 ^{c}	0.41±0.04 ^{b}
18:3n-3	1.48±0.16 ^{b}	4.75±0.73 ^{a}	3.13±0.24 ^{ab}	4.47±0.48 ^{a}	4.27±0.52 ^{a}	1.87±0.14 ^{b}
22:6n-3	-	-	-	-	-	1.43±0.16
SFA	54.22±1.97 ^{b}	58.13±3.40 ^{ab}	68.95±0.80 ^{a}	57.98±4.11 ^{ab}	61.74±1.89 ^{ab}	57.74±2.41 ^{ab}
MUFA	25.50±2.17 ^{a}	18.90±1.81 ^{ab}	14.83±0.42 ^{b}	19.62±2.55 ^{ab}	18.69±0.91 ^{ab}	19.75±1.55 ^{ab}
PUFA n-6	5.33±0.24 ^{ab}	4.47±0.49 ^{b}	3.58±0.17 ^{b}	4.65±0.44 ^{b}	4.43±0.35 ^{b}	6.46±0.55 ^{a}
PUFA n-3	1.63±0.14 ^{b}	4.90±0.70 ^{a}	3.18±0.23 ^{ab}	4.77±0.48 ^{a}	4.28±0.51 ^{a}	3.87±0.25 ^{a}
n-6/n-3	3.45±0.42 ^{a}	0.93±0.03 ^{b}	1.14±0.07 ^{b}	0.99±0.04 ^{b}	1.06±0.04 ^{b}	1.68±0.11 ^{b}

Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$ among each value.

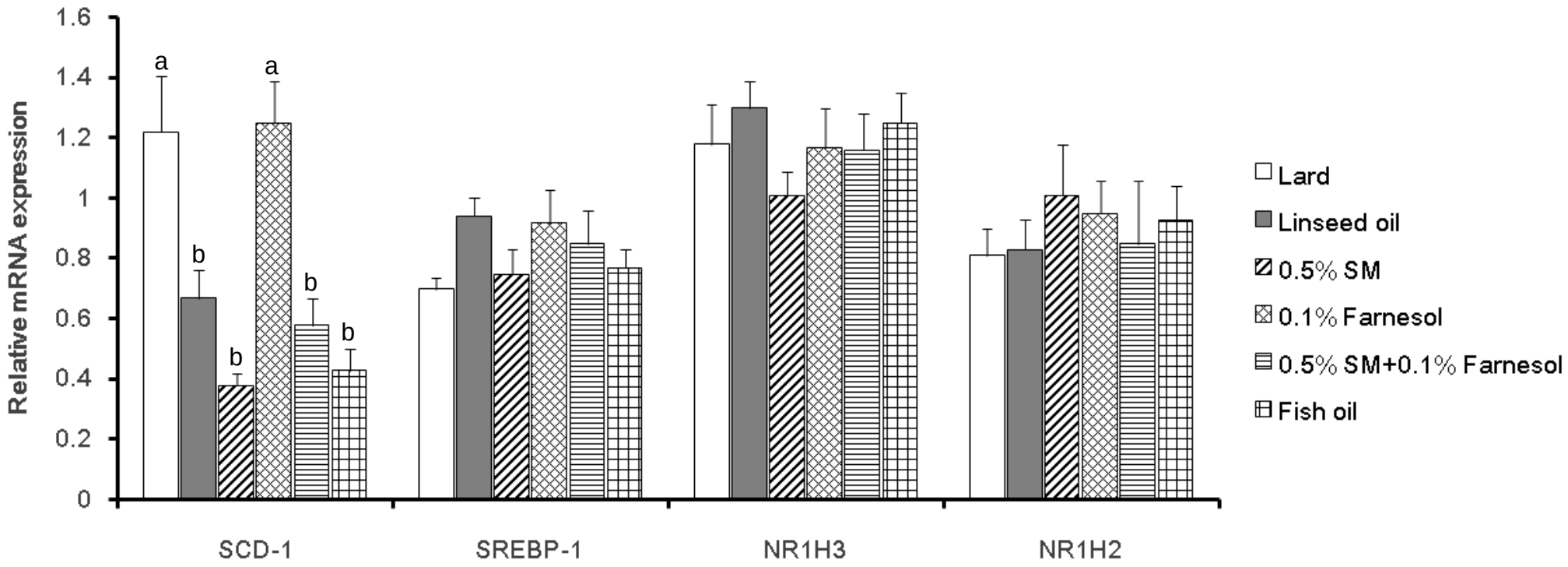

Figure 3-14 Effect of farnesol and SM on mRNA expressions related to the hepatic MUFA metabolism of KK-A^y mice.

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. Values are expressed as mean ± SEM, different letters indicate significant difference at $P < 0.05$ among each value.

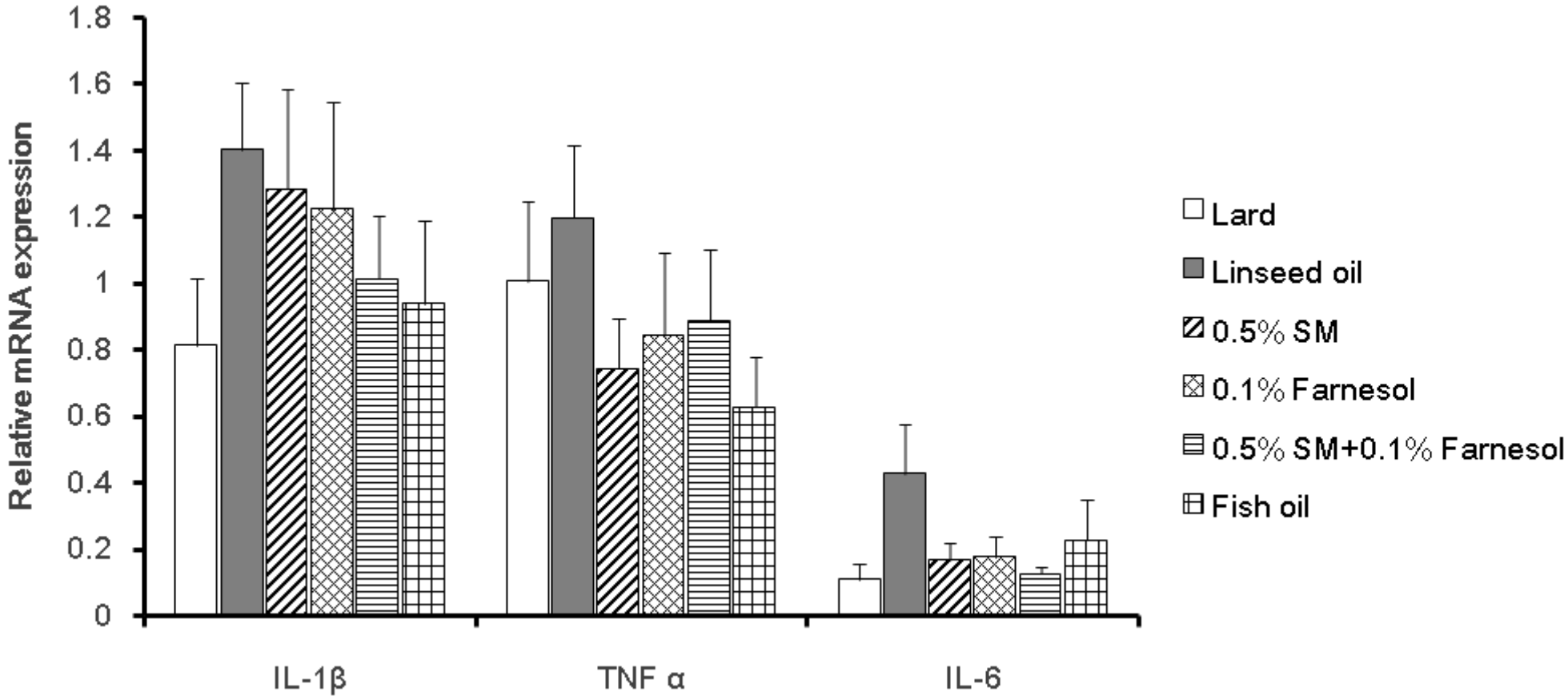

Figure 3-15 Effect of farnesol and SM on the mRNA expressions related to hepatic inflammation of KK-A^y mice.

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. Values are expressed as mean ± SEM.

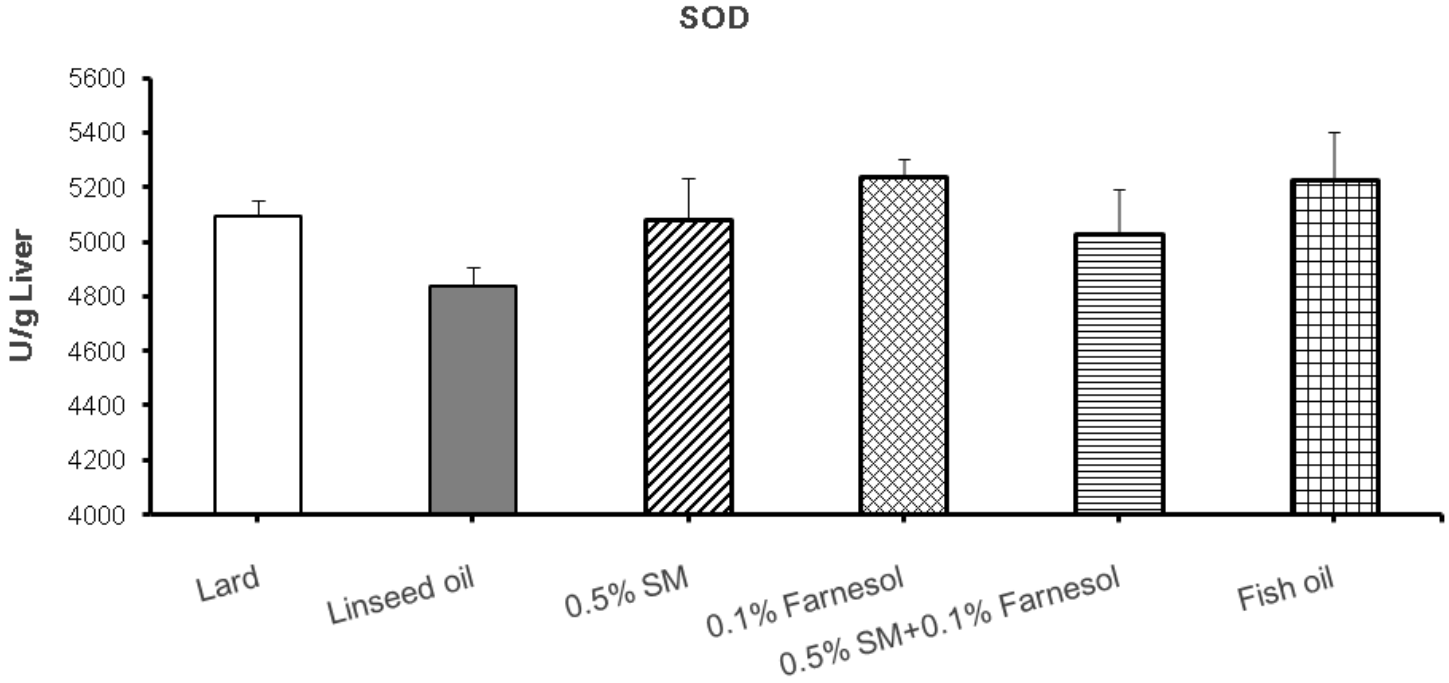

Figure 3-16 Effect of farnesol and SM on the content of SOD in the liver of KK-A^y mice.

SOD activity was measured by using the SOD Assay Kit- WST (Dojindo Laboratories, Kumamoto, Japan). Values are expressed as mean ± SEM.

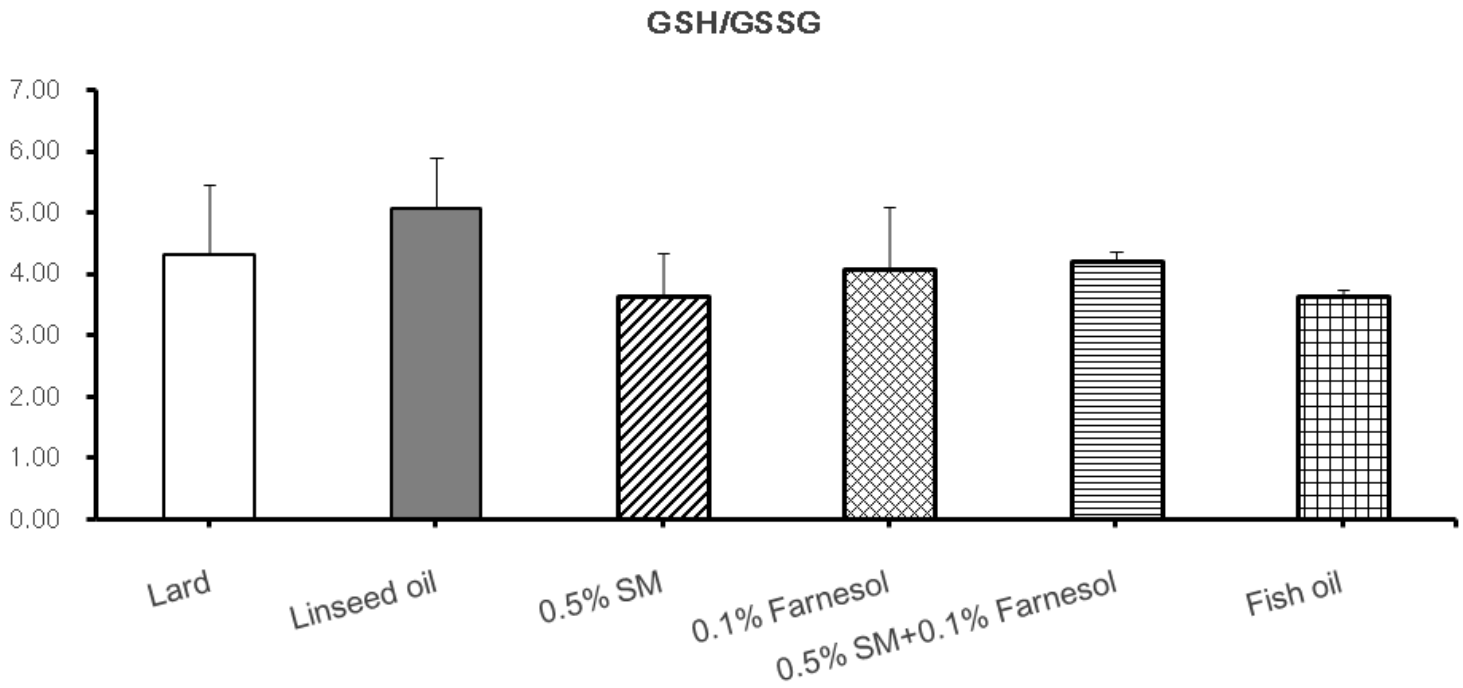

Figure 3-17 Effect of farnesol and SM on the content of GSH and GSSG in the liver of KK-A^y mice.

GSH and GSSG were quantified respectively by using GSSG/GSH Quantification Kit (Dojindo Laboratories, Kumamoto, Japan). Values are expressed as mean ± SEM.

3.4 Discussion

HMG CoA reductase, a rate- limiting enzyme in cholesterol biosynthesis pathway, is the target of a class of cholesterol-lowering drugs. Previous work has shown that farnesol increases the hepatic level of HMG-CoA reductase mRNA but reduced plasma TC levels [157]. In other reports, farnesol accelerates degradation of the HMG CoA reductase in cultured cells and intact cells [161; 162]. However, another report refuted this opinion and showed that farnesol was not the regulator mediating degradation of the HMG-CoA reductase [163]. These previous results indicate that the effect of farnesol on cholesterol biosynthesis pathway is controversial. Here we showed that plasma and hepatic TC levels increased by 0.1% farnesol feeding to KK-A^y male mice. Farnesol is the building block of farnesyl pyrophosphate, the precursor of squalene in the cholesterol biosynthetic pathway [161]; therefore, our results may indicate that farnesol is only as a substrate of the cholesterol synthesis.

Hepatic steatosis is considered to be a significant risk factor for many metabolic and cardiovascular diseases, such as atherosclerosis and nonalcoholic fatty liver disease (NAFLD). Reduction of cholesterol absorption is thought to be negatively related with CVD [164; 165], and risk of NAFLD in mice [166]. A number of *in vivo* [84; 86; 167; 168] and *in vitro* [85] studies has shown the inhibition effect of cholesterol absorption by SM. The reduced intestinal cholesterol absorption would be strongly associated with lower hepatic cholesterol levels and plasma cholesterol levels. This effect may be also due to the down-regulation of mRNA expressions involved in hepatic cholesterol export, such as ABCA1, ABCG1, ATP-binding cassette sub-family G member 5 (ABCG5) and ATP-binding cassette sub-family G member 8 (ABCG8) [169].

In the present study, we showed the tendency of the decrease in plasma and hepatic TC and nonHDL-C levels by SM administration; however, the effect was not

significant as compared with linseed oil group. Furthermore, plasma HDL-C, an independent risk predictor of CVD, decreased together with plasma TC level in this case. As several prospective studies on different racial and ethnic groups worldwide have confirmed that HDL-C is a strong, consistent and independent predictor of incident cardiovascular events [23; 170], administration of SM may not be a good factor for cardio-protection.

A number of studies have shown that synthesis of FA and TAG is modulated in response to changes in levels of metabolites derived from the cholesterol biosynthetic pathway in culture cells or *in vivo* [158; 159; 171; 172]. Treatment with farnesol and its derivatives reduced TAG biosynthesis in hepatocytes [159], and to lower serum TAG levels in several animal models [158; 171]. In HepG2 hepatocarcinoma cells, levels of farnesol and other intermediates of the cholesterol biosynthetic pathway was lowered by HMG-CoA reductase inhibitors, whereas opposite results were observed with the TAG production [172]. Lipid accumulation was also inhibited by farnesol in 3T3-L1 adipocytes [173]. However, when we treated KK-A^y mice with 0.1% farnesol, there was a trend towards increased plasma NL and accumulated hepatic TL. These opposite results may be due to the model of mice used. It has been reported that obesity and hyperglycemia of KK-A^y mice were observed from young ages (6-8 weeks of age) [174]. The 20% fat treatment may not be enough for the obesity development to measure the effect of farnesol.

Farnesol, a catabolite in the isoprenoid/cholesterol pathway, is possible to inhibit cholesterol formation due to down-regulation of HMG-CoA reductase [162]. However, a controversial result was observed in this study in which plasma TC and HDL-C levels significantly increased by 0.1% farnesol administration. Another report also showed no significant change in the cholesterol levels by the administration of 1.5% farnesol for a period of 10 weeks [175]. The difference in these studies may be derived from the different feeding period. Four weeks feeding in the present study may not be sufficient to make clear the effect of farnesol intake on the metabolism of

cholesterol. But, one should be noticed that administration of 0.1% farnesol ameliorated the down-regulation effect on plasma HDL-C level by SM (**Table 3-3**), which may confirm the up-regulation effect of farnesol on plasma HDL-C level. Therefore, farnesol may also show the anti-atherosclerotic effect on subjects with metabolic disorders as shown in SQ administration (**Chapter 1**).

Stearoyl-CoA desaturase (SCD) is a key and highly regulated endoplasmic reticulum (ER) enzyme that catalyzes the biosynthesis of MUFA from saturated fatty acids [176]. Palmitoyl- and stearoyl-CoA are the preferred substrates, which are then converted to palmitoleoyl- and oleoyl-CoA, the most abundant MUFA in animal, respectively. Then the MUFA can serve as substrates for the synthesis of various kinds of lipids, including PL, TAG, cholesteryl esters, wax esters, and alkyldiacylglycerols [177; 178]. SCD-1 gene transcription can be regulated positively or negatively by the transcription factor binding sites in its promoter. Sterol regulatory element-binding protein 1 and liver X receptor α and β, as the positive transcription factors of SCD-1, induced the SCD-1 expression [176]. In an effort to understand the up-regulation of the composition of MUFA by farnesol, we first tested whether farnesol caused any changes in expression of SCD-1. Hepatic SCD-1 expression was significantly increased in farnesol treated mice. When SREBP-1c, NR1H3 and NR1H2 expressions were determined, no significant change was observed. Regretfully, protein levels of the nuclear form of SREBP-1c, NR1H3 and NR1H2 were not measured in this study. Other reports revealed that endogenously synthesized MUFA by SCD most likely serve as the main substrates for the synthesis of hepatic TG and cholesterol esters [179; 180]. A possible physiological explanation for the requirement of SCD expression in the synthesis of the TAG and cholesterol esters is the production of more easily accessible MUFA within the vicinity of ER enzymes acyl-CoA:cholesterol acyltransferas, diacylglycerol acyltransferase and microsomal glycerol phosphate acyltransferase [176]. In our study, not only TL and cholesterol but also the composition of MUFA was increased in farnesol treatment mice. These

results suggest that farnesol may induce the synthesis of MUFA, NL and cholesterol through the SCD-1.

In humans, oxidative stress is thought to be involved in the development of many diseased, such as cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, etc. Previous reports have shown that farnesol treatment can modulate the impaired redox homeostasis status through increasing the level of antioxidant enzymes in the cortex and hippocampus of Swiss albino mice [160] and renal of rats [156]. Here we show that hepatic SOD level was up-regulated by farnesol treatment, although no significant difference was found with linseed oil group. This result may indicate that farnesol is capable to attenuate oxidative stress *in vivo*.

General discussion

As introduced in general introduction, CVD have become a number 1 cause of death globally since 31% of all global deaths dues to CVD in 2016 [1]. 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 [105]. Although the exact motive of atherosclerosis is still unknown, modification and deposition of lipids in the vascular wall can induce this event.

HDL-C has a causal relation to atheroprotection. Through the data from test tubes, model systems, and even some human studies, HDL has been shown to have various properties that might reasonably be supposed to confer CVD protection [181]. In the various properties, the HDL-C efflux capacity in cells and the related complex physiological process of reverse cholesterol transport are best studied [182-185]. Other properties that could be atheroprotective include anti-inflammatory and anti-oxidant effects, anti-apoptotic effects and NO-promoting effects [181; 186-188].

Olive oil intake is also known to show health beneficial effects including cardioprotection [98-101] and these effects have been recognized to be, in part, derived from olive oil minor compounds, mainly phenolic compounds. In addition, due to the relatively high content in olive oil as compared with other vegetable oils, SQ has been also regarded as a contributing factor for the observations of reduced risk of diseases associated with olive oil intake. Up to date, SQ has been reported to show anticancer, anti-inflammatory, anti-oxidant, skin protection, liver protection, neuroprotection, and cadioprotection activities. Olive oil is known to be rich in SQ, while shark has been reported to contain much more SQ. Thus, the present study has been conducted to make clear the effect of SQ on lipid metabolism, especially plasma lipids, and FA metabolism in animal models.

For the protection of CVD, much attention has been paid to many other food factors. Among them, EPA and DHA, two important n-3 long-chain PUFA, have been reported to be protective against major coronary disorders and myocardial infarction. It is well-known that α-linolenic acid (ALA, 18:3n-3) can be converted to EPA and DHA through the desaturation/elongation pathway *in vivo*. However, the conversion is small. Therefore, much attention has been paid to up-regulation factors on the conversion of ALA to EPA and DHA. In the present study, the interest has been focused on farnesol and SM from this viewpoint.

In **Chapter 1** and **Chapter 2**, the comparison has been done on the effect of SQ on the obese/diabetes KK-A^y mice and normal C57BL/6J mice.

Epidemiological studies have revealed an inverse correlation between HDL-C levels and the risk of CVD and of atherosclerosis. Every 1 mg/dL increase in HDL is associated with a 2% to 3% decrease in CVD risk, independent of LDL-C and TAG levels [122]. In the present study, we have found a significant increase in plasma HDL-C of obese/diabetic KK-A^y mice fed SQ with no significant difference in the TC and nonHDL-C levels. The increase in HDL-C was also 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 on subjects with metabolic disorders.

In the present study, different results on TC level were observed between the obese/diabetic KK-A^y mice and normal C57BL/6J mice. In KK-A^y mice, SQ administration decreased TC level in the liver and almost did not affect plasma TC. In C57BL/6J mice, both hepatic and plasma TC were increased. Few studies in animals and human had revealed discrepant effects of SQ on plasma cholesterol [107; 110; 135]. Further research will be needed to clarify the discrepant influence of SQ on animals and humans. On the other hand, significant increase in hepatic NL, TAG and TL by SQ intake was observed in KK-A^y mice, and a same increase tendency was also observed in C57BL/6J mice. TAG can be accumulated in liver via the process of

hepatic steatosis and hepatic steatosis has been proposed to be associated with liver disease, type 2 diabetes mellitus, arterial hypertension and metabolic syndrome [136; 137]. The results on hepatic NL, TAG and TL by SQ may illustrate that SQ administration can aggravate hepatic steatosis. Although SQ administration could reduce the risk of CVD by increase in HDL-C, it is noteworthy that SQ may also increase the risk of hepatic steatosis.

To know the effect of SQ on cholesterol metabolism, HMG-CoA reductase activity and its gene expression were analyzed. In KK-A^y mice, 1% SQ intake significantly decreased HMGCR mRNA expression and 2% SQ intake significantly decreased HMG-CoA reductase activity. In C57BL/6J mice, HMG-CoA reductase activity was not affected by SQ administration. As one of the key factor in cholesterol synthesis, the down-regulation of HMG-CoA reductase activity and its gene expression might explain the decrease tendency in the TC level in the liver of KK-A^y mice. On the other hand, although HMG-CoA reductase activity was not affected by SQ intake, increase in hepatic TC level was observed in C57BL/6J mice. This may be due to the compensation of other pathways of cholesterol metabolism and/or the effect of cholesterol supplementation from other tissues.

FA in hepatic NL and PL are also determined. In KK-A^y mice, the contents of SFA, MUFA and PUFA in hepatic NL were significantly higher in SQ supplementation groups, probably as a result of increment of liver TAG and NL. However, FA in hepatic PL was affected slightly or was not affected by SQ supplementation. In C57BL/6J mice, 2% SQ supplementation significantly increased the contents of SFA, MUFA and PUFA in hepatic PL, while FA in hepatic NL was affected slightly or was not affected by SQ supplementation. Further research will be needed to clarify this completely opposite results found between two animal models.

In **Chapter 3**, farnesol and SM were used as experiment sample to assess their effects on lipid metabolism of KK-A^y mice.

Farnesol, a catabolite in the isoprenoid/cholesterol pathway, is possible to inhibit cholesterol formation due to down-regulation of HMG-CoA reductase [162]. However, a controversial result was observed in the present study where plasma TC and HDL-C levels were significantly increased by 0.1% farnesol administration. These results may show that farnesol can also increase plasma HDL-C levels as seen in SQ supplementation, and farnesol may also be a positive factor for cardioprotection. An increase tendency in hepatic NL, and TL by farnesol intake was observed in KK-A^y mice. This effect was also found in SQ supplementation to KK-A^y mice.

Administration of 0.5% SM down-regulated plasma and hepatic TC levels. However, plasma HDL-C level also decreased by SM supplementation. As several prospective studies on different racial and ethnic groups worldwide have confirmed that HDL-C is a strong, consistent and independent predictor of incident cardiovascular events, administration of SM may not be a good factor for cardioprotection.

SCD is a key and highly regulated ER enzyme that catalyzes the biosynthesis of MUFA from SFA [176]. In this study, plasma and hepatic MUFA composition has been increased by farnesol treatment. In an effort to understand the up-regulation of the composition of MUFA by farnesol, we first tested whether farnesol caused any changes in expression of SCD-1. Hepatic SCD-1 expression was significantly increased in farnesol treated mice. However, significant changes on SREBP-1c, NR1H3 and NR1H2 expression were not observed. This result may illustrate that farnesol serve as a SCD-1 activator in liver of KK-A^y male mice. In addition, the increase in EPA and DHA has been expected to be found in the farnesol and SM supplementation in the present study; however, the promotion effect on conversion of ALA to EPA and DHA of these food factors was not observed in the KK-A^y mice.

Conclusion

The present study showed that SQ supplementation to the obese/diabetes KK-A^y mice and normal C57BL/6J mice increased plasma HDL-C level, important and independent anti-atherosclerotic factor. It is noteworthy that this effect of SQ was found more clearly in obese/diabetes model mice as compared with normal mice. Although more research is needed to make clear the effect of SQ on the lipid metabolism and dynamics related to atherosclerosis, the present study suggests the anti-atherosclerotic effect of SQ especially on subjects with metabolic disorders. In addition, the effect of farnesol has been also examined in the present study, since farnesol is known to be an important intermediary metabolite in SQ biosynthesis. As a result, it has been found that farnesol could also have ability to increase plasma HDL-C level as seen in SQ. Furthermore, farnesol supplementation to KK-A^y mice up-regulated the SFA conversion to corresponding MUFA. This effect would be due to the promotion of SCD-1 expression in the mice.

References

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