Development of New Healthy Edible Oil for the Management of High Blood Cholesterol and Obesity

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Development of New Healthy Edible Oil for the Management of High Blood Cholesterol and Obesity

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CONTENTS

ABBREVIATIONS

Apo	apolipoprotein
ABC	adenosine triphosphate-binding cassette transporter
ALT	alanine aminotransferase (= glutamic pyruvic transaminase: GPT)
AST	aspartate aminotransferase (= glutamic oxaloacetic transaminase: GOT)
BMI	body mass index (kg/m^2)
CHD	coronary heart disease
CT	computed tomography
CVD	cardiovascular disease
DAG	diacylglycerol
FDA	Food and Drug Administration
FOSHU	Food for Specified Health Uses
γ-GTP	γ-glutamyltranspeptidase
HDL	high-density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
LDL	low-density lipoprotein
LPL	lipoprotein lipase
Lp(a)	lipoprotein(a)
NPC1L1	Nieman-Pick C1 Like 1

PS	phytosterols (plant sterols, sterol esters, stanols and stanol esters)
RLP	remnant-like lipoprotein
SD	standard deviation
SE	standard error
SFA	subcutaneous fat area
TAG	triacylglycerol
TBA	total bile acid
TFA	total fat area
VFA	visceral fat area
VLDL	very low-density lipoprotein
WHO	World Health Organization

To convert for cholesterol from SI unit (mmol/L) to mg/dL, multiply by 1/0.02586 (= 38.67).

To convert for triacylglycerol from SI unit (mmol/L) to mg/dL, multiply by 1/0.01129 (= 88.57).

To convert for glucose from SI unit (mmol/L) to mg/dL, multiply by 1/0.05551 (= 18.0).

CHAPTER 1

General introduction

In 21st century society, cardiovascular disease (CVD) represents a growing threat to global health. According to WHO (2011), the mortality due to CVD in the world that is silently progressive in the heart and brain reached approximately one in three people (**Fig. 1-1**). Several epidemiological studies have identified elevated blood cholesterol as a strong risk factor for CVD (Key, 1970; Kannel et al., 1971; Martin et al., 1986). Low-density lipoprotein (LDL) is a main transporter of cholesterol, but readily oxidizes and deposits plaques on artery walls, triggering an ischemic heart attack or stroke. Nowadays, cholesterol-lowering medication (e.g., statin) has been established as an effective therapy for the prevention of CVD (Shepherd et al., 1995; Downs et al., 1998; 4S Group, 1994; Sacks et al., 1996; LIPID study Group, 1998; Nakamura et al., 2006).

Recently, contributions of dietary factors to blood cholesterol concentration are estimated in the U.S., (Grundy, 2016). Current dietary cholesterol (Key et al., 1965; Hopkins 1992; Berger et al., 2015), overnutrition (obesity) and low intake of dietary fiber (Chandalia et al., 2000) raise blood cholesterol concentrations by 5%, 5% and 5%, respectively, compared to that of recommended intakes. Also, dietary saturated & trans fatty acids found in animal fat, shortening, and deep-fried foods (Mustad et al., 1997; Mensink & Katan, 1990) raise blood cholesterol concentration by 10%. Meanwhile, a limitation of individual dietary intakes of cholesterol and animal fat can help reduce

blood LDL cholesterol concentration by up to 40% and slow or even reverse atherosclerosis (Ornish et al., 1998). Also, moderately lowering blood cholesterol by 10% contributes to a reduction in the incidence of heart disease in men within 5 years by 50% at age 40 and 20% at age 70 (Law et al., 1994), however changing eating habits is very hard in this age of plenty. Hence, universally accepted 'functional food' that provides a lifetime of a healthy blood cholesterol should be explored to prevent CVD.

In Japan, the concept of 'functional food' comprised of 1) nutritional function, 2) sensory function such as flavor and texture, and 3) physiological function was systematically defined early in the 1980s (Arai, 1996). The Japanese Ministry of Health, Labour, and Welfare (MHLW) established 'Foods for Specified Health Use' (FOSHU) in 1991 as a regulatory system to approve the health claims based on the scientific evidence of the food on the human body. Nowadays, the market of FOSHU pass the US$ 6 billion mark (≥ 1,100 items in 2017). Most of the statements of the Japanese FOSHU are similar to the structure/function claims in the U.S. or the enhanced function claims of the Codex. The concrete evidence including mechanism and responsiveness of the functional foods is crucial as well as that of medicines. To extend the human health span, clinical data on the functional foods should be verified in different populations. Additionally, a great deal of thought should be given to the real effectiveness of physiological function, as well as basic food features such as nutritional and sensory functions, in a daily practical field.

Sterols are universally present in animal and plant kingdoms, not most bacteria and viruses.

Cholesterol is a vital element in the cell membrane and as precursor of steroid hormones, vitamin D and bile salts, in humans. In general, people gain cholesterol from animal foodstuff (~300-500 mg/day) and from de novo synthesis (~700-1,000 mg/day). By contrast, phytosterols (PS) have similar chemical structures to cholesterol (**Fig. 1-2**), and function in the cell membrane like cholesterol. Dietary PS intake from fruits, vegetables and nuts is ~150- 400 mg/day (Hirai et al., 1986; Katan et al., 2003); ~1-1.4 g/day in a vegetarian diet resembling our early ancestral diets (Jenkins et al., 2001).

Accumulating evidence collected over the last half century indicates that PS consisting of Δ5-type ('sterols') or 5α-reduced type ('stanols') help reduce blood cholesterol concentration. PS potentially inhibit the intestinal absorption of both dietary and biliary cholesterols by displacing cholesterol from micelles, facilitating the excretion of cholesterol in the feces (**Fig. 1-3**). Daily amount of PS intake is near to that of cholesterol, whereas PS are poorly absorbed via intestinal transporters including Niemann-Pick C1 Like 1 (NPC1L1) (Altmann et al., 2004) and adenosine triphosphate-binding cassette (ABC) G5 and ABCG8 (Berge et al., 2000). In general, conventionally purified plant edible oils contain small amount of PS (<1%). Food and drink manufactures have developed a variety of foods and drinks (e.g., margarine and milk) enriched with PS extracted from soybean oil or tall oil. The US National Cholesterol Education Program Adult Treatment Panel III (NCEP, 2002) endorses PS (2 g/day) as an essential feature of a therapeutic lifestyle change along with diet modifications, weight reduction, intake of viscous fibers, and increased physical activity to reduce the risk of coronary heart disease. Meanwhile, because such high dose of PS may decrease the

absorption of fat-soluble vitamins and carotenoids, intakes of fruits and vegetables are recommended. To attain the secure cholesterol-lowering effect of PS, it is needed to explore an effectively active form of PS even at low-dose (< 1 g/day) especially in Asian with less information than Caucasian.

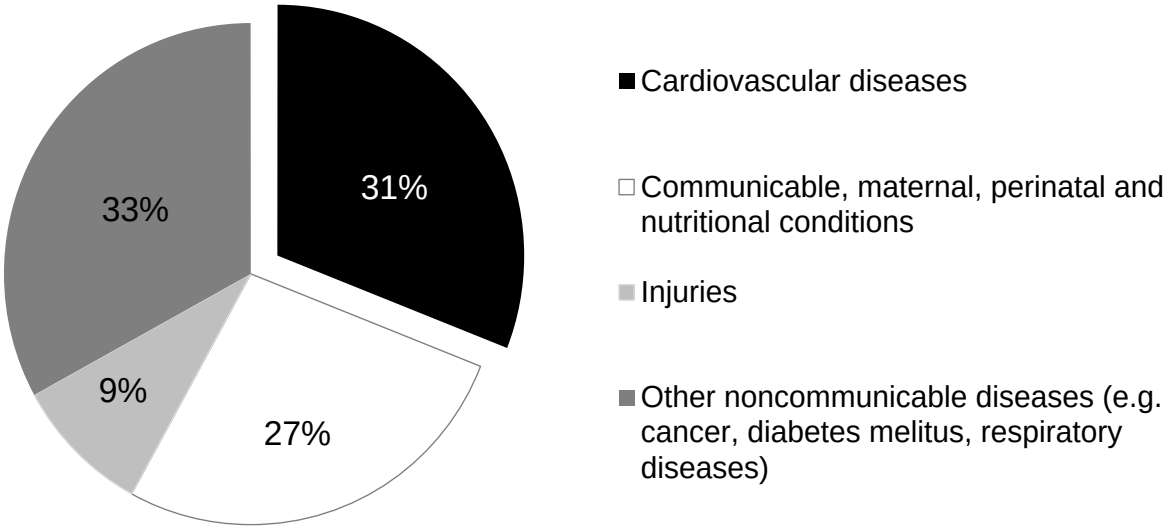


Fig. 1-1 Distribution of major causes of death including CVD

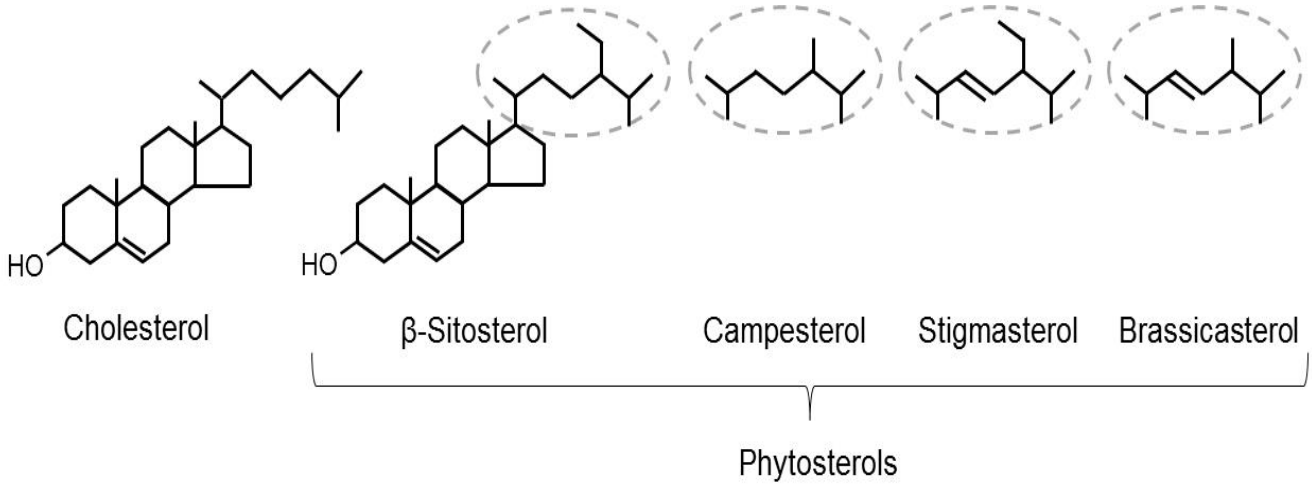


Fig. 1-2 Chemical structure of cholesterol and PS

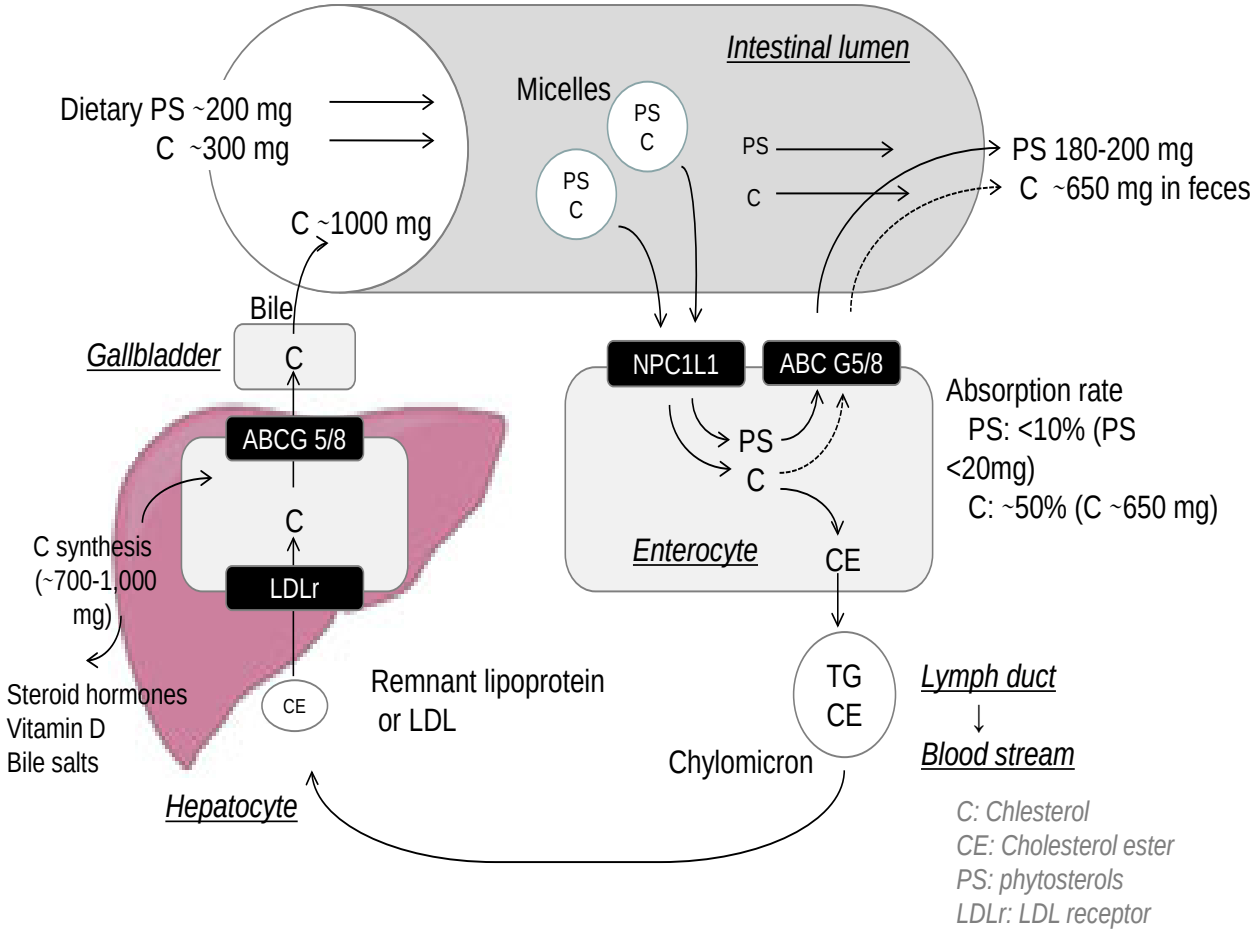


Fig. 1-3 Transport of cholesterol (C) and phytosterols (PS) from the intestinal lumen.

Dietary C and PS mix with biliary C from the gallbladder and form micelles in the intestinal lumen. Specific transporters, Nieman-Pick C1 Like 1 (NPC1L1), transport the majority of C and PS into the enterocyte. Then, nearly all of the PS turn back to the intestinal lumen by adenosine triphosphate-binding cassette (ABC) transporter G5 and ABCG8 for excretion from the body. Some C may also turn back into the intestinal lumen by ABCG5/G8. About half of the C is packaged into chylomicrons and are delivered to the liver through the lymphatic system and the bloodstream.

More recently, modern sedentary habits accompanied with consuming high-fat diets and overeating contribute to the epidemic of obesity (WHO, 2011). Especially, visceral fat obesity (Matsuzawa et al., 1987), namely augmented intra-abdominal adipose tissue, is recognized to be a leading cause for metabolic syndrome (Kissebah et al., 1982; Reaven, 1988; Kaplan, 1989; Grundy et al., 2005; JMSDCC, 2005; IDF, 2006), which is a cluster of multiple cardiovascular risk factors such as dyslipidemia (Zilversmit, 1979), hypertension (Hayashi et al., 2004), and glucose intolerance (Hayashi et al., 2003) linked to type 2 diabetes (Boyko et al., 2000) and CVD (Fujimoto et al., 1999). Then, the prevalence of lifestyle-related diseases has encouraged the food industry to develop new functional fatty acids (e.g., medium chain-rich triacylglycerol) or non-absorbed fats and oils.

Diacylglycerol (DAG) is a partial hydrolysate of triacylglycerol (TAG), and naturally occurs in edible oils and fats (Abdel-Nabey et al., 1992; D'alonzo et al., 1982), as shown in **Table 1-1**. In the Mediterranean area, olive oil has been consumed for a long time period. It was reported that some traditional olive oils obtained in Majorca in Spain contained high DAG content (~20 mol%) (Barceló Mairata & Barceló Mairata, 1985). DAG is classified as glycerol positional isomers (1,3:1,2-isomer ratio is 7:3), as shown in **Fig. 1-4**, and is also a component of popular food emulsifiers; however, its nutrition was poorly understood. Then, through devoted studies over the quarter of a century, it has been demonstrated that DAG oil (≥ 80 wt%) has the taste, texture and energy value of ordinarily used TAG oils, as shown in **Table 1-2** (Nishide et al., 2004; Taguchi et al., 2001). The major amount of DAG in the DAG oil is the 1,3-configuration, not the 1(3),2- configuration (the ratio of 1,3-DAG to

1(3),2-DAG was approximately 7:3). The cooking properties (Katsuta et al., 2008; Nakatsugawa et al., 2001; Ohno, 2002; Ohno, 2003) and sensory evaluation (Ogawa et al., 2001a; Ogawa et al., 2001b) of DAG oil were studied compared to those of TAG oil, resulting in almost same between oils.

Basically, lipases secreted in the intestinal lumen hydrolize dietary TAG to free fatty acids and 2-monoacylglycerol (MAG). 2-MAG is absorbed by enterocytes and resynthesized back to TAG. Then, most of the TAG generated in the small intestine is secreted as chylomicron particle. The most latest research has found out that main 1,3-DAG isomer in DAG is hydrolyzed to 1-MAG or 3-MAG in the gut, not 2-MAG like TAG (Kondo et al., 2003). Consequently, since 1-MAG or 3-MAG is not effectively utilized to reproduce TAG (chylomicron) in epithelial cells of the small intestine by DGAT, DAG oil is less likely to increase postprandial responses of TAG (Tada et al., 2005) and fasting TAG concentration in the blood (Yamamoto et al., 2006), and be stored as body fat via the enhanced fat oxidation (Nagao et al., 2000), compared to TAG oil. Thus, the metabolism of DAG oil differs from those of TAG oil (**Fig. 1-5**). Then, the Ministry of Health, Labour and Welfare of Japan has approved DAG cooking oils as a FOSHU in 1999.

Furthermore, it was found that because of the basic physiochemical (amphipathic) properties, DAG oil could also dissolve free forms of PS much more easily than TAG oil, as shown in **Fig. 1-6**. Maximum solubility of PS is ～6% in TAG oil vs. ～1.3% in DAG oil, respectively (Meguro et al., 2001). Recent short-term studies indicate that PS-rich DAG oil has enhanced cholesterol-lowering activity compared to PS-rich TAG oil (Goto et al., 1999; Meguro et al., 2001;

Saito et al., 2006a), suggesting DAG oil works as an advantageous delivery vehicle for PS (**Table 1-3**). Also, the free forms of PS are more economical and lower in calories than ester forms of PS (PSE). Therefore, PS-rich DAG oil enriched with two active ingredients that are originally contained at low levels in natural plant fats and oils, respectively, may be new advanced functional edible oil with multiple health benefits.

Table 1-1 Contents of acylglycerols in various edible oils (wt%)

Edible oils	TAG	DAG	MAG	Others
Soybean oil	97.9	1.0	-	1.1
Palm oil	93.1	5.8	-	1.1
Cottonseed oil	87.0	9.5	0.2	3.3
Corn oil	95.8	2.8	-	1.4
Safflower oil	96.0	2.1	-	1.9
Olive oil	93.3	5.5	0.2	1.0
Rapeseed oil	96.8	0.8	0.1	2.3
Cocoa butter	96.0	2.2	0.2	1.5
Tallow	89.6	3.8	-	6.6
Lard	97.9	1.3	-	0.8

Oils contain triacylglycerol, TAG; diacylglycerol, DAG; monoacylglycerol, MAG.

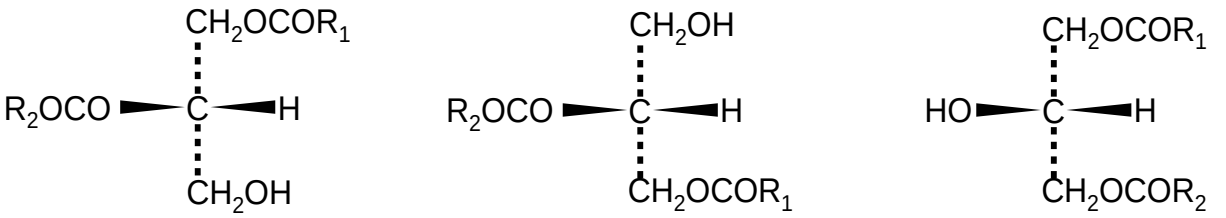
Table 1-2 Chemical and biological properties between TAG and DAG oils (wt%)

	TAG oil	DAG oil
Acylglycerol (wt%)		
TAG	97.2	10.7
DAG	1.1	87.0
1(or 3), 2-DAG	ND	27.8
1,3-DAG	ND	59.2
MAG	< 0.05	0.82
Free fatty acid	ND	ND
Fatty acid composition (wt%)		
C16:0	6.0	2.4
C18:0	2.2	0.7
C18:1	29.1	28.0
C18:2	57.8	60.3
C18:3	2.5	5.6
C20:0	0.4	< 0.05
C20:1	0.6	0.2
C22:0	0.3	< 0.05
C22:1	0.2	< 0.05
C24:1	0.2	< 0.05
Others	0.7	2.8
Energy value (kJ/g oil) *	39.6	38.9
Fat absorption coefficient (%) **	96.3 ± 0.3	96.3 ± 0.4

Oils contain triacylglycerol, TAG; diacylglycerol, DAG; monoacylglycerol, MAG.

* Energy values of TAG and DAG oils were measured by bomb calorimeter.

** Fat intake and excretion in rats fed either TAG or DAG diets for 3days were analyzed (mean ± SEM).



sn-1,2-Diacylglycerol sn-2,3-Diacylglycerol sn-1,3-Diacylglycerol

Fig. 1-4 Stereospecific isomers of DAG

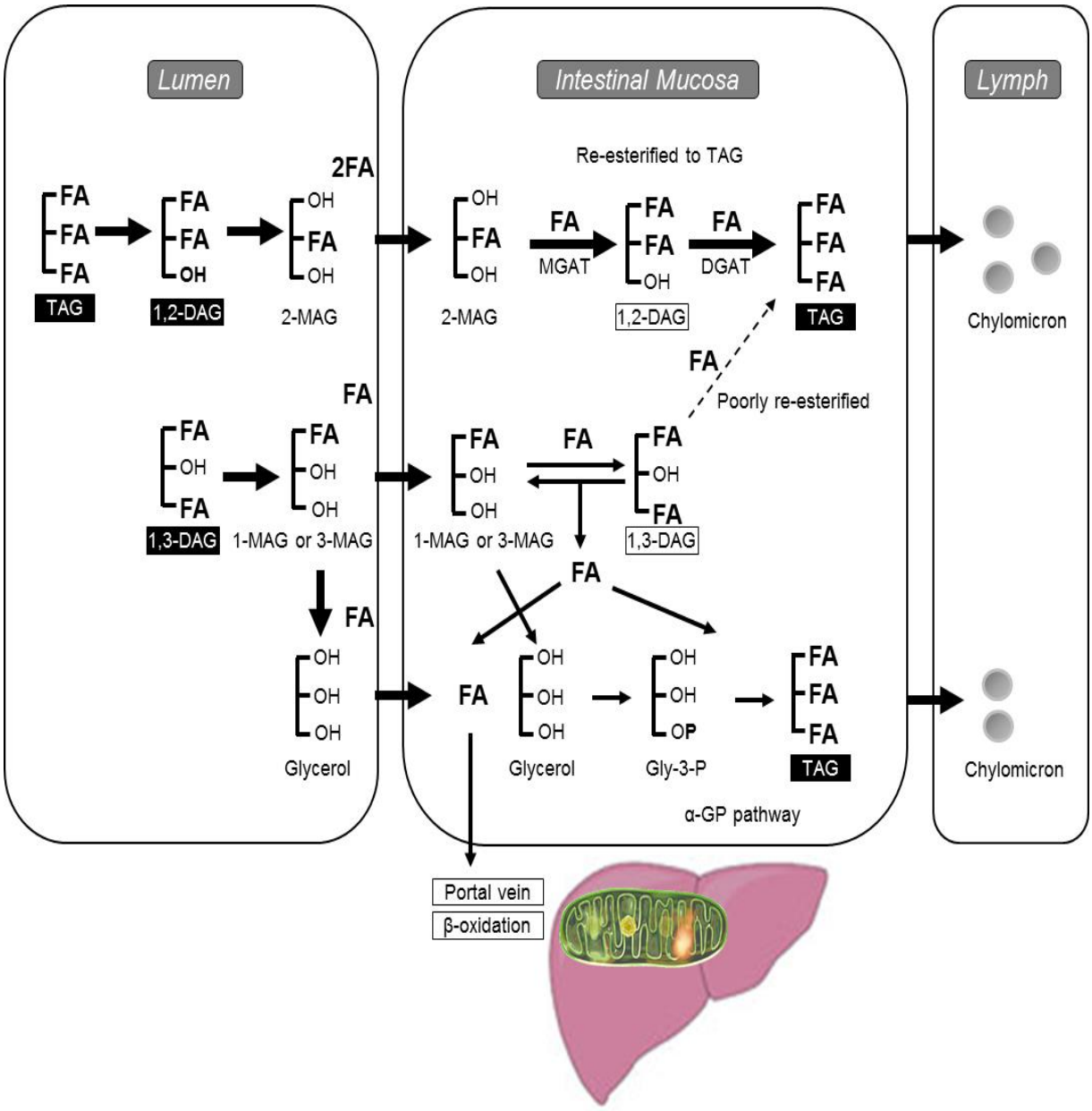


Fig. 1-5 Possible metabolic fate of TAG and DAG in the small intestine.

MGAT: monoacylglycerol O-acyltransferase, DGAT: diacylglycerol O-acyltransferase

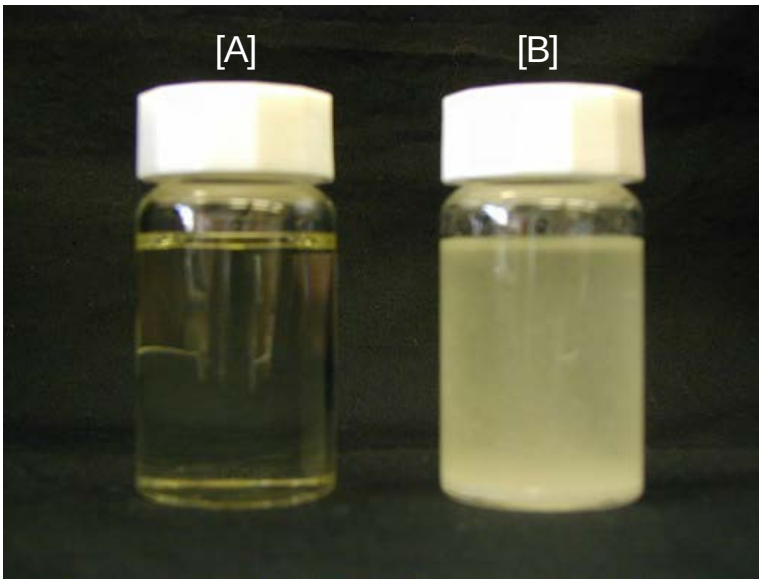


Fig. 1-6 Solubility of PS (4 wt%) in DAG oil [A] and TAG oil [B], respectively

Table 1-3 Dose differences of PS between DAG oil and TAG oil

Articles	Dose of PS (as free form g/day)	Types of PS	Solvent
Goto et al. (1999)	0.4	PS_{free} / edible oil	DAG (dissolved)
Saito et al. (2006a)	0.4	PSE / mayonnaise	DAG (dissolved)
Meguro et al. (2001)	0.5	PS_{free} / mayonnaise	DAG (dissolved)
Vanhanen et al. (1992)	0.63	PSE /mayonnaise	TAG (dissolved)
Miettinen et al. (1994)	0.7	PS_{free} / mayonnaise	TAG (dissolved)
Pelletier et al. (1995)	0.74	PS_{free} / butter	TAG (dissolved)
Hendriks et al. (1999)	0.83-3.24	PSE/ spread	TAG (dissolved)
Weststrate et al. (1998)	1.6-3.3	PSE / margarine	TAG (dissolved)
Jones et al. (2000)	1.84	PSE / margarine	TAG (dissolved)

Main oils are triacylglycerol: TAG, or diacylglycerol: DAG.

PS belong to free forms of phytosterols: PS_{free} or ester forms of phytosterols: PSE.

Here, this thesis presents that replacing common TAG oil with PS/DAG oil (PS: 4 wt%) in a daily diet was simple and safe, and may therefore be a useful dietary approach to prevent CVD in the high cholesterol and/or overweight/obese populations associated with modern diet, unfavorable lifestyle, aging, and genetic background. Chapter 2 shows that long-term *ad libitum* consumption of PS/DAG cooking oil without dietary restriction resulted in a robust cholesterol-lowering effect throughout the test period, without rebound phenomena or adverse effects, in subjects with moderate to hypercholesterolemia. No significant effect was observed in subjects with normocholesterolemia (Takeshita et al., 2001). This suggests that practical use of PS/DAG oil will support a long-term strategy for the prevention of CVD. Chapter 3 shows that the 4-week PS/DAG cooking oil consumption reduced elevated atherogenic blood LDL cholesterol and Lp(a) concentrations in postmenopausal women (Takeshita et al., 2007a). These findings suggest that PS/DAG oil has also potential benefits for reducing risk factors for CVD even in age-related hypercholesterolemia due to estrogen depletion. Chapter 4 discusses the effects of well-regulated consumption of DAG oil or PS/DAG oil as a mayonnaise-type food on body fat accumulation and blood lipids in overweight/obese middle-aged men. Interestingly, consumption of DAG oil alone slightly, but significantly, reduced blood LDL cholesterol concentrations depending on a reduction in abdominal fat. Thus, combined consumption of dietary DAG oil with PS may exert mutually complementary cholesterol-lowering effects, respectively (Takeshita et al., 2007b). These findings suggest that DAG oil functions not only as an advantageous delivery vehicle for PS, but also as an antiatherogenic

ingredient mainly in a population with metabolic syndrome. Chapter 5 shows that the consumption of PS/DAG cooking oil reinforced low-dose therapy of a major hypocholesterolemic agent, statin (cholesterol biosynthesis inhibitors) in persistent hypercholesterolemic outpatients (Takeshita et al., 2008). This finding suggests that the use of PS/DAG cooking oil in combination with a conservatively low dose of statin may play more beneficial role for the prevention of CVD than statin monotherapy in hypercholesterolemic patients with a low response to statin due to increased cholesterol absorption.

CHAPTER 2

Effectiveness and safety of long-term *ad libitum* consumption of dietary phytosterol-rich diacylglycerol oil

2.1 Introduction

A lifetime of healthy blood cholesterol concentration is a key strategy in the prevention of CVD (Law et al., 1994; Ference et al., 2012). Also, the management of other atherosclerotic parameters are very important. On the other hand, PS-rich DAG oil may synergistically induce a transient cholesterol-lowering effect (400 mg/day) (Goto et al., 1999; Saito et al., 2006a) due to a good vehicle for PS, but it is not known whether the addition of PS/DAG oil to the daily diet will ensure the long-term maintenance of lowered blood cholesterol concentrations.

This chapter discusses the effects of 6-month *ad libitum* consumption of PS/DAG cooking oil without dietary restriction on mainly blood lipids. It was demonstrated that the sufficient use of PS/DAG oil resulted in a robust cholesterol-lowering effect throughout the test period, without rebound phenomena or adverse effects, in subjects with moderate to hypercholesterolemia. No significant effect was observed in subjects with normocholesterolemia. These findings suggest that the practically daily use of PS/DAG oil will support a long-term strategy for the primary prevention of CVD.

2.2 Material and methods

Study design

The study design is presented in **Fig. 2-1**. During the first -1 month of the run-in period, subjects consumed TAG cooking oil. Then, at the beginning of 6-month test period, subjects replaced their dietary TAG cooking oil with PS/DAG cooking oil and consumed only PS/DAG cooking oil for the subsequent 6 months. All subjects were informed of the study procedures and requirements and gave written consent to participate. The study was approved by the Ethics Committee of the Kao Corporation, and was conducted according to the Declaration of Helsinki under the supervision of an occupational health physician

Subjects

Seventy-one healthy male and female volunteers with no history of coronary heart disease (CHD) or strokes were recruited for the study in the Kao Corporation in Japan, who were 20-year-old and over.

Experimental methods

Subjects were encouraged to purchase their lunches in the company cafeteria where the experimental cooking oil (PS/DAG oil) was used exclusively for weekday meal preparation up before the last +1 month of the wash-out period at which time TAG cooking oil was again used. Subjects were also asked to maintain their normal lifestyles and dietary patterns without dietary restriction, and to

consume assigned oils in the form of cooking oil during the entire study period. On the day prior to blood sampling, all subjects were instructed to finish dinner by 9:00 pm, to not consume anything other than water after this time, and to abstain from alcohol for the 24 hours. A fasting blood sample was obtained prior to breakfast. As shown in **Fig. 2-1**, fasting blood sampling and physical measurements were performed at 6 separate time points: -1 month before the test period, at 0 (baseline), 1, 3, and 6 months during the test period, and +1 month after the wash-out. Total energy and intake levels of fat, carbohydrate, protein, fiber, cholesterol, and cooking oil were calculated from a diet diary in which subjects recorded dietary intake for 3 consecutive days prior to blood draws at the 0-, 1-, 3-, 6-, and +1-week time points. Calculations were performed at the Health & Nutrition Institute Snow Brand Milk Products Co., Ltd. based on the 4th Revision of Standard Tables of Food Composition in Japan (JST, 1998).

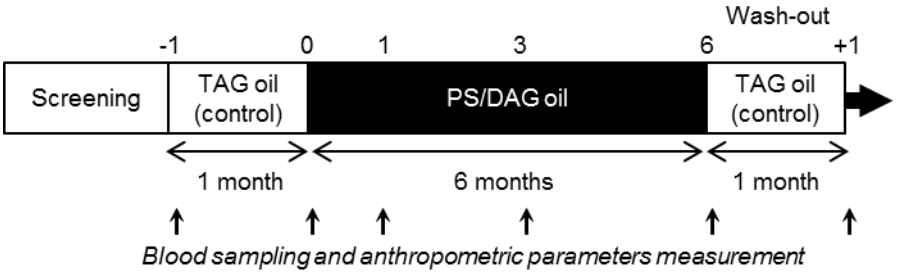


Fig. 2-1 Diagram of the study design

Test oil

DAG rich cooking oil (DAG oil) with an 84 % weight was prepared from soybean oil under the immobilized lipase according to the method of Huge-Jensen et al. (1988). The ratio of 1,3-DAG to 1(3),2-DAG in DAG oil was 66:34 wt/wt. The major fatty acids of DAG oil were oleic acid and linoleic acid (C14:0, 0.1; C16:0, 2.5; C18:0, 0.7; C18:1, 28.1; C18:2, 61.1, C18:3, 7.3; C20:0, 0.1; C20:1, 0.1 wt%). PS purified from the distillates of soybean and rapeseed oils was dissolved in DAG oil at a ratio of 4:96 (wt:wt). One hundred grams of the experimental oil contained β-sitosterol 1.85 g, campesterol 1.01 g, stigmasterol 1.20 g, and brassicasterol 0.11 g.

Blood Analyses and anthropometetry measurements

Plasma and serum were obtained by centrifugation at 1,500 ×g for 15 minutes at 4°C and stored at 4°C or -80°C, and then analyzed by SRL, Inc. (Tokyo, Japan). Serum triacylglycerol concentrations were measured using an LPL-GK-GPO-POD assay kit (Pure Auto S TG-N, Daiichi Pure Chemicals, Tokyo, Japan). Serum total cholesterol concentrations were measured using an enzyme assay kit (L-type Wako CHO H, Wako Pure Chemical Industries, Osaka, Japan). Serum HDL cholesterol concentrations were measured using an enzymatic assay kit (Cholestest N HDL, Daiichi Pure Chemicals, Tokyo, Japan). Serum LDL cholesterol concentrations were calculated using Friedewald's equation (LDL cholesterol = total cholesterol - HDL cholesterol - triacylglycerol x 0.2; 0.16 was used instead of 0.2 in cases where serum triacylglycerol was over 400 mg/dl). Serum

remnant-like lipoprotein particles cholesterol (RLP-C) concentrations, in a serum lipoprotein fraction binding neither to anti-human apo-B100 antibody nor anti-human apo-A1 antibody, were measured using a RLP-Cholesterol Immunoseparation Assay Kit (RLP Cholesterol JIMROII, Japan Immunoresearch Laboratories Co., Ltd., Gunma, Japan). Serum apolipoprotein (apo) AI, B, CII, CIII, and E concentrations were measured using a kit (Auto・N KIT series, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). Plasma PAI-1 concentrations were measured using an immunoreaction kit (LPIA・tPAI test, Mitsubishi Kagaku Medical, Inc., Ibaraki, Japan). Serum leptin concentrations were measured using a Human Leptin Radioimmunoassay kit (HUMAN LEPTIN RIA KIT, LINCO Research, Inc., Missouri, USA). Hematological variables were measured by routine clinical techniques (SE-9000, Sysmex Corporation, Hyogo, Japan). Serum phospholipids (PL) concentrations were measured using a kit (L-type Wako Phospholipids, Wako Pure Chemical Industries, Osaka, Japan). Serum non-esterified fatty acid (NEFA) concentrations were measured using an ACS-ACOD-POD assay kit (NEFA-SS ”EIKEN”, Eiken Chemical, Tokyo, Japan). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured using enzymatic assay kits (Transaminase-HR II, Wako Pure Chemical Industry, Osaka, Japan). Serum γ-glutamyltranspeptidase (γ-GTP) activity was measured using an enzymatic assay kit (Cicaliquid gamma-GT J, Kanto Chemical, Tokyo, Japan). Serum campesterol, β-sitosterol, and lathosterol concentrations were analyzed at Kao using gas-liquid chromatography on DB-5ht columns (15 m × 0.25mm, J&W Co.) from saponified serum extract as trimethylsilyl derivatives for

subjects with serum stocks -80°C (n = 66). These sterols were quantified from a single run using 5α-cholestane as the internal standard (Gylling et al., 1999). Anthropometric measurements were performed immediately before blood sampling to assess body weight, body mass index (BMI), waist circumference, hip circumference, and subcutaneous fat thickness using a slide caliper. Subcutaneous fat thickness was estimated by adding the upper arm and dorsal fat measurements.

Statistical analyses

Data for each of the serum lipid, apo, PS, lathosterol, PAI-1, leptin, hematological/metabolic, and anthropometric parameters were analyzed using paired t-tests to assess the differences from the baseline. Correlation coefficients were assessed using Pearson's correlation test. A type I error rate of < 0.05 was considered to be statistically significant. The parameters measured in the nutrition test, blood test, and physical examinations were expressed as the mean ± standard deviation (SD).

2.3 Results

Subjects and profile

Subjects were comprised of 71 healthy adult volunteers (23 - 58 years). Based on the guideline of Japan Atherosclerosis Society for hyperlipidemias in adults (JAS, 1997), all subjects were categorized and evaluated (**Table 2-1**). The characteristics of subjects, according to their baseline levels of serum total cholesterol, are presented in **Table 2-2.** The subjects are equally divided into three groups (normal: < 5.17 mmol/L, 35 %; borderline: 5.17 - 5.68 mmol/L, 28 %; high: ≥ 5.69 mmol/L, 37 %).

Diet survey

Total energy, fat, cholesterol, and experimental cooking oil intakes and the n-6/n-3 ratios are presented in **Table 2-3**. No remarkable changes were observed in any of the groups with respect to total energy and fat intake levels or n-6 / n-3 ratios from the test period to the wash-out period. In addition, there were no significant changes in experimental PS/DAG cooking oil intake levels, which was stable at 15-17 g/day during the study. PS/DAG cooking oil was ingested on average at a rate of 16.3 g/day (total PS consumption: 680 mg/day) in all subjects and 16.0 g/day (total PS consumption: 667mg/d) in the high group. No adverse events were observed throughout the study. Cholesterol intake slightly increased from baseline during the test period in all subjects. However, this increase did not reach

statistical significance between groups despite a significantly higher cholesterol intake in the high group. No remarkable changes were observed (data not shown) with respect to other nutritional parameters such as protein, carbohydrate, and dietary fiber intake. The percentage of energy derived from fat was approximately 31.4%, which was somewhat higher than Japanese average (26.3%). Dietary record showed that subjects were more likely to have fatty foods. No subjects withdrew their consent due to the test oil.

Table 2-1 Diagnosis of blood lipids levels in Japanese adults

Parameters	(Unit)	Low	Normal	Borderline	High
Triacylglycerol	(mmol/L)	-	< 1.69	-	≥ 1.69
	(mg/dL)	-	< 150	-	≥ 150
Total cholesterol	(mmol/L)	-	< 5.17	5.17-5.68	≥ 5.69
	(mg/dL)	-	< 200	200-219	≥ 220
LDL cholesterol	(mmol/L)	-	< 3.10	3.10-3.61	≥ 3.62
	(mg/dL)	-	< 120	120-139	≥ 140
HDL cholesterol	(mmol/L)	< 1.03	≥ 1.03	-	-
	(mg/dL)	< 40	≥ 40	-	-

Table 2-2 Characteristics of subjects according to the baseline levels of serum TC [1]

	All subjects	Normal	Borderline	High
Age (years)	41.1 ± 9.1	41.4 ± 9.3	39.5 ± 8.0	42.1 ± 9.9
n (male / female)	71 (66 / 5)	25 (24 / 1)	20 (17 / 3)	26 (25 / 1)
Body weight (kg)	66.9 ± 7.8	65.0 ± 7.3	65.0 ± 7.2	70.2 ± 7.9
BMI (kg/m^2)	23.3 ± 2.3	22.6 ± 1.7	22.7 ± 2.0	24.4 ± 2.5
Total cholesterol (mmol/L)	5.40 ± 0.75	4.63 ± 0.44	5.35 ± 0.13	6.15 ± 0.44
LDL cholesterol (mmol/L)	3.34 ± 0.59	2.82 ± 0.41	3.36 ± 0.28	3.85 ± 0.49
HDL cholesterol (mmol/L)	1.37 ± 0.31	1.32 ± 0.34	1.45 ± 0.26	1.37 ± 0.31
Triacylglycerol (mmol/L)	1.50 ± 1.08	1.12 ± 0.71	1.15 ± 0.43	2.13 ± 1.40

[1] Values are means ± SD. TC: total cholesterol. Normal, serum TC < 5.17 mmol/L; borderline, serum TC 5.17-5.68 mmol/L; high, serum TC ≥ 5.69 mmol/L.

Table 2- 3 Dietary composition thoughout the test periods[1]

	Study months	All subjects (n = 71)	Normal (n = 25)	Borderline (n = 20)	High (n = 26)
Energy	0	9255 ± 1552	9213 ± 1657	9368 ± 1418	9209 ± 1607
（kJ/day）	1	9259 ± 1607	9364 ± 1707	9163 ± 1397	9230 ± 1707
	3	9029 ± 1615	8979 ± 1761	9100 ± 1205	9021 ± 1791
	6	9196 ± 1611	9297 ± 1770	9129 ± 1351	9146 ± 1695
	+1	9268 ± 1749	8954 ± 1971	9795 ± 1644	9163 ± 1573
Fat	0	75.4 ± 18.8	77.6 ± 18.1	77.3 ± 15.0	71.8 ± 21.9
(g/day)	1	75.8 ± 20.5	75.7 ± 20.0	79.9 ± 22.9	72.8 ± 19.4
	3	77.0 ± 18.0	79.2 ± 18.9	79.3 ± 15.7	73.3 ± 18.6
	6	76.1 ± 18.0	79.5 ± 17.1	74.1 ± 15.3	74.3 ± 20.8
	+1	77.2 ± 20.7	72.9 ± 19.2	87.9 ± 26.3	73.1 ± 14.0
Cholesterol	0	396 ± 139	400 ± 162	439 ± 129	360 ± 116
（mg/day）	1	411 ± 140	382 ± 118	440 ± 178	416 ± 127*
	3	431 ± 144	427 ± 161	465 ± 159	409 ± 111*
	6	429 ± 146	429 ± 134	408 ± 175	445 ± 135*
	+1	434 ± 157	357 ± 126	502 ± 179	454 ± 140**
n-6 / n-3	0	4.4 ± 1.7	4.9 ± 1.9	3.9 ± 1.2	4.3 ± 1.6
(ratio/day)	1	4.7 ± 1.9	5.1 ± 1.8	4.9 ± 2.1	4.1 ± 1.6
	3	4.0 ± 1.5	3.9 ± 1.4**	4.5 ± 1.7	3.7 ± 1.4
	6	4.6 ± 1.9	4.7 ± 1.7	4.6 ± 1.8	4.6 ± 2.2
	+1	4.5 ± 1.7	4.6 ± 1.6	4.7 ± 1.7	4.1 ± 2.0
Test oil	0	15.6 ± 7.4	15.4 ± 6.9	16.9 ± 7.9	14.7 ± 7.7
(g/day)	1	16.9 ± 7.3	15.9 ± 6.9	18.1 ± 7.7	16.9 ± 7.6
	3	16.8 ± 7.4	16.2 ± 8.6	18.1 ± 6.4	16.4 ± 7.0
	6	15.1 ± 6.5	15.8 ± 5.5	14.9 ± 7.9	14.6 ± 6.5
	+1	16.9 ± 7.8	16.7 ± 7.9	20.5 ± 8.7	14.5 ± 6.2

[1] Values are means ± SD. Significantly different from baseline (= 0week) determined by Student's t-test : *P < 0.05. **P < 0.01. TC: total cholesterol. Normal, serum TC < 5.17 mmol/L; borderline, serum TC 5.17-5.68 mmol/L; high, serum TC ≥ 5.69 mmol/L.

Blood lipids and apos analyse

At first, the subjects were divided into 2 to 3 groups according to baseline values of each serum lipid concentration, and each group was evaluated separately (**Fig. 2-2**). During the run-in period (-1 month to the beginning of the test period) when all subjects consumed conventional TAG cooking oil, no significant changes in total cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol concentrations were observed.

Next, **Table 2-4** presents details about the effects of the experimental oil on serum lipids in the three groups of subjects that were categorized based on their serum total cholesterol baseline concentrations. No significant changes in total cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol concentrations or LDL cholesterol / HDL cholesterol ratios were observed in any of the study groups during the control period (-1 month to the beginning of the test period) when subjects consumed conventional TAG cooking oil (data not shown). Significant decreases in total cholesterol and LDL cholesterol were observed at 1 and 3 months for all subjects. In the high group, total cholesterol and LDL cholesterol concentrations decreased significantly from baseline values by 7.1% ($P < 0.001$) and 8.1% ($P < 0.01$) at 1-month, respectively. Subjects in the high group experienced a cholesterol-lowering effect of the experimental oil on total cholesterol and LDL cholesterol concentrations at 1-month in 81% and 73%, respectively. The statistically significant difference from baseline cholesterol concentration persisted until the end of the 6-month period and was still present during the final wash-out period. Although statistically not significant, total

cholesterol and LDL cholesterol concentrations in the borderline group also decreased up to the end of the 6-month period. Neither the total cholesterol nor LDL cholesterol concentrations showed an apparent change in the normal group. HDL cholesterol concentrations tended to increase gradually following continued use of the experimental cooking oil even though significant decreases were observed in all groups except the high group at 1 month. HDL cholesterol concentrations at 6 months significantly increased for all subjects combined and those in the normal group by 3.8% ($P < 0.01$) and 3.9% ($P < 0.05$), respectively. LDL cholesterol / HDL cholesterol ratios in all subjects significantly decreased by 6.5% ($P < 0.001$) by the end of the 6-month period. This effect was especially evident in the high group where the ratios decreased by 6.3 - 9.3% during the test period. HDL cholesterol concentrations and LDL cholesterol / HDL cholesterol ratios after the wash-out period were not significantly different from those observed at baseline. RLP-C and triacylglycerol concentrations particularly decreased in the high group, however the differences from baseline did not reach statistical significance. On the other hand, changes in triacylglycerol concentrations were strongly correlated with changes in RLP-C concentrations ($R = 0.839$, $P < 0.001$), as shown in **Fig. 2-3**. In addition, there is a negative correlation between changes in serum triacylglycerol concentrations and HDL cholesterol concentrations ($R = 0.430$, $P < 0.001$).

Table 2-5 shows changes in the apos that were measured from the baseline test period to +1 month after the wash-out period. ApoAI concentrations in each group except the borderline group at 3 months, significantly increased by an average of 4-5% ($P < 0.05$) by the end of 6 months. This

statistically significant increase in apoAI concentrations was maintained in all subjects even after the wash-out period. ApoB concentrations in all subjects significantly decreased by 4.6% ($P < 0.001$) and 5.6% ($P < 0.001$) at the 3- and 6-month points, respectively. This effect was particularly evident in the high group where a 6.3% decrease was observed. The apoB / apoAI ratios in each group significantly decreased by an average of 8-10% ($P < 0.001$) at the end of 6 months. There were no significant differences from the baseline concentrations of apoB and apoB / apoAI ratios after the wash-out period. Although no significant changes in apoCII were observed in any groups during the PS/DAG treatment period, the level significantly increased after the washout period in the normal group and in all subjects. ApoCIII concentrations in each group significantly decreased by between 9-10% ($P < 0.01$) at the end of 6 months. Similarly, apoCII / apoCIII ratios significantly increased during the test period in all except the borderline group and the increases were maintained during the washout period. ApoE concentrations in all subjects significantly decreased by 10.9% ($P < 0.001$) at the end of the 6-month period and by 7.5% ($P < 0.01$) and 12.3% ($P < 0.001$) in the borderline and high groups, respectively. Furthermore, changes in apoCIII and apoE concentrations were positively correlated with changes in serum triacylglycerol concentrations ($R = 0.588$, $P < 0.001$; $R = 0.769$, $P < 0.001$), respectively, as shown in **Fig. 2-3**.

Anthropometry related parameters analyses

In the present study, an average value of BMI was the almost same as Japanese national average (male: 23.1; female: 22.5), and normal size. No remarkable changes in BMI or weight were observed during the test period (**Table 2-6**). However, at 6 months, subcutaneous fat thickness and serum leptin concentrations in male subjects significantly reduced by 3.2% ($P < 0.05$) and 6.9% ($P < 0.05$), respectively. Changes in serum leptin concentrations were weakly correlated with changes in subcutaneous fat thickness ($R = 0.284$, $P = 0.021$). In addition, plasma PAI-1 concentration for subjects with a baseline concentration > 30 ng/mL ($n = 10$) was significantly reduced from baseline by 37.5% at the end of 1 month ($P < 0.001$) and this reduction was maintained until the end of the 6-month period.

Other blood parameters analyses

Serum β-sitosterol and campesterol concentrations in each group significantly increased by an average of 21-27% and 35-45%, respectively, by the end of 6 months (**Table 2-7**). After the wash-out period (+1 month), β-sitosterol and campesterol concentrations in each group returned to near baseline concentrations. Lathosterol did not increase by the end of the 6-month period. Changes in serum total cholesterol concentrations were moderately correlated with baseline values (0 month) in β-sitosterol concentrations (a cholesterol absorption marker) ($R = 0.308$, $P < 0.05$) (Tilvis & Miettinen, 1986), and also mildly correlated with baseline values (0 month) in lathosterol

concentrations (a cholesterol synthesis marker) ($R = 0.204$, $P = 0.100$) (Miettinen et al., 1990).

No significant changes in white blood cell (WBC), haemoglobin, NEFA, GPT, or γ-GTP were observed in any subjects. Red blood cell (RBC), hematocrit (in the high group only), PL, and AST changed significantly in all subjects; however, levels for each of these parameters remained within their normal ranges (**Table 2-8**).

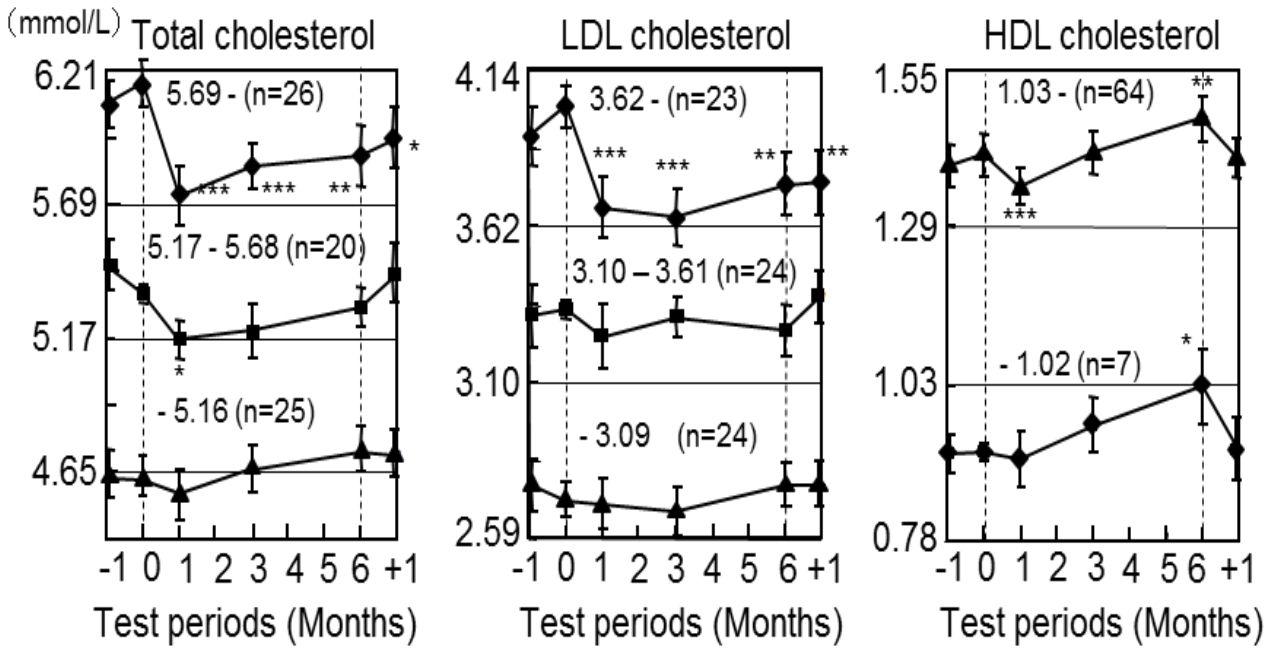


Fig. 2-2 Effects of dietary PS/DAG oil consumption on serum cholesterols concentrations (n=71)

Values are means ± SE, Significant difference from baseline: * P < 0.05, ** P < 0.01, *** P < 0.001.

Table 2-4 Effects of PS/DAG oil on serum lipids by group classified by baseline TC [1]

Parameters	Study months	All subjects (n=71)		Normal (n=25)		Boderline (n=20)		High (n=26)	
Total	-1	5.40 ± 0.78		4.63 ± 0.52		5.46 ± 0.44		6.08 ± 0.47	
cholesterol	0	5.40 ± 0.75		4.63 ± 0.44		5.35 ± 0.13		6.15 ± 0.44	
(mmol/L)	1	5.15 ± 0.70 ***	-4.8%	4.55 ± 0.54	-1.7%	5.17 ± 0.34	-3.4%	5.72 ± 0.57 ***	-7.1%
	3	5.25 ± 0.70 **	-2.9%	4.68 ± 0.52	1.1%	5.20 ± 0.47	-2.9%	5.84 ± 0.47 ***	-5.0%
	6	5.33 ± 0.70	-1.4%	4.76 ± 0.49	2.8%	5.30 ± 0.34	-1.0%	5.87 ± 0.59 **	-4.6%
	+1	5.38 ± 0.75	-0.5%	4.71 ± 0.47	1.7%	5.43 ± 0.52	1.4%	5.95 ± 0.59 *	-3.4%
LDL	-1	3.31 ± 0.672		2.79 ± 0.49		3.44 ± 0.54		3.75 ± 0.54	
cholesterol	0	3.34 ± 0.59		2.82 ± 0.41		3.36 ± 0.28		3.85 ± 0.49	
(mmol/L)	1	3.21 ± 0.62 **	-3.9%	2.82 ± 0.54	0.0%	3.28 ± 0.39	-2.3%	3.54 ± 0.65 **	-8.1%
	3	3.21 ± 0.57 **	-3.9%	2.82 ± 0.47	0.0%	3.23 ± 0.47	-3.8%	3.57 ± 0.49 **	-7.4%
	6	3.26 ± 0.59	-2.3%	2.87 ± 0.47	1.8%	3.31 ± 0.39	-1.5%	3.62 ± 0.57 **	-6.0%
	+1	3.31 ± 0.595	-0.8%	2.87 ± 0.47	1.8%	3.41 ± 0.54	1.5%	3.62 ± 0.52 **	-6.0%
HDL	-1	1.34 ± 0.31		1.27 ± 0.28		1.45 ± 0.28		1.37 ± 0.31	
cholesterol	0	1.37 ± 0.31		1.32 ± 0.34		1.45 ± 0.26		1.37 ± 0.31	
(mmol/L)	1	1.32 ± 0.28 ***	-3.8%	1.24 ± 0.26 *	-5.9%	1.37 ± 0.28 **	-5.4%	1.34 ± 0.28	-1.9%
	3	1.37 ± 0.31	0.0%	1.34 ± 0.34	2.0%	1.42 ± 0.26	-1.8%	1.37 ± 0.34	0.0%
	6	1.42 ± 0.31**	3.8%	1.37 ± 0.31 *	3.9%	1.53 ± 0.31	5.4%	1.42 ± 0.34	3.8%
	+1	1.37 ± 0.31	0.0%	1.32 ± 0.28	0.0%	1.45 ± 0.28	0.0%	1.34 ± 0.31	-1.9%
LDL	-1	2.58 ± 0.78		2.33 ± 0.77		2.49 ± 0.73		2.88 ± 0.75	
cholesterol	0	2.57 ± 0.76		2.29 ± 0.73		2.42 ± 0.67		2.96 ± 0.72	
/ HDL	1	2.55 ± 0.75	-0.8%	2.37 ± 0.74	3.2%	2.50 ± 0.72	3.2%	2.77 ± 0.77 *	-6.3%
cholesterol	3	2.46 ± 0.72 **	-4.3%	2.23 ± 0.69	-3.0%	2.3 9 ± 0.66	-1.3%	2.74 ± 0.72 **	-7.3%
	6	2.41 ± 0.74 ***	-6.5%	2.20 ± 0.71	-4.0%	2.30 ± 0.74 *	-5.1%	2.68 ± 0.71 ***	-9.3%
	+1	2.56 ± 0.81	-0.4%	2.32 ± 0.73	1.1%	2.49 ± 0.89	2.9%	2.85 ± 0.77	-3.6%
Triacylglycerol	-1	1.61 ± 1.02		1.28 ± 0.81		1.26 ± 0.67		2.21 ± 1.16	
(mmol/L)	0	1.50 ± 1.08		1.12 ± 0.71		1.15 ± 0.43		2.13 ± 1.40	
	1	1.42 ± 0.98	-5.3%	1.15 ± 0.96	3.0%	1.11 ± 0.49	-3.9%	1.94 ± 1.11	-9.0%
	3	1.47 ± 1.08	-2.3%	1.13 ± 0.86	1.0%	1.19 ± 0.54	2.9%	2.02 ± 1.37	-5.3%
	6	1.35 ± 0.87	-9.8%	1.10 ± 0.75	-2.0%	1.04 ± 0.49	-9.8%	1.84 ± 1.02	-13.8%
	+1	1.58 ± 1.16	5.3%	1.19 ± 0.72	6.1%	1.20 ± 0.52	3.9%	2.25 ± 1.52	5.3%
RLP	0	6.1 ± 5.1		4.1 ± 2.7		4.6 ± 1.4		9.2 ± 6.9	
cholesterol	3	6.1 ± 4.9	0.0%	4.3 ± 3.1	4.9%	5.0 ± 2.2	8.7%	8.7 ± 6.7	-5.4%
(mg/dL)	6	5.3 ± 3.6	-13.1%	4.2 ± 2.7	2.4%	4.4 ± 2.0	-4.3%	7.1 ± 4.5	-22.8%
	+1	6.9 ± 6.2	13.1%	4.8 ± 2.7	17.1%	5.4 ± 3.0	17.4%	10.0 ± 8.9	8.7%

[1]Mean ± SD. Significantly different from baseline (= 0week) determined by Student's t-test: $^{*}P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$. TC: total cholesterol. Normal, serum TC < 5.17 mmol/L; borderline, serum TC 5.17-5.68 mmol/L; high, serum TC ≥ 5.69 mmol/L.

Table 2-5 Effects of PS/DAG oil on serum apos by group classified by baseline TC [1]

Parameters	Study months	All subjects (n=71)		Normal (n=25)		Boderline (n=20)		High (n=26)	
ApoAI	0	141 ± 21		134 ± 24		145 ± 18		145 ± 21	
(mg/dL)	3	146 ± 21***	3.5%	141 ± 22 ***	5.2%	147 ± 17	1.4%	149 ± 24 *	2.8%
	6	147 ± 21***	4.3%	139 ± 19 *	3.7%	152 ± 20 *	4.8%	151 ± 21 *	4.1%
	+1	145 ± 20*	2.8%	139 ± 21	3.7%	148 ± 20	2.1%	148 ± 19	2.1%
ApoB	0	108 ± 22		91 ± 17		103 ± 12		127 ± 18	
(mg/dL)	3	103 ± 20***	-4.6%	89 ± 16	-2.2%	99 ± 15	-3.9%	119 ± 16 ***	-6.3%
	6	102 ± 22***	-5.6%	88 ± 17	-3.3%	99 ± 12 *	-3.9%	119 ± 21 ***	-6.3%
	+1	109 ± 24	0.9%	93 ± 19	2.2%	106 ± 16	2.9%	126 ± 22	-0.8%
ApoB / ApoA I	0	0.78 ± 0.21		0.70 ± 0.20		0.73 ± 0.18		0.90 ± 0.19	
	3	0.72 ± 0.18 ***	-7.5%	0.65 ± 0.17 **	-6.8%	0.69 ± 0.15 *	-5.9%	0.82 ± 0.18 ***	-9.1%
	6	0.71 ± 0.19 ***	-9.1%	0.65 ± 0.17 ***	-7.6%	0.67 ± 0.17 ***	-8.9%	0.81 ± 0.19 ***	-10.3%
	+1	0.77 ± 0.22	-1.7%	0.69 ± 0.20	-1.3%	0.74 ± 0.20	0.3%	0.87 ± 0.21	-3.1%
ApoCII	0	4.2 ± 2.4		3.3 ± 1.5		3.5 ± 1.2		5.5 ± 3.2	
(mg/dL)	3	4.2 ± 2.3	0.0%	3.5 ± 1.7	6.1%	3.4 ± 1.3	-2.9%	5.4 ± 2.9	-1.8%
	6	4.0 ± 2.2	-4.8%	3.4 ± 1.7	3.0%	3.4 ± 1.3	-2.9%	5.1 ± 2.6	-7.3%
	+1	4.4 ± 2.2 *	4.8%	3.8 ± 1.6 **	15.2%	3.6 ± 1.2	2.9%	5.6 ± 2.8	1.8%
ApoCIII	0	10.8 ± 3.8		8.9 ± 2.8		9.8 ± 1.5		13.4 ± 4.4	
(mg/dL)	3	10.1 ± 4.0 **	-6.5%	8.3 ± 3.0 *	-6.7%	9.0 ± 2.1 *	-8.2%	12.7 ± 4.7	-5.2%
	6	9.8 ± 3.4 ***	-9.3%	8.1 ± 2.7 **	-9.0%	8.8 ± 1.6 **	-10.2%	12.2 ± 3.8 **	-9.0%
	+1	10.5 ± 3.6	-2.8%	8.8 ± 2.8	-1.1%	9.3 ± 1.6	-5.1%	13.0 ± 4.1	-3.0%
ApoCII	0	0.37 ± 0.11		0.36 ± 0.08		0.36 ± 0.12		0.39 ± 0.12	
/ ApoCIII	3	0.40 ± 0.11 ***	8.6%	0.41 ± 0.11 ***	13.8%	0.37 ± 0.09	3.7%	0.42 ± 0.13 **	7.5%
	6	0.40 ± 0.11 ***	7.2%	0.40 ± 0.10 **	11. 7%	0.38 ± 0.10	5.0%	0.41 ± 0.12 *	4.9%
	+1	0.41 ± 0.11 ***	11.5%	0.43 ± 0.09 ***	17.5%	0.39 ± 0.10 *	8.4%	0.42 ± 0.13 *	8.3%
ApoE	0	4.6 ± 1.6		3.8 ± 0.9		4.0 ± 0.7		5.7 ± 1.9	
(mg/dL)	3	4.3 ± 1.5 **	-6.5%	3.7 ± 1.0	-2.6%	3.8 ± 0.8 *	-5.0%	5.3 ± 1.8 *	-7.0%
	6	4.1 ± 1.3 ***	-10.9%	3.6 ± 0.8	-5.3%	3.7 ± 0.7 **	-7.5%	5.0 ± 1.6 ***	-12.3%
	+1	4.4 ± 1.4	-4.3%	3.8 ± 0.9	0.0%	4.0 ± 0.8	0.0%	5.5 ± 1.6	-3.5%

[1] Mean ± SD. Significantly different from baseline (= 0 week) determined by Student's t-test: *P < 0.05. **P < 0.01, ***P < 0.001. TC: total cholesterol. Normal, serum TC < 5.17 mmol/L; borderline, serum TC 5.17-5.68 mmol/L; high, serum TC ≥ 5.69 mmol/L.

Table 2-6 Effects of PS/DAG oil on anthropometrics parameters, serum leptin and plasma PAI-1 [1]

Parameters	Study months	All subjects (n=71)	Changes
BMI (kg/m^2)	-1	23.3 ± 2.3	-
(All subjects, n = 71)	0	23.3 ± 2.3	-
	1	23.3 ± 2.3	-0.1%
	3	23.4 ± 2.3	0.3%
	6	23.4 ± 2.3	0.5%
	+1	23.5 ± 2.3**	0.9%
Subcutaneous fat	-1	27.4 ± 9.6	-
thickness (mm)	0	28.0 ± 9.7	-
(Male subjects, n = 66)	1	27.5 ± 9.5	-1.7%
	3	27.3 ± 9.7	-2.4%
	6	27.1 ± 9.6*	-3.2%
	+1	27.5 ± 9.5	-1.7%
Leptin (ng/mL)	0	4.3 ± 2.3	-
(Male subjects, n = 66)	6	4.0 ± 2.2*	-6.9%
	+1	4.3 ± 2.2	1.4%
PAI-1 (ng/mL)	-1	45.6 ± 25.1	-
(High levels, n = 10) [2]	0	46.1 ± 11.6	-
	1	28.8 ± 10.1***	-37.5%
	3	37.3 ± 16.9	-19.1%
	6	33.3 ± 13.3**	-27.8%
	+1	39.4 ± 15.4	-14.5%

[1] Values are means ± SD. Significantly different from baseline (= 0week) determined by Student's t-test: $^{*}P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$. [2] Subjects with the high level of baseline (PAI-1 > 30 ng/mL).

Table 2-7 Effects of dietary PS/DAG oil on serum noncholesterol sterols by group classified by baseline of total cholesterol [1]

Parameters	Study months	All subjects (n=71)		Normal (n=25)		Borderline (n=20)		High (n=26)	
β-Sitosterol	0	8.11 ± 3.89	-	6.99 ± 2.19	-	9.04 ± 5.41	-	8.48 ± 3.72	-
(μmol/L)	6	10.12 ± 4.70 ***	24.8%	8.47 ± 2.93***	21.2%	11.45 ± 6.68**	26.7%	10.69 ± 4.02***	26.0%
	+1	8.20 ± 3.99	1.1%	6.93 ± 2.18	-0.8%	9.46 ± 6.20	4.7%	8.45 ± 2.96	-0.4%
Campesterol	0	11.90 ± 4.45	-	12.31 ± 3.97	-	11.34 ± 5.59	-	11.91 ± 4.07	-
(μmol/L)	6	16.56 ± 5.59***	39.2%	16.59 ± 4.76***	34.7%	16.43 ± 7.15***	44.9%	16.63 ± 5.27***	39.6%
	+1	12.14 ± 4.36	2.1%	12.57 ± 3.74	2.1%	11.19 ± 4.53	-1.3%	12.44 ± 4.81	4.4%
Lathosterol	0	10.69 ± 4.97	-	8.70 ± 4.36	-	11.68 ± 5.00	-	11.80 ± 5.10	-
(μmol/L)	6	10.34 ± 4.66	-3.3%	8.37 ± 3.06	-3.8%	10.40 ± 3.83	-11.0%	12.11 ± 5.73	2.6%
	+1	9.47 ± 5.01 *	-11.4%	7.92 ± 4.21	-9.0%	9.41 ± 3.56	-19.4%	10.95 ± 6.18	-7.2%

[1] Values are means ± SD. Significantly different from baseline (= 0week) determined by Student's t-test: *P < 0.05. **P < 0.01, ***P < 0.001. TC: total cholesterol. Normal, serum TC < 5.17 mmol/L; borderline, serum TC 5.17-5.68 mmol/L; high, serum TC ≥ 5.69 mmol/L.

Table 2-8 Changes in hematological and other serum indices[1]

	Study months	All subjects (n = 71)	Normal (n = 25)	Borderline (n = 20)	High (n = 26)
Hematology					
RBC (10^4/μL)	0	489 ± 40	488 ± 29	470 ± 49	506 ± 34
	6	494 ± 38 *	496 ± 26 *	478 ± 49 *	505 ± 35
WBC (/μL)	0	6006 ± 1700	6020 ± 1589	5700 ± 2109	6227 ± 1470
	6	5742 ± 1703	5728 ± 1496	5150 ± 1484	6212 ± 1947
HB (g/dL)	0	14.9 ± 1.2	14.9 ± 0.8	14.3 ± 1.6	15.4 ± 0.9
	6	15.0 ± 1.2	15.0 ± 0.7	14.7 ± 1.6	15.4 ± 1.1
HT (%)	0	45.3 ± 3.4	44.8 ± 2.3	43.9 ± 4.4	46.8 ± 2.8
	6	45.0 ± 3.1	45.0 ± 2.2	43.9 ± 4.0	45.8 ± 3.0 *
Others					
NEFA (μEq/L)	0	507 ± 209	462 ± 163	470 ± 203	579 ± 238
	6	526 ± 235	471 ± 224	601 ± 235 *	523 ± 238
PL (mmol/L)	0	3.11 ± 0.48	2.75 ± 0.31	3.06 ± 0.18	3.51 ± 0.47
	6	3.01 ± 0.39 **	2.73 ± 0.26	2.97 ± 0.23	3.32 ± 0.37 *
AST (IU/L)	0	22 ± 8	21 ± 6	22 ± 12	23 ± 7
	6	24 ± 13 *	22 ± 7	25 ± 17	26 ± 15
ALT (IU/L)	0	27 ± 18	25 ± 17	23 ± 15	33 ± 19
	6	30 ± 21	25 ± 16	26 ± 21	37 ± 23
γ-GPT (IU/L)	0	51 ± 42	45 ± 50	36 ± 25	68 ± 38
	6	57 ± 57	46 ± 60	41 ± 37	79 ± 61

[1] Values are means ± SD. Significantly different from baseline (= 0 week) determined by Student's t-test: *P < 0.05. TC: total cholesterol. Normal, serum TC < 5.17 mmol/L; borderline, serum TC 5.17-5.68 mmol/L; high, serum TC ≥ 5.69 mmol/L.

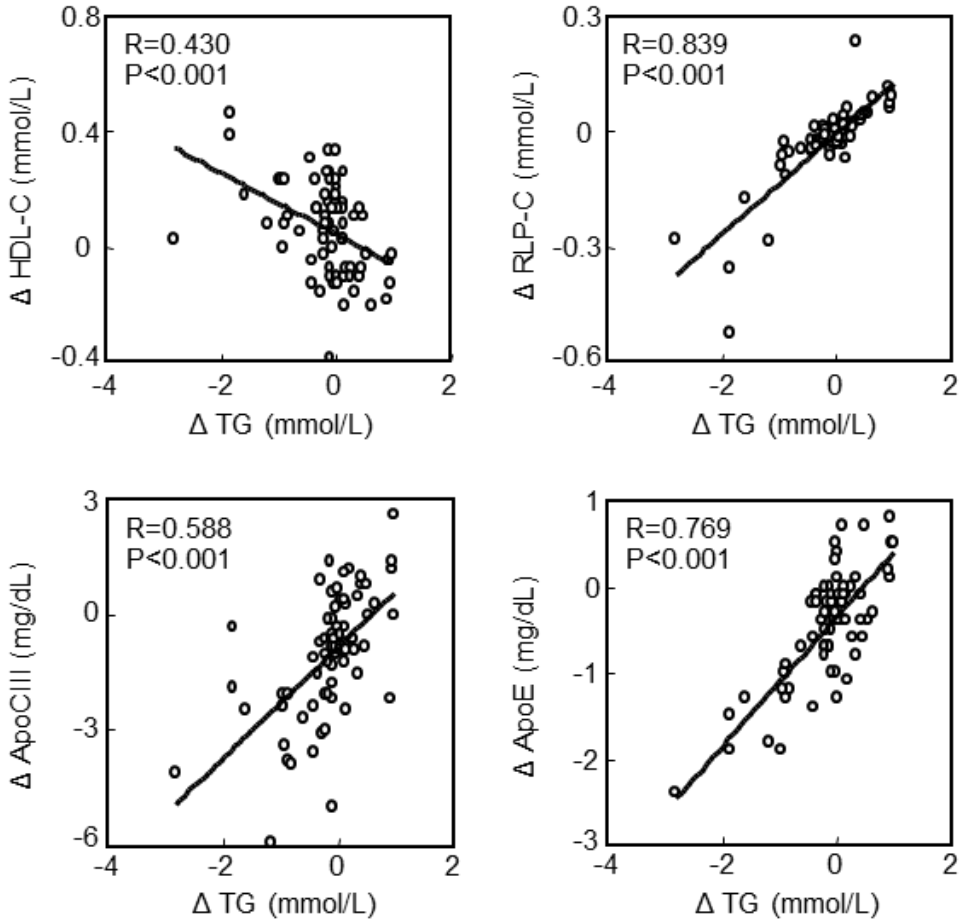


Fig. 2-3 Correlation between changes of serum TG and changes of the other lipid parameters for 24 weeks in male subjects (n=66).

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2.4 Discussion

The present study investigated the long-term effects of PS in combination with DAG oil on levels of serum lipids, apos and anthropometric parameters among healthy adults. The subjects substituted their normal cooking oil with PS/DAG oil during the test period after a specified control period.

In the previous controlled studies of DAG oil, the cholesterol-lowering effect has not directly shown in DAG oil group similar to TAG oil group (Nagao et al., 2000; Maki et al., 2002; Yamamoto et al. 2001). Thus, with addition of PS to DAG oil, the serum total cholesterol and LDL cholesterol lowering effect was expected to become clearly evident. In addition, it has been reported that PS is more soluble in DAG oil (6.0%) than it is in TAG oil (1.3%) (Meguro et al., 2001), and the low daily ingestion of 400-500 mg of PS dissolved in DAG oil achieved a cholesterol-lowering effect (Goto et al., 1999; Meguro et al., 2001; Saito et al., 2006a).

In the present study, significant reductions in serum total and LDL cholesterols concentrations were also shown in the high group at 1-month of PS/DAG oil consumption and maintained to the end of the 6-month period, even though the average intake of PS (667 mg/day) in high group was lower than those of previous human studies with PS esters dissolved in TAG oil (PS 740 and 830 mg/day) (Pelletier et al., 1995; Hendriks et al., 1999). Moreover, the cholesterol-lowering effect by PS was clearly shown in the subjects with increased cholesterol absorption in the small intestine (high level of β-sitosterol in serum) rather than those with increased cholesterolgenesis in the liver (high level of lathosterol in serum). Those results indicate that the

inhibition of dietary cholesterol absorption is basically effective for the management of cholesterol in general.

Recent report has revealed that a soy lecitihin formulation remarkably elevated the solubility of β-sitostanol in bile acid micelles and significantly reduced cholesterol adsorption at lower doses (300 and 700 mg) (Ostlund et al., 1999). Thus, DAG might be expected to be one of the effective solvents such as lecithin, could facilitate to dissolve high levels of PS in the bile acid micelles of the intestine and that those micelles could significantly reduce cholesterol absorption in the small intestine at lower doses.

The cholesterol-lowering effects gradually appeared to decline with time (**Table 2-4**). This effect might be attributed to an increase of dietary cholesterol intake levels obtained from special meals and a decrease of the frequency of test oil ingestion, because the later stages of study overlapped with a celebratory season (December and January) when subjects were more likely to dine out and eat nourishing meals (**Table 2-3**). On the other hand, serum lathosterol concentrations did not remarkably change during the test period (**Table 2-7**). These findings suggest that a gradual decline in the cholesterol-lowering effect in this study can be attributed to elevated consumption of dietary cholesterol rather than the activation of cholesterol synthesis in the liver.

DAG oil composed mainly of 1,3-species has been shown to suppress the post-prandial elevation of serum triacylglycerol concentrations and decrease serum fasting triacylglycerol concentrations (Yamamoto et al., 2001). The triacylglycerol resynthesis in small intestinal epithelial

cells may be delayed after the ingestion of DAG (mainly 1,3-species of DAG) (Hara et al., 1993; Murata et al., 1994; Taguchi et al., 2000; Tada et al., 2001; Tada et al., 2005). In this study, the serum triacylglycerol concentration tended to greatly decrease at the end of 6-month test period, although not significant. Thus, the decrease in triacylglycerol concentrations in this study can also be attributed to the ingestion of DAG oil rather than PS as has previously been reported (Yamamoto et al., 2001). Moreover, the decrease in triacylglycerol concentrations by DAG oil contributed to increases in HDL cholesterol concentration (**Fig. 2-3**), since it has not been shown that PS change HDL cholesterol concentration so far (Jones et al., 1997). This effect suggests that DAG oil may be effective in preventing arteriosclerosis and thrombosis.

It was especially showed that various parameters related with HDL cholesterol, LDL cholesterol, and remnant particles in serum such as apoAI, B, CIII and E, were significantly improved in all subject groups with PS/DAG oil. Specifically, the remarkable decreases in apoCIII at fasting strongly associated with decreased serum triacylglycerol concentrations by DAG oil consumption ($R = 0.588$, $P < 0.001$) can indicate that DAG oil is effective for the prevention of arteriosclerosis in hypertriglyceridemia, since it has reported in the Cholesterol and Recurrent Events (CARE) Trial that apoCIII concentration in very low-density lipoprotein (VLDL) + LDL is one of the significant markers of progression of coronary atherosclerosis rather than LDL cholesterol or triacylglycerol concentration (Sacks et al., 2000). In addition, the significantly increased ratio of apoCII / apoCIII, which is a predictor of lipoprotein lipase (LPL) activation, in all groups (**Table 2-5**)

might suggest that LPL, which is typically associated with lipid metabolism, is moderately activated by DAG oil consumption.

DAG oil has reduced the abdominal fat in humans (Nagao et al., 2000; Maki et al., 2002). In studies with animals, DAG oil was suggested to have a higher capacity for hepatic lipid oxidation and a decrease in leptin levels (Murata et al., 1997; Murase et al., 2001). In this study, PS/DAG oil did not affect the body weights of the subjects within the normal range for BMI, however subcutaneous fat thickness and serum leptin concentrations, which are known to correlate closely with obesity (Considine et al., 1996), significantly decreased by 3.2% and 6.9% by the end of a 6-month consumption period in male subjects (n = 66). Further, plasma PAI-1 concentrations, which are known to correlate with visceral fat mass (Shimomura et al., 1996), also decreased in subjects who had high baseline PAI-1 concentrations. These combined results suggest that the long-term use of PS/DAG oil as cooking oil could contribute to the prevention of or reduction in central obesity by DAG oil, in addition to cholesterol-lowering effects of PS.

In healthy humans, the absorption rate of β-sitosterol in the small intestine was reported modest 5.0% (Kritchevsky, 1997). However, high levels of PS in serum might become a risk of arteriosclerosis, because in phytosterolemia, an extremely rare inherited disease, high levels of PS in the blood vessels induce arteriosclerosisis (Salen et al., 1992). In this study, serum PS concentrations were within the 1% of serum total cholesterol concentration and quickly returned to those of baseline after a 1-month wash-out period (**Table 2-7)**. This observation indicates that PS solubilized in DAG

oil were normally absorbed in the small intestine, were immediately eliminated through billiary secretion and did not accumulate in the body similar to PS in TAG oil. Furthermore, the rates of those levels, although increased, were remarkably lower compared to a previous report that showed a 70% increment with a difference in doses of PS (Weststrate & Meijer, 1998). On the other hand, no significant change in serum lathosterol concentrations was observed. It is concluded that a low intake of PS in the present study can't induce the cholesterol synthesis in the liver, resulting from the feedback effect of PS. Changes in hematological results and serum chemistry fell within normal ranges and were not statistically significant (**Table 2-8**) suggesting that PS/DAG oil ingestion has a safe profile in humans.

In conclusion, western diets have involved with increases in the risk factors associated with arteriosclerosis. In the present study, PS/DAG oil consumption play a role to ameliorate blood concentrations of total and LDL cholesterols, HDL cholesterol, triacylglycerol, remnant, PAI-1, and body fat parameters without adverse effects. This indicates that PS/DAG oil could be beneficial in the primary prevention of arteriosclerosis and obesity if used daily in place of conventional cooking oils.

CHAPTER 3

Potential impact of dietary phytosterol-rich diacylglycerol oil on atherogenic lipoproteins in postmenopausal women

3.1 Introduction

Estrogen has a protective effect against atherosclerosis due to a decreased blood concentration of "bad" low-density lipoprotein (LDL) cholesterol and increased "good" high-density lipoprotein (HDL) cholesterol. Because of estrogen depletion, the onset of menopause dramatically increases atherogenic blood lipoprotein concentrations, especially LDL and lipoprotein (a) [Lp(a)], resulting in a precipitous climb in CVD rates (Jenner et al., 1993; Davidson et al., 2002; Welty, 2004;Thom et al., 2006). Large-scale clinical trials indicate that lowering cholesterol is beneficial for preventing morbidity and death associated with CVD, irrespective of sex (Sacks et al., 1996; Downs et al., 1998; Heart Protection Study Collaborative Group, 2002; Athyros et al., 2002). As a first-line therapy for the management of cholesterol in postmenopausal women, diet and other lifestyle changes are recommended (Expert panel on detection, evaluation, and treatment of high blood cholesterol in adults, 2001).

Chapter 2 showed that the 6-month *ad libitum* consumption of PS/DAG cooking oil substantially and reduces blood total and LDL cholesterol concentrations in almost male subjects with moderate to hypercholesterolemia without dietary restriction (Takeshita et al, 2001). In this chapter, it

is demonstrated that a month consumption of PS/DAG cooking oil further reduces elevated atherogenic blood LDL cholesterol and Lp(a) concentrations in postmenopausal women with elevated blood cholesterol concentrations, compared to DAG oil. These findings suggest that PS/DAG oil has potential benefits for reducing risk factors for CVD, even in women with age-related hypercholesterolemia**.**

3.2 Materials and methods

Study design

The study was a randomized, double-blind, controlled study, performed in a crossover manner comprising two 4-week intervention periods (**Fig. 3-1)**. The study was performed under the supervision of an occupational health physician, in accordance with the regulations of the Kao Corporation Ethics Committee for Internal Clinical Studies and in conformity with the Helsinki Declaration. The conditions and procedures used in this study were reviewed with all subjects previous to obtaining their written informed consent.

Subjects

Twenty-five healthy female employees of the Kao Corporation who were 45-year-old and over were recruited for the study. Eleven individuals were not eligible to participate at that moment because they

were judged as pre-menopausal or whose serum total cholesterol concentrations were less than 5.17 mmol/L (200 mg/dL). In Japan, the recommended total cholesterol goal for postmenopausal women for the primary prevention of CVD is less than 200 mg/dL (5.17 mM) and dietary therapy is advised for postmenopausal women whose levels are above the borderline (JAS, 1997). Fourteen subjects after spontaneous menopause, who were more than a year away from menopause, were enrolled in the study. All the subjects were healthy and were taking no medication for blood glucose levels, lipid levels, blood pressure, or hormone replacement therapy.

Experimental methods

All subjects were randomly assigned to one of two groups and each group consumed either PS/DAG oil or DAG oil for a period of 4 weeks. The subjects were then given a four-week "wash-out period" before crossing over and receiving the other oil for an additional 4 weeks. The test oils were labelled with a blind code and were consumed as a replacement for the edible oils. The oils were used mainly as a cooking (fried, deep-fried) and were also used partly in the domestic use of handmade dressings such as salad dressing and mayonnaise, and curry at each subject's home. The target dose of the test oils was 10 g/day, equivalent to the average intake of an edible oil in Japan (JMHLW, 2002). The subjects were instructed to maintain their usual daily isocaloric diets, nonessential groceries (coffee and tobacco) and physical activities, and to limit their alcohol consumption no more than 30 g/day during the study period. In addition, the subjects were instructed to abstain from alcohol for 2 days

prior to each blood sampling and to fast for 12 hours before the blood sampling which was conducted in the morning at 4 week intervals. The physicians checked the health conditions of the subjects at every blood sampling and gave advice. The subjects were instructed to keep a dietary record for a three-consecutive-day before the blood sampling and to submit it at each visit. The mean daily intake of total energy, fat, fiber, cholesterol, and test oil were calculated by a dietician from the dietary record based on the 4th revised Japanese Food Composition Table (JST, 1998) . In addition, the actual frequency, application (fried, deep-fried, dressings and the others) and consumption of test oils at home was verified by a questionnaire survey throughout the period of the test oil treatments.

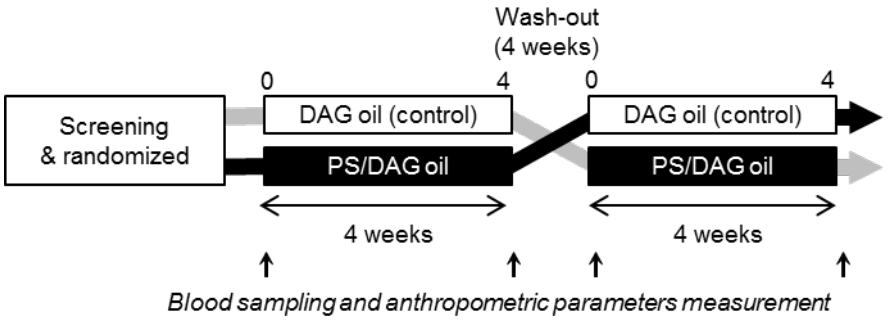


Fig. 3-1 Diagram of the study design

Test oils

The test oils used in the study were functional cooking oils manufactured in Japan and permitted as FOSHU Use by The Ministry of Health, Labour and Welfare of Japan. DAG oil (Econa Cooking Oil ®, Kao Corporation, Tokyo Japan) contained ~80 g DAG / 100 g and the major fatty acids of this oil were linoleic and oleic acids (**Table 3-1**). PS/DAG oil (Econa H&H Cooking Oil ®, Kao Corporation, Tokyo Japan) was prepared from DAG oil and PS diluted from soy bean and rapeseed oils. The PS/DAG oil contained 4.20 g of free forms of PS in 100 g, while the DAG oil contained 0.28 mg of PS in 100 g (**Table 3-2**). The test oils were stable during cooking and very similar to conventional cooking oils (TAG oil) in taste, flavour and appearance and were indistinguishable from each other. In the United States, the Food and Drug Administration has listed DAG oil as a "Generally Recognized as Safe" substance.

Table 3-1 Composition of acylglycerol and fatty acids in DAG oil

	DAG oil
Acylglycerol Species (wt%)	
Triacylglycerol	15.8
1(or 3), 2-Diacylglycerol	28.8
1,3-Diacylglycerol	54.8
Monoacylglycerol	0.6
Fatty acids composition (wt%)	
C16:0	2.4
C18:0	0.7
C18:1	28.1
C18:2	61.2
C18:3	7.3

Table 3-2 Composition of plant sterols in DAG oil and PS/DAG oil

	DAG oil	PS/DAG oil
β-Sitosterol (g)	0.16	1.86
Stigmasterol (g)	0.05	1.22
Campesterol (g)	0.06	1.01
Brassicasterol (g)	0.01	0.11
Total (g)	0.28	4.20

(g/100 g oil)

Blood analyses

Blood samples were collected from fasting subjects and serum was obtained by centrifugation at 1500 ×g for 15 minutes at 4°C, stored at 4°C or -80°C. All analyses except for noncholesterol sterols were conducted by SRL, Inc. (Tokyo, Japan). Serum total, LDL and HDL cholesterols and triacylglycerol concentrations were measured using an enzymatic assay. Serum apoAI and apoB concentrations were measured using a turbidimetric immunoassay. Serum AST, ALT and γ-GTP activities were measured using an enzymatic assay. Lp(a) concentrations were measured using an antihuman Lp(a) monoclonal antibody-coated latex assay. Serum total bile acid (TBA) concentrations were measured using an enzymatic assay kit (ENZABILE-Auto, Daiichi Pure Chemicals Co., Ltd.). Estradiol concentrations were measured using a radioimmunoassay. Follicle-stimulating hormone activity was measured using a chemiluminescent enzyme immunoassay. Serum campesterol, β-sitosterol, and lathosterol concentrations were analyzed at the Kao Corporation by a Hewlett Packard (Agilent Technologies) 5890A Gas chromatograph with the flame ionization detector with a DB-5HT column (15 m × 0.25 mm, J&W Co., USA) using saponified serum extract as their trimethylsilyl derivatives. These sterols were quantified using 5α-cholestane as the internal standard (Gylling et al., 1999). The serum concentrations of noncholesterol sterols were standardized by the ratio to serum total cholesterol concentration and multiplied by 10^2 and are expressed as mmol/mol of cholesterol.

Statistical analyses

A power calculation was performed to determine the required number of subjects. A sample size of 12 subjects would have a power of 80% to detect a difference of ≈ 10 % in LDL cholesterol with $\alpha = 0.05$. Values are expressed as means ± SD. Statistical differences between the diet periods were tested with the use of the nonparametric Wilcoxon matched paired signed ranks test (two-tailed). The changes within the diet periods (start - end) were used for comparison of variables. The significant levels were set at $P < 0.05$ for all variables. Correlation coefficients were assessed using the Spearman's rank test. No period or carryover effects were observed in this study. The statistical analyses were performed with SPSS version 11.0 (SPSS Inc., Chicago, IL).

3.3 Results

Subjects and profile

The baseline physical and blood profiles for the 14 subjects were as follows: age, 52.2 ± 5.0 years (45-62); height, 153.3 ± 5.4 cm (146-166); weight, 54.2 ± 8.8 kg (40-79); BMI, 23.0 ± 2.7 kg/m^2 (19-29); serum triacylglycerol, 1.13 ± 0.54 mmol/L (0.41-2.33); total cholesterol, 6.06 ± 0.75 mmol/L (5.15-7.47); LDL cholesterol, 3.69 ± 0.65 mmol/L (2.71-4.65); HDL cholesterol, 1.62 ± 0.37 mmol/L (0.93-2.59). No changes in body weight were found throughout the study (data not shown).

Diet survey

The mean total energy, fat, fiber, cholesterol and intake of the test cooking oil of all subjects at the start and end of the two diet periods are shown in **Table 3-3**. There were no significant differences in total energy, fat, fiber, and cholesterol intake between the two diet periods. During each period, the percentage of energy derived from fat was approximately 30% and the ratios of saturated-, mono-unsaturated-, and poly-unsaturated-fats were 1:1.1:0.8. The average intake of PS from the PS/DAG and control oils were 563 and 50 mg/day, respectively ($P = 0.001$). Three smokers (two subjects < 20 cigarettes/day; one subject ≥ 20 cigarettes/day) participated in the study, and tobacco consumption remained unchanged throughout the study. In addition, 12 subjects had coffee constantly, but not more than 3 cups per day in the study.

Table 3-3 Dietary intake of the subjects in the two diet periods [1]

	Control period (DAG oil)		Test period (PS/DAG oil)		P [2]
	Start	End	Start	End	
Energy (MJ/day)	7.55 ± 1.98	7.56 ± 1.08	7.20 ± 1.17	7.23 ± 1.19	NS
Fat (% of energy)	32.0 ± 9.7	31.9 ± 6.5	29.9 ± 7.2	30.6 ± 6.9	NS
Saturated	11.4 ± 5.9	11.2 ± 2.5	11.6 ± 4.3	11.1 ± 4.0	NS
Mono-unsaturated	12.5 ± 5.9	12.4 ± 2.5	11.2 ± 3.3	12.1 ± 4.3	NS
Poly-unsaturated	8.2 ± 2.9	8.4 ± 2.4	7.0 ± 1.7	7.3 ± 2.0	NS
Fiber (g/day)	17.0 ± 4.6	15.0 ± 3.1	15.1 ± 5.3	15.3 ± 4.8	NS
Cholesterol (mg/day)	321 ± 113	360 ± 184	320 ± 125	361 ± 114	NS
Test oil (g/day)	16.7 ± 6.1	17.8 ± 5.9	12.6 ± 5.1	13.4 ± 6.0	NS
PS from test oil (mg/day)	47 ± 17	50 ± 16	35 ± 14	563 ± 250	0.001

[1] Values are means ± SD (n=14). [2] Difference between the changes in the two periods using the Wilcoxon matched paired signed ranks test.

Blood lipids analyses

There was no significant difference between the starting levels of serum lipids in the two diet periods (**Table 3-4).** Serum total cholesterol concentration was reduced by 10.2% ($P < 0.004$) in the PS/DAG oil period compared to that of the control period (DAG oil). Serum total cholesterol concentration reduced by 7.6% during the PS/DAG oil period, and an increase of 2.5% was observed during the DAG oil period. LDL cholesterol was 12.1% ($P < 0.003$) lower after the PS/DAG oil treatment. Serum LDL cholesterol concentration reduced by 9.0% and increased by 3.1% in the PS/DAG oil and the DAG oil treatments, respectively. Serum HDL cholesterol and triacylglycerol did not change significantly after the PS/DAG oil. ApoB and Lp(a) were 9.7% lower ($P < 0.001$) and 18.6% lower ($P = 0.023$) after the PS/DAG oil, whereas no change was observed in apoAI.

Individual data for changes in serum LDL cholesterol and Lp(a) concentrations between the two periods are shown in **Fig. 3-2**. In addition, a significant negative correlation was found between the changes in serum Lp(a) concentrations in the PS/DAG oil period and the baseline serum Lp(a) concentrations ($R = -0.661$, $P < 0.05$, **Fig. 3-3**).

Table 3-4 Blood lipids and lipoproteins in the two diet period [1]

	Control period (DAG oil)		Test period (PS/DAG oil)		Difference [2]		P [3]
	Start	End	Start	End			
Total cholesterol (mmol/L)	5.87 ± 0.85	6.01 ± 0.92	6.03 ± 0.72	5.57 ± 0.82	-0.60 ± 0.51	(-10.2 ± 8.7)	0.004
LDL cholesterol (mmol/L)	3.55 ± 0.81	3.67 ± 0.93	3.53 ± 0.71	3.24 ± 0.91	-0.41 ± 0.40	(-12.1 ± 11.6)	0.003
HDL cholesterol (mmol/L)	1.63 ± 0.41	1.64 ± 0.45	1.69 ± 0.41	1.61 ± 0.44	-0.09 ± 0.23	(-5.1 ± 13.6)	NS
Triacylglycerol (mmol/L)	1.44 ± 0.67	1.32 ± 0.77	1.24 ± 0.60	1.31 ± 0.78	0.18 ± 0.63	(21.8 ± 70.9)	NS
ApoAI (mg/dL)	153 ± 23	155 ± 25	157 ± 26	152 ± 22	-6.8 ± 17.7	(-4.2 ± 9.8)	NS
ApoB (mg/dL)	114 ± 25	115 ± 26	117 ± 25	107 ± 26	-11.0 ± 7.1	(-9.7 ± 6.6)	0.001
Lp (a) (mg/dL)	23.1 ± 18.6	23.6 ± 17.4	24.8 ± 19.2	22.5 ± 16.9	-2.8 ± 4.1	(-18.6 ± 26.8)	0.023
LDL cholesterol / HDL cholesterol	2.30 ± 0.76	2.42 ± 0.93	2.24 ± 0.80	2.18 ± 0.89	-0.19 ± 0.58	(-8.2 ± 20.7)	NS
ApoB / ApoAI	0.77 ± 0.22	0.77 ± 0.23	0.78 ± 0.25	0.72 ± 0.22	-0.05 ± 0.09	(-5.9 ± 10.8)	0.001

[1] Values are means ± SD (n=14). Percentages in parentheses. NS: not significant.

[2] Difference relative to control oil (DAG oil) is based on individual data.

[3] Difference between the changes in the two periods using the Wilcoxon matched paired signed ranks test.

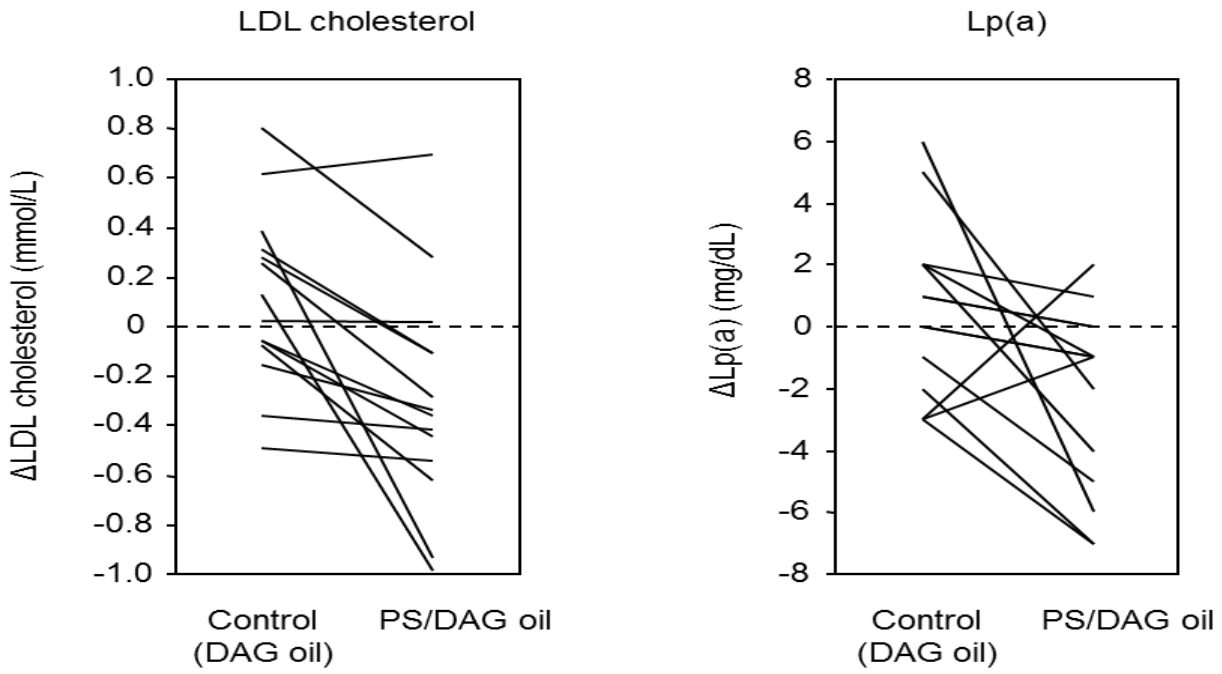


Fig. 3-2 Individual data for changes in serum LDL cholesterol and Lp(a) concentrations in the two diet periods (n = 14)

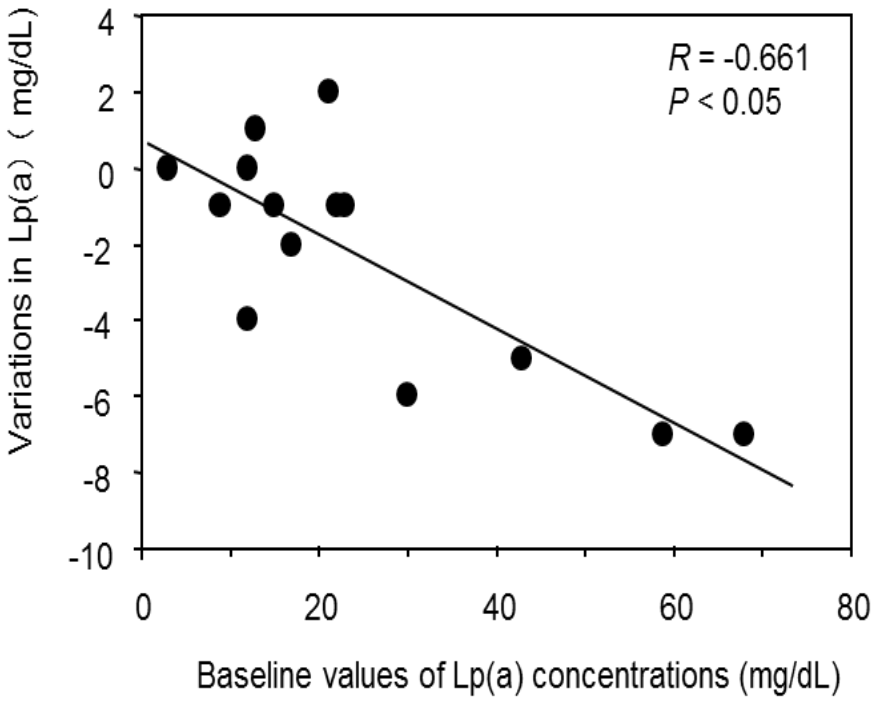


Fig. 3-3 Correlation between baseline serum Lp(a) concentrations and variations in serum Lp(a) during the PS/DAG oil period (n = 14)

Other blood parameters analyses

Serum concentrations of noncholesterol sterols and their levels relative to total cholesterol are shown in **Table 3-5**. During the PS/DAG oil period, serum β-sitosterol, campesterol and lathosterol concentrations were not significantly changed, while the relative-to-total cholesterol levels were significantly increased. The correlation between serum cholesterol reduction by the PS/DAG oil treatment and baseline characteristics of the subjects with regard to intestinal absorption and de novo synthesis levels was investigated. The changes in serum total cholesterol concentrations were negatively correlated with baseline ratios of serum lathosterol to total cholesterol, an index of cholesterol synthesis ($R = -0.608$, $P < 0.05$), although not significant with baseline ratios of serum campesterol to total cholesterol, an index of intestinal cholesterol absorption ($R = 0.273$, $P = 0.345$). No significant changes in serum TBA concentrations between two treatments were observed.

There were no adverse indications in any of the physical or blood examinations: the indices of hepatic functions including AST, ALT, and γ-GTP (**Table 3-6)**, and the female hormone parameters including estradiol and follicle-stimulating hormone (data not shown).

Table 3-5 Serum concentrations of noncholesterol sterols in the two diet periods [1]

	Control period (DAG oil)		PS/DAG oil period		P [2]
	Start	End	Start	End	
β-Sitosterol (μmol/L)	7.01 ± 2.72	7.30 ± 2.57	6.58 ± 2.32	8.31 ±3.02	NS
Campesterol (μmol/L)	14.92 ± 4.83	15.27 ± 5.16	13.27 ± 3.89	18.00 ±6.49	NS
Lathosterol (μmol/L)	10.42 ± 4.19	10.63 ± 5.86	10.79 ± 5.94	10.79 ±5.47	NS
β-Sitosterol/ TC (mmol/mol)	1.19 ± 0.43	1.21 ± 0.38	1.09 ± 0.37	1.47 ±0.45	0.004
Campesterol/TC (mmol/mol)	2.54 ± 0.81	2.53 ± 0.75	2.21 ± 0.63	3.20 ±1.00	0.016
Lathosterol/TC (mmol/mol)	1.80 ± 0.73	1.78 ± 0.94	1.77 ± 0.91	2.00 ±1.14	0.041

[1] Values are means ± SD. TC: total cholesterol.

[2] Difference between the changes in the two periods using the Wilcoxon matched paired signed ranks test.

Table 3-6. Serum concentrations of hepatic function parameters in the two diet periods [1]

	Control period (DAG oil)		PS/DAG oil period		P [2]
	Start	End	Start	End	
AST (IU/L)	23 ±8	23 ±8	23 ± 6	23 ±7	NS
ALT	24 ±14	23 ±13	25 ± 13	24 ±12	NS
γ-GTP (IU/L)	30 ±18	28 ±16	32 ± 23	31 ±20	NS
TBA (μmol/L)	2.3 ±1.1	2.6 ±1.5	2.3 ± 1.1	2.2 ±1.1	NS

[1] Values are means ± SD. [2] Difference between the changes in the two periods using the Wilcoxon matched paired signed ranks test.

3.4 Discussion

The primary objective of the present study was to examine the effect of the PS/DAG oil as a practical first-line therapy for the management of atherogenic blood lipoproteins in postmenopausal women (not obesity) before resorting to drug treatments. At the end of a four-week period of ingesting of PS/DAG oil, not only reductions in serum total (10.2%) and LDL (12.1%) cholesterols and apoB (9.7%) concentrations but also a reduction in serum Lp(a) (18.6%) concentration was induced (**Table 3-4, Fig. 3-2**). DAG oil itself did not induce a cholesterol-lowering effect which is consistent with previous studies (Maki et al., 2002; Teramoto et al., 2004; Yamamoto et al., 2006).

A large number of studies have used PS in a dosage range from 700 to 3,000 mg/day (Katan et al., 2003; St-Onge & Jones, 2003) and the effective minimum dose of PS dissolved in TAG oil has been estimated to be 800 mg/day or higher, worldwide (U.S. Department of Health and Human Services, 2000). Although it has been recommended that 2 g of PS/day should be consumed to achieve a cholesterol-lowering effect, Ostlund et al. (2002) reported that a unique formula of free stanols emulsified with lecithin was more effective in reducing cholesterol absorption at a small dose (300 mg/day) than previously believed. DAG can be found in hydrolysates of TAG and has been used to emulsify oils in processed foods in small amounts. Solubilization of PS in DAG oil has recently been proposed as a newer method of incorporating PS into food, compared to TAG oil with a similar fatty acid composition. Meguro et al. reported that PS, when solubilized in DAG oil, significantly reduced serum total and LDL cholesterols concentrations (PS: 500 mg/day) in men

(Meguro et al., 2001) and prevented the development of atherosclerosis in cholesterol-fed rabbits (Meguro et al., 2003) compared to a similar formulation in TAG oil, suggesting that the effect may be the result of the higher solubility of PS in DAG oil compared to TAG oil (6 vs. 1.3%, respectively). Furthermore, the effective minimum dosage of PS or PSE dissolved in DAG oil has been reported to be 400 mg/day (free PS equivalent) in Japanese men (Goto et al., 1999; Saito et al., 2006a). However, the effect of PS dissolved in 1-monoacylglycerol, a major component of the hydrolysate of DAG oil (Kondo et al., 2003) that possesses a strong emulsifying activity, on cholesterol and PS incorporation to biliary micelles remains to be addressed.

In the present study, the cholesterol-lowering effect of the PS/DAG oil was more closely correlated with baseline cholesterol synthesis than cholesterol absorption, contrary to the previous sitostanol ester margarine study in postmenopausal women with CHD (Gylling et al., 1997). Based on a study by Rajaratnam et al. (2001), the cholesterol metabolism balance in postmenopausal women without CHD was reported to involve a higher baseline synthesis and a decreased absorption of cholesterol, compared to postmenopausal women with CHD. The differences in cholesterol balance due to the different of the two groups appear to be involved in the above discrepancy. The identification of mutations in ATP binding cassette transporters in sitosterolemia and experiments using transgenic mice has greatly expanded our knowledge of cholesterol transport in the small intestine and liver. Plat and Mensink (2002b), using Caco-2 cells, proposed that PS possibly enhance cholesterol efflux back into the intestinal lumen, but other effects on cholesterol efflux from liver and

bile metabolism are unknown. In general, the bile is comprised of ~82% of water, 12% of bile acids, 4% of phospholipids, 1% of free form of cholesterol and 1% of proteins in humans. The primary bile acids are C24-sterol derivatives synthesized from cholesterol (Bloch et al., 1943) such as cholic acid and chenodeoxy-cholic acid (trimming 3 carbons from the side chain of cholesterol), and are conjugated especially with glycine or taurine (Zwicker & Agellon, 2013). Bile acids including conjugated forms are released from the gallbladder after each meal, and help absorb fat including fat soluble vitamins as detergents in the intestine. Subsequently, more than 95% of the bile acids are reabsorbed in the intestinum ileum. Because of the main pathway of cholesterol metabolism (cholesterol elimination from the body), the synthesis of bile acids from cholesterol in the liver is tightly regulated to recover a loss due to the excretion to the stool (~0.5 g/day).

A famous cholesterol-lowering resin such as cholestyramine (e.g. Questran) strongly binds bile acids in the intestine and excretes them into the stool. As a result of this removal of bile acids, more cholesterol is converted to bile acids and normalises blood cholesterol concentration. In the present study, serum TBA concentration did not change, suggesting that there were no impacts of PS/DAG oil on enterohepatic circulation of bile acids (total bile acid pool) and absorption of fat soluble vitamins (Chapter 4). Therefore, the results in the present study imply that cholesterol efflux from the liver depends on cholesterol synthesis and the subsequent effective inhibition of only cholesterol reabsorption (not affect bile acids) in the small intestine by PS may contribute to the reduction in blood cholesterol concentrations; however, the exact mechanism of action of PS on ATP

binding cassette transporters needs to be investigated further.

Recently, it has been reported that bile acids interact with a G protein-coupled receptor, TGR5, expressed in various tissues including brown adipose tissue, immune system, skeletal muscle, nervous tissue and large intestine, linked to signalling pathway for the energy metabolism (Watanabe et al., 2006). Bile acids also promote Glucagon-like peptide 1 (GLP-1) release from the intestinal L-cells via the TGR5 activation, which contributes to the antiobesity effect due to inhibitions of gastric emptying and food appetite (Pols et al., 2011). In the present study, it is assumed that the DAG oil consumption did not change serum TBA concentration since the test period was too short to evaluate the antiobesity effect (4 weeks). Thus, further long-term studies are needed to explore a relationship between food ingredients intake and biomarkers such as bile acids and TGR5 levels.

Lp(a) has been linked to an increased risk of heart disease and stroke (Armstrong et al., 1986). Upon reaching menopause, serum Lp(a) concentrations become sharply elevated by 15% to 25% compared to that of premenopausal women, which partially correlates with the incidence of CHD in elderly women, since Lp(a) may interfere with the thrombolytic process (Jenner et al., 1993). A lack of estrogen suppresses the expression of LDL receptors in the liver, resulting in a sudden elevation in serum LDL particles; however, the mechanism responsible for this change in Lp(a) metabolism is not clearly understood. Teramoto et al. reported that the long-term ingestion of DAG oil improved serum Lp(a) concentrations in patients on hemodialysis, which may be a consequence of decreased hepatic fat, as evidenced by reduced levels of abdominal fat (Teramoto et al., 2004). On the other hand,

Thomsen et al. (2004) reported that serum Lp(a) concentration was not affected by the consumption of PS (1.2 and 1.6 g/day) for 4 weeks in subjects with hypercholesterolemia, although the baseline serum Lp(a) concentration was relatively higher (39 mg/dL). In the present study, a slight but significant reduction in serum Lp(a) concentration was observed during the PS/DAG oil period alone without reducing body weight, especially, in subjects with higher levels of baseline serum Lp(a) (>20 mg/dL), analogous to nicotinic acid (Teramoto et al., 1996) and fish oil (Beil et al., 1991) (**Fig. 3-3**), although this was not found during the DAG oil period. These results suggest that the reduction in serum Lp(a) in the present study may be caused by a synergistic effect between PS and the DAG oil; however, whether a combination of PS and the DAG oil affects serum Lp(a) concentrations through modification in catabolism or synthesis of Lp(a) particles, needs to be investigated.

The normal range of serum PS concentration (β-sitosterol and campesterol) relative to the total cholesterol concentration (the ratio of PS to total cholesterol) is known to be less than 1% (Hidaka et al., 1990; Salen et al., 1992; Stalenhoef et al., 2001). In the present study the ratios of serum β-sitosterol/total cholesterol and campesterol/total cholesterol were mildly but significantly increased by 26% and 31% during the PS/DAG oil period (**Table 3-5**); however, the ratio of serum PS to total cholesterol at the end of PS/DAG period was also within the normal range (< 1%). There were no significant changes in liver functions (**Table 3-6)**, female hormone parameters, although serum lathosterol/total cholesterol ratio (cholesterol synthesis) was slightly but significantly increased by 13% as a feed back action via the inhibition of intestinal cholesterol absorption by the PS/DAG oil

(**Table 3-5**) as previous described (Gylling et al., 1999). These results indicate that the 4-week ingestion of PS/DAG oil pose no safety issues in postmenopausal women, although the cholesterol-lowering effect observed in the present study may be relatively mild compared to a previous study using the margarine (TAG oil) with PSE or plant stanol esters (Katan et al., 2003; St-Onge & Jones, 2003).

Previous nutritional studies of DAG oil have shown unique characteristics such as antiobesity and an improvement in fasting hypertriglyceridemia (Nagao et al., 2000; Maki et al., 2002; Teramoto et al., 2004; Yamamoto et al., 2006); however, no significant changes in these parameters were observed in the present study. The reason for this may be that subjects with hypertriglyceridemia or an obese population were a small minority in the present study and the test period was relatively short.

In conclusion, the present findings indicate that the PS/DAG oil is effective in reducing serum Lp (a) concentration as well as total and LDL cholesterols, apoB concentrations and apoB/AI ratio in postmenopausal women with mild to moderate hypercholesterolemia, without harmful influence on bile acids in the intestine that transport fat soluble vitamins. PS/DAG oil may be a useful adjunct to first-line therapy for the management of atherogenic lipoproteins in women after menopause.

CHAPTER 4

Reciprocal effects of combined use of diacylglycerol oil and phytosterols on cholesterol metabolism in abdominal obesity

4.1 Introduction

Obesity, especially intra-abdominal (visceral) obesity, is a multiple risk factor for lipidemia, type 2 diabetes and CVD (Matsuzawa et al., 1996). Visceral fat accumulation also may enhance blood atherogenic lipoprotein concentrations such as low-density lipoprotein (LDL) via hepatic lipoprotein overproduction. Although diacylglycerol (DAG) oil consumption reduces visceral fat accumulation, its effects on blood LDL concentrations have yet to be evaluated.

Chapter 2 and 3 show the widely cholesterol-lowering effects of PS/DAG cooking oil in men and postmenopausal women with elevated blood cholesterol. In this Chapter, it is discussed the reciprocal benefits of well-regulated combined consumption of dietary DAG oil and PS as a mayonnaise-type food on body fat accumulation and blood lipids in overweight/obese middle-aged men. Compared to dietary TAG oil (control), DAG oil and PS/DAG oil significantly reduce body weight and visceral fat area at the umbilical level as measured by computed tomography (CT). Consumption of DAG oil alone slightly, but significantly, reduces blood cholesterol concentrations depending on a reduction in abdominal fat. These findings suggest that DAG oil functions not only as an advantageous delivery vehicle for PS, but also as an anti-atherogenic ingredient in combination

with PS especially in an overweight/obese population with increased visceral abdominal fat levels.

4.2 Material and methods

Study design

This study was a randomized, double-blind, placebo-controlled, 3-arm intervention parallel trial (**Fig. 4-1**). The study was performed under the supervision of an occupational health physician, in accordance with regulations of the Kao Corporation Ethics Committee for Internal Clinical Studies and in conformity with the Helsinki Declaration. The conditions and procedures of the investigation were reviewed with all subjects before they gave written informed consent.

Subjects

In Kao Corporation, 70 healthy overweight/obese middle-aged male volunteers (25-51 y) with no history of CHD or strokes were recruited. Subjects were excluded if they had poor blood sugar control, type 2 diabetes, hepatobiliary disorders, intolerance to PS or drug therapies for hyperlipidemia and diabetes.

Experimental methods

Prior to a test period, all subjects underwent a 2-week run-in period, and were instructed to have a 15

g package of control mayonnaise made of TAG oil daily as training. Then, the subjects were randomly assigned to one of three groups who ingested a 15 g package of mayonnaise comprised of either TAG oil, DAG oil, or DAG oil containing a mixture of PSE and PS (4 wt% as a free form, PS/DAG oil) for 16 weeks.

The subjects were instructed to maintain 20-25% of fat energy ratio and not to exceed the recommended Japanese daily dietary energy (JMHLW, 1999). Daily alcohol intake was also limited to less than 30 g/day. During the study period, their physical habit and the activities were continued at the same levels as before the study. As shown in **Fig. 4-1**, fasting blood samples were collected from each subject a total of 6 times, prior to the run-in period (-2 week), at week 0, 4, 8, 12, 16 of the the treatment period. The subjects refrained from alcohol for 2 days prior to the examination. On the day before the examination, the subjects finished eating dinner by 21:00 except for drinking water. The blood sampling was conducted during fasting of 12 hours and more. CT scan was conducted at 0, 8, 12 and 16 weeks. Compliance during the test period was checked by the remnants in mayonnaise packages before each examination visit. Daily dietary intake during the study was assessed by a national registered dietician, with 7-day record, based on the 5th revised Japanese Food Composition Table (JST, 2002).

Test food

The DAG oil was prepared from soybean and rapeseed oils according to the method of Huge-Jensen et al (1988), and contained more than 80 g DAG (1,3-DAG:1(3),2-DAG = 7:3 wt/wt)/ 100 g of oil (Saito et al., 2006b). The TAG oil (control) was prepared by mixing rapeseed, safflower and perilla oils to give a final acid composition that was similar to that of the DAG oil (**Table 4-1**). No appreciable differences in combustion energy between the DAG and TAG oils were previously reported (Taguchi et al., 2001). The PS/DAG oil was prepared to add in PSE acids and PS at a molar ratio of 3:1 (mol/mol) on the basis of the DAG oil and contained 4.2 wt% of PS in terms of a free form (β-Sitosterol: 2.1, Stigmasterol: 0.7, Campesterol: 1.2, Brassicasterol: 0.2 in wt%), while the DAG and TAG oils contained 0.3 and 0.5 wt% of PS, respectively. The three mayonnaise-type product used was prepared with TAG oil, DAG oil, or PSDAG oil according to the method of Saito et al. (2006b). All mayonnaise samples were packed at 15 g/package containing 10 g of test oil and were not distinguishable by appearance or taste. Each subject consumed one package containing 15 g/day at any time of the day for 16 wk.

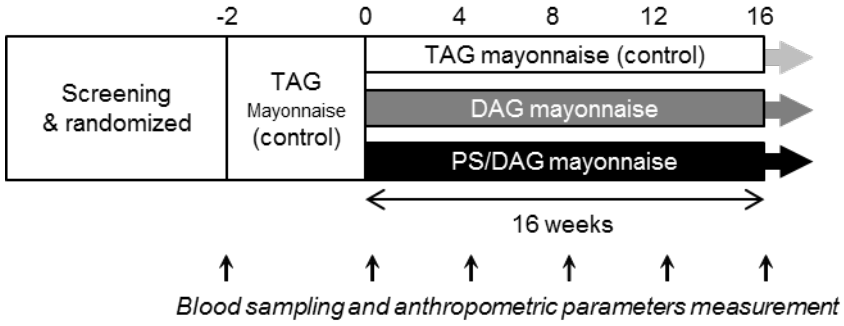


Fig. 4-1 Diagram of the study design

Table 4-1 Composition of acylglycerols and fatty acids in test oils (wt%)

		TAG oil	DAG oil
Acylglycerols	TAG	98.4	11.9
	DAG	1.4	87.1
	MAG	0.2	1.0
Fatty acids	C16:0	5.7	3.1
	C18:0	2.2	1.3
	C18:1	36.2	37.8
	C18:2	46.7	48.6
	C18:3	8.2	8.5
	C20:1	0.9	0.7

Oils contain triacylglycerol, TAG; diacylglycerol, DAG; monoacylglycerol, MAG.

Anthropometry measurements

Body weight, waist circumference, and hip circumference were measured in each visit. Waist circumference at the umbilical level was measured while the subjects were standing. Within 3 days after the anthropometric measurements, the subjects underwent CT imaging (PRATICO, Hitachi Medical Corporation, Tokyo) of the abdominal transverse section at the umbilical level (L4-L5) at Wakayama Wellness Foundation (Wakayama, Japan) (Tokunaga et al., 1983). The X-ray conditions were tube voltage of 120 kVp and mAs of 200; the film was processed at a window level of 0 and a window width of 1000. CT imaging was performed by CT value in the range of -70 to -150 (scan time: 1 sec). The visceral fat area (VFA) and subcutaneous fat area (SFA) were obtained from the abdominal CT image, and these areas were summed to obtain the total fat area (TFA). The investigator who performed the scans and analyzed the data was blind to treatment assignment and clinical status of all subjects throughout the study.

Blood Analyses

Blood samples were collected from fasting subjects via a vein on the flexor side of the arm between 09:00 and 10:00. Serum were obtained by centrifugation at 1,500 ×g for 15 minutes at 4°C, and stored at 4°C or -80°C. All anlyses, except for serum noncholesterol sterols, were conducted by SRL Inc. (Tokyo, Japan) Serum total, LDL and HDL cholesterols and triacylglycerol were measured using an enzyme assay, respectively. Serum apoB was measured using an immunonephelometric kit. Plasma

fibrinogen, serum AST, ALT and γ-GTP activities and creatinin were measured using an enzyme assay, respectively. Serum urea nitrogen was measured by urease UV method. Serum campesterol (an index of intestinal cholesterol absorption levels) (Miettinen et al., 1995; Tilvis & Miettinen, 1986), β-sitosterol, and lathosterol (an index of hepatic cholesterol synthesis levels) (Miettinen et al., 1990) concentrations were analyzed by gas-liquid chromatography on a DB-5HT column (15 m × 0.25 mm, J&W Co., USA) using saponified serum extract as trimethylsilyl derivatives (Saito et al., 2006a). These sterols were quantified using 5α-cholestane as the internal standard. Serum fat-soluble vitamins (retinol and α-tocopherol) and (α+β)-carotene concentrations were analyzed according to the method of Saito et al. (2006b). All parameters were measured in a blind manner.

Statistical analyses

A power calculation was performed to determine the required number of subjects. A sample size of 18 subjects would have a power of 80% to detect a difference of ≈ 10 cm^2 in visceral fat area with $\alpha = 0.05$. Data are presented as means ± standard error (SE) for descriptive purpose and mean [95% confidence interval (CI)] for inference. Baseline data are defined as the average of week -2 and randomized visit. The end point was defined as the average of the weeks 12 and 16. Changes and percent changes from baseline for each parameter were analyzed as below. Differences from baseline to end-point within the group were analyzed by Student's paired t-test. Differences among three groups for body and blood lipid parameters at each point (4, 12, 8 and 16 weeks) and end-point were

analyzed using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (LSD) test. Differences within the treatment periods from baseline to 16 weeks among three groups for variables were analyzed using two-way repeated ANOVA followed by Fisher's protected least significant difference (LSD) test. Correlation coefficients were assessed using Pearson’s correlation test. A type I error rate of < 0.05 was considered to be statistically significant. The statistical analyses were performed using SPSS (11.0 for Windows, SPSS Inc., Chicago, IL).

4.3 Results

Subjects and profile

A total of 70 overweight/obese middle-aged male subjects participated in the study. Three moderate hypercholesterolemic subjects with lack of stability in blood cholesterol concentrations during run-in period and a subject with type 2 diabetes were excluded. Five subjects with the marked changes in their living environment and four subjects with low compliance for the test mayonnaises (frequency in use < 75%) during the study were also excluded. There were no significant differences between before and after the exclusion for the baseline profiles. Finally, data for 57 subjects were analyzed as a per protocol. The baseline profiles for the subjects in each group are shown in **Table 4-2**. There were no significant differences at baseline variables among the groups.

Diet survey

Frequency in use of the test mayonnaise packages in each group was ≥ 95%. Mean daily energy intakes at baseline and end-point were TAG group, 8.5 kJ and 8.9; DAG group, 8.5 and 8.5; PS/DAG group, 8.7 and 8.5. Mean fat intake of the energy at baseline and end-point were TAG group 26% and 26%; DAG group, 27% and 26%; PS/DAG group, 27% and 28%. The energy and fat intakes were close to the standard of Japanese men (20-50 y) (JMHLW, 2004). No significant differences were shown in nutritional variables among groups throughout the study period (**Fig. 4-2**). No significant differences among groups were shown in others such as protein, carbohydrate, fiber and cholesterol.

Table 4- 2 Clinical characteristics of the subjects at baseline [1]

	TAG	DAG	PS/DAG
Age (years)	37.1 ± 2.0	36.3 ± 1.3	37.8 ± 1.4
Male	18	21	18
Body weight (kg)	69.2 ± 1.4	71.9 ± 1.6	73.4 ± 2.4
BMI (kg/m^2)	24.0 ± 0.5	24.1 ± 0.5	24.8 ± 0.7
Waist (cm)	83.0 ± 1.2	84.5 ± 1.3	85.8 ± 1.8
TFA (cm^2)	189.0 ± 17.1	204.2 ± 13.3	210.6 ± 23.9
VFA (cm^2)	69.8 ± 8.6	67.8 ± 5.4	68.1 ± 8.3
SFA (cm^2)	119.3 ± 11.8	136.4 ± 10.5	142.5 ± 17.2
Total cholesterol (mmol/L)	5.36 ± 0.23	5.23 ± 0.15	5.21 ± 0.20
LDL cholesterol (mmol/L)	3.33 ± 0.16	3.31 ± 0.14	3.34 ± 0.16
HDL cholesterol (mmol/L)	1.45 ± 0.08	1.33 ± 0.05	1.34 ± 0.06
Triacylglycerol (mmol/L)	1.17 ± 0.12	1.35 ± 0.14	1.22 ± 0.16

[1] Values are the means ± SE. At baseline, there were no significant differences among groups in all variables using ANOVA followed with Fisher's protected least significant difference (LSD).

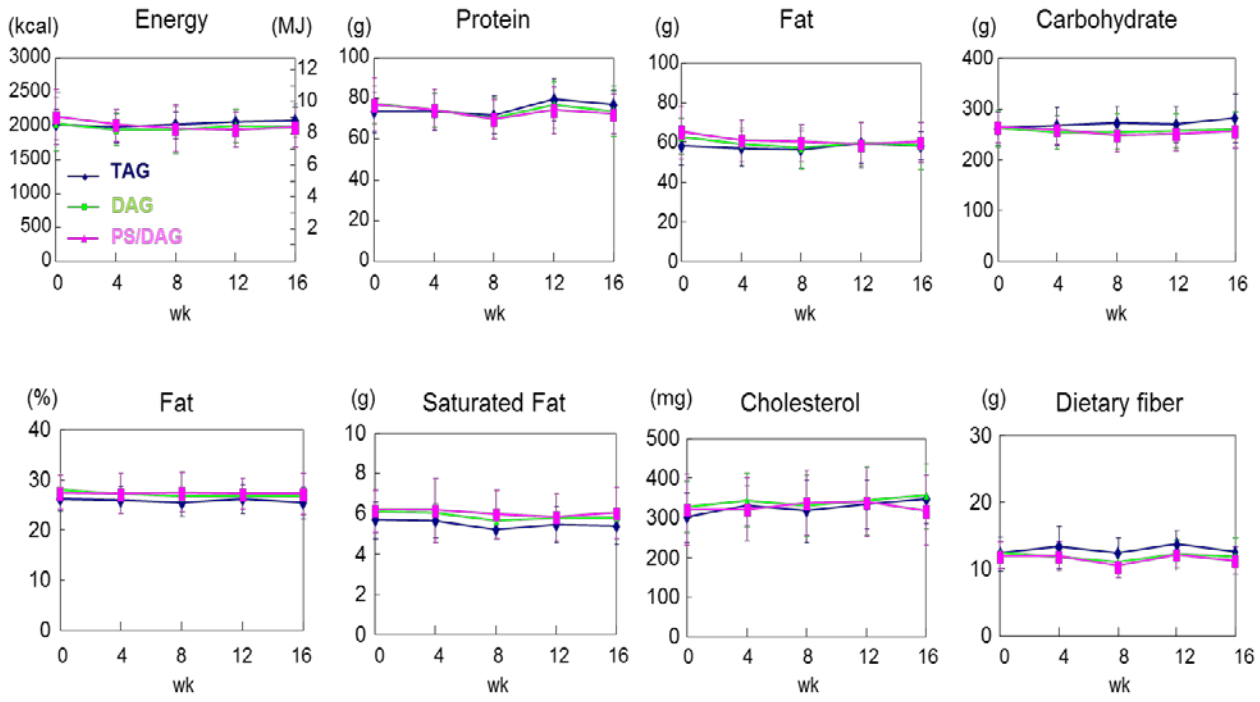


Fig. 4-2 Dietary composition throughout the test periods

Anthropometry parameters analyses

Anthropometric values and body composition at baseline and the percentage changes from baseline to each time point (week 4, 8, 12 and 16) and the end-point (average of week 12 and 16) of the study according to study group are shown **Table 4-3** & **4-4** and **Fig. 4-3 & 4-4**. At baseline, anthropometric values and body composition were not significantly different among three groups. At weeks 12, the responses for the major variables in the DAG and PSE/DADG groups appeared plateau. In the DAG and PS/DAG groups, body weight were 2.6% ($P < 0.01$) lower and 1.6% ($P = 0.069$) lower than values in TAG group at the end-point, respectively. At the end-point, abdominal TFA of the DAG and PS/DAG groups were 12.7% ($P < 0.01$) lower and 9.4% ($P < 0.05$) lower than values in TAG group, respectively. VFA of the DAG and PS/DAG groups were 10.9% ($P < 0.05$) lower and 10.3% ($P = 0.058$) lower than values in TAG group, respectively. SFA of DAG and PS/DAG groups were reduced by 14.2% ($P <0.01$) lower and 8.5% ($P = 0.086$) than values in TAG group, respectively.

Blood lipids

Serum concentrations of lipids and apos at baseline and the percentage changes from baseline to each time point (week 4, 8, 12 and 16) and the end-point are shown **Table 4-5**. At baseline, all serum concentrations of lipids and apos were not significantly different among three groups. The responses for serum total and LDL cholesterols and apoB concentrations in the PSE/DADG and DAG groups appeared plateau around at week 12. Their responses at the end-point among three groups were highly

significant in the PS/DAG and DAG groups. Pairwise comparison showed that serum LDL cholesterol and apoB concentrations were significantly lower by -11.2 ($P < 0.01$) and -8.4% ($P < 0.05$) in the PS/DAG group than TAG group. In the DAG group, serum LDL cholesterol and apoB concentrations were lower by -6.5% ($P = 0.066$) and by -4.1% (not significant) than control group at the end-point. No significant differences were shown in serum HDL cholesterol and triacylglycerol concentration in each group during the test period.

Furthermore, at the end-point, changes in serum cholesterol parameters of the PS/DAG group were closely correlated with baseline serum campesterol concentrations, index of cholesterol absorption (total cholesterol: $R = -0.811$, $P < 0.001$; LDL cholesterol: $R = -0.437$, $P = 0.070$; apoB: $R = -0.600$, $P < 0.01$), not baseline serum lathosterol, index of cholesterol synthesis (total cholesterol: $R = -0.324$, $P = 0.189$; LDL cholesterol: $R = -0.292$, $P = 0.240$; apoB: $R = -0.115$, $P = 0.649$). In contrast, no relationship between the changes in apoB and the changes in VFA was shown in PS/DAG group ($R = -0.338$, $P = 0.170$). Interestingly, at the end-point, changes in serum cholesterol parameters of the DAG group were not correlated with baseline serum campesterol or lathosterol concentrations; however, positive correlation between the changes in apoB and changes in VFA was observed ($R = 0.484$, $P < 0.05$) (**Table 4-6**).

Other blood parameters analyses

The effects of DAG and PS/DAG oils on the biochemical marker including liver function, fat-soluble vitamins and noncholesterol sterols were examined in **Table 4-7**. No significant differences between groups were observed in the blood safety markers including liver and kidney functions, fat-soluble vitamins (retinol, α-tocopherol and carotenes). Serum PS (sum of campesterol and β-sitosterol) concentrations in the PS/DAG oil group were maintained at < 0.045 mmol/L, and the ratios of serum PS to total cholesterol concentrations were also <1% throughout the present study. Serum lathosterol concentration was not significant among the groups. Fat-soluble vitamin concentrations in serum were within reference ranges at baseline and after treatment and were not affected by all of the treatments (**Table 4-7**). There were no significant differences among groups for hematology, general biochemical indexes marker. No adverse events were observed for any of the subjects.

Table 4-3 Changes in anthropometric variables [1]

	Treatment	Test period					End-point (Avg. of Wks 12 and 16)			Statistical significance	
		Baseline	Week 4	Week 8	Week 12	Week 16	Mean (95% CI)	Difference (95% CI) vs. Control		Test period [2] vs. Control	End-point [3] vs. Control
Weight (kg) [B), C)]	TAG	69.2 ± 1.4	68.6 ± 1.5	68.6 ± 1.5	68.5 ± 1.5	68.7 ± 1.5	68.6 (65.5 to 71.7) [†]				
	DAG	71.9 ± 1.6	70.6 ± 1.5	69.8 ± 1.4	69.5 ± 1.4	69.2 ± 1.4	69.3 (66.4 to 72.3) [‡]	0.7	(-4.2 to 5.6)	0.547	NS)
	PS/DAG	73.4 ± 2.4	72.4 ± 2.3	71.8 ± 2.3	71.5 ± 2.3	71.6 ± 2.3	71.6 (66.7 to 76.4) [‡]	3.0	(-2.1 to 8.1)	0.185	
% Δ from baseline [A), B), C), D)]	TAG		-0.9 ± 0.4	-0.8 ± 0.2 [a]	-1.0 ± 0.4 [a]	-0.7 ± 0.4 [a]	-0.9 (-1.7 to -0.1)				
	DAG		-1.8 ± 0.3	-2.9 ± 0.4 [b]	-3.4 ± 0.6 [b]	-3.7 ± 0.7 [b]	-3.5 (-4.8 to -2.3)	-2.6	(-4.1 to -3.2)	< 0.001	< 0.001
	PS/DAG		-1.4 ± 0.3	-2.2 ± 0.4 [b]	-2.6 ± 0.4 [b]	-2.5 ± 0.6 [b]	-2.5 (-3.6 to -1.5)	-1.6	(-3.2 to -0.1)	< 0.05	< 0.05
BMI (kg/m^2) [B), C)]	TAG	24.0 ± 0.5	23.8 ± 0.5	23.8 ± 0.5	23.8 ± 0.5	23.8 ± 0.5	23.8 (22.7 to 24.9) [†]				
	DAG	24.1 ± 0.5	23.6 ± 0.4	23.4 ± 0.4	23.2 ± 0.4	23.1 ± 0.4	23.2 (22.3 to 24.1) [‡]	-0.6	(-2.1 to 0.9)	0.643	NS)
	PS/DAG	24.8 ± 0.7	24.4 ± 0.7	24.2 ± 0.7	24.1 ± 0.7	24.1 ± 0.7	24.1 (22.7 to 25.5) [‡]	0.3	(-1.2 to 1.9)	0.539	
% Δ from baseline [A), B), C), D)]	TAG		-0.9 ± 0.4	-0.8 ± 0.2 [a]	-1.0 ± 0.4 [a]	-0.7 ± 0.4 [a]	-0.9 (-1.7 to -0.1)				
	DAG		-1.8 ± 0.3	-2.9 ± 0.4 [b]	-3.4 ± 0.6 [b]	-3.7 ± 0.7 [b]	-3.5 (-4.8 to -2.3)	-2.6	(-4.1 to -1.2)	< 0.001	< 0.001
	PS/DAG		-1.4 ± 0.3	-2.2 ± 0.4 [b]	-2.6 ± 0.4 [b]	-2.5 ± 0.6 [b]	-2.5 (-3.6 to -1.5)	-1.6	(-3.2 to -0.1)	< 0.05	< 0.05
Waist (cm) [B)]	TAG	83.0 ± 1.2	82.5 ± 1.3	82.5 ± 1.3	82.2 ± 1.3	82.1 ± 1.3	82.1 (79.4 to 84.9) [†]				
	DAG	84.5 ± 1.3	82.6 ± 1.2	82.5 ± 1.2	81.4 ± 1.2	81.2 ± 1.2	81.3 (78.8 to 83.7) [‡]	-0.9	(-4.8 to 3.1)	0.917	NS)
	PS/DAG	85.8 ± 1.8	84.8 ± 1.8	84.7 ± 1.8	84.0 ± 1.8	83.8 ± 1.7	83.9 (80.2 to 87.6) [‡]	1.7	(-2.3 to 5.8)	0.275	
% Δ from baseline [A), B), C), D)]	TAG		-0.6 ± 0.3 [a]	-0.6 ± 0.3	-1.0 ± 0.4 [a]	-1.1 ± 0.4 [a]	-1.0 (-1.9 to -0.2)				
	DAG		-2.1 ± 0.4 [b]	-2.3 ± 0.6	-3.6 ± 0.6 [b]	-3.8 ± 0.7 [b]	-3.7 (-5.1 to -2.3)	-2.6	(-4.2 to -1.1)	< 0.01	< 0.01
	PS/DAG		-1.1 ± 0.5 [ab]	-1.3 ± 0.5	-2.1 ± 0.5 [ab]	-2.3 ± 0.5 [ab]	-2.2 (-3.2 to -1.1)	-1.1	(-2.7 to 0.5)	0.190	0.167
Hip (cm) [B)]	TAG	94.0 ± 0.8	94.2 ± 0.8	94.1 ± 0.8	94.0 ± 0.8	93.5 ± 0.9	93.8 (92.0 to 95.5)				
	DAG	96.4 ± 1.1	95.4 ± 0.8	95.2 ± 0.8	94.8 ± 0.8	94.4 ± 0.9	94.6 (92.9 to 96.3) [†]	0.8	(-1.8 to 3.4)	NS)	NS)
	PS/DAG	96.7 ± 1.2	95.9 ± 1.1	95.8 ± 1.2	95.5 ± 1.1	95.3 ± 1.1	95.4 (93.0 to 97.7) [‡]	1.6	(-1.1 to 4.3)		
% Δ from baseline [B)]	TAG		0.3 ± 0.3	0.1 ± 0.3	0.0 ± 0.4	-0.5 ± 0.4	-0.2 (-1.0 to 0.6)				
	DAG		-0.8 ± 0.8	-1.1 ± 0.8	-1.5 ± 0.7	-1.9 ± 0.8	-1.7 (-3.3 to -0.1)	-1.5	(-3.1 to 0.1)	NS)	NS)
	PS/DAG		-0.8 ± 0.4	-0.9 ± 0.3	-1.2 ± 0.3	-1.4 ± 0.3	-1.3 (-1.9 to -0.7)	-1.1	(-2.7 to 0.5)		

[1] Values are the means ± SE. Control group, n=18; DAG group, n=21; PS/DAG group, n=18. Means in a row without a common letter differed significantly among groups using ANOVA followed with Fisher's protected least significant difference (LSD) test at each point (baseline, week 4, 8, 12 and 16). [2] Measured values and percentage changes in each variable among groups during 16 weeks were analyzed using two way repeated-measures ANOVA followed with the Fisher's protected LSD test; [A)] significant effect of treatment, $P < 0.05$; [B)] significant effect of time, $P < 0.05$; [C)] significant effect of interaction, $P < 0.05$; [NS)] no significant effect of treatment or interaction. [3] At the end-point, measured values and percentage changes in each variable among groups were analyzed using one way ANOVA followed with the Fisher's protected LSD test; [D)] significant effect of treatment, $P < 0.05$; [NS)] no significant effect of treatment. [†, ‡] Significantly different from the baseline value using the paired t-test: [†] $P < 0.05$, [‡] $P < 0.001$.

Table 4-4 Changes in abdominal fat areas [1]

	Treatment	Test period				End-point (Avg. of Wks 12 and 16)				Statistical significance	
		Baseline	Week 8	Week 12	Week 16	Mean (95% CI)		Difference (95% CI) vs. Control		Test period [2] vs. Control	End-point [3] vs. Control
TFA (cm^2) [B)]	TAG	189.0 ± 17.1	182.2 ± 16.7	179.6 ± 17.6	178.9 ± 16.9	179.2	(142.9 to 215.5) †				
	DAG	204.2 ± 13.3	179.3 ± 12.5	167.5 ± 11.6	166.9 ± 11.3	167.2	(143.4 to 190.9) ‡	-12.0	(-58.9 to 34.8)	NS)	NS)
	PS/DAG	210.6 ± 23.9	192.9 ± 23.2	183.8 ± 21.4	184.2 ± 21.5	184.0	(138.8 to 229.2) ‡	4.8	(-43.8 to 53.3)		
% Δ from baseline [A), B), C), D)]	TAG		-2.5 ± 2.2 [a]	-5.3 ± 2.1 [a]	-4.6 ± 3.0 [a]	-4.9	(-10.0 to 0.2)				
	DAG		-12.3 ± 2.2 [b]	-17.6 ± 2.6 [b]	-17.7 ± 2.8 [b]	-17.6	(-23.1 to -12.2)	-12.7	(-20.2 to -5.2)	< 0.01	< 0.01
	PS/DAG		-9.8 ± 2.2 [b]	-14.2 ± 2.8 [b]	-14.4 ± 3.3 [b]	-14.3	(-20.6 to -8.1)	-9.4	(-17.2 to -1.6)	< 0.05	< 0.05
VFA (cm^2) [B)]	TAG	69.8 ± 8.6	68.7 ± 9.4	66.7 ± 9.3	64.5 ± 8.5	65.6	(46.9 to 84.3)				
	DAG	67.8 ± 5.4	60.3 ± 5.6	55.4 ± 4.3	55.4 ± 4.9	55.4	(45.9 to 64.9) ‡	-10.2	(-28.9 to 8.5)	NS)	NS)
	PS/DAG	68.1 ± 8.3	60.1 ± 7.5	54.8 ± 6.4	56.4 ± 6.6	55.6	(42.0 to 69.2) ‡	-10.0	(-29.4 to 9.4)		
% Δ from baseline [A), B), D)]	TAG		-2.7 ± 2.7	-5.2 ± 3.9 [a]	-7.4 ± 4.2	-6.3	(-14.4 to 1.82)				
	DAG		-11.2 ± 3.6	-16.9 ± 3.2 [b]	-17.5 ± 3.6	-17.2	(-23.8 to -10.5)	-10.9	(-19.9 to -1.9)	< 0.05	< 0.05
	PS/DAG		-10.3 ± 3.4	-17.7 ± 2.7 [b]	-15.5 ± 3.0	-16.6	(-21.7 to -11.4)	-10.3	(-19.7 to -0.9)	< 0.05	< 0.05
SFA (cm^2) [B), C)]	TAG	119.3 ± 11.8	113.5 ± 10.8	112.9 ± 11.2	114.4 ± 11.1	113.6	(90.1 to 137.1) †				
	DAG	136.4 ± 10.5	119.0 ± 9.5	112.1 ± 9.2	111.4 ± 9.1	111.8	(92.7 to 130.8) ‡	-1.9	(-36 to 32.2)	0.787	NS)
		142.5 ± 17.2	132.7 ± 17.0	129.0 ± 16.1	127.8 ± 16.0	128.4	(94.7 to 162.1) ‡	14.8	(-20.6 to 50.2)	0.323	
% Δ from baseline [A), B),C), D)]	TAG		-2.5 ± 2.7 [a]	-4.8 ± 1.8 [a]	-2.5 ± 3.0 [a]	-3.7	(-8.4 to 1.1)				
	DAG		-12.8 ± 2.0 [b]	-17.7 ± 2.6 [b]	-18.0 ± 2.8 [b]	-17.9	(-23.3 to -12.4)	-14.2	(-22.3 to -6.1)	< 0.001	< 0.001
	PS/DAG		-9.0 ± 2.4 [ab]	-11.8 ± 3.7 [ab]	-12.6 ± 3.9 [b]	-12.2	(-20.0 to -4.4)	-8.5	(-17.0 to -0.1)	< 0.05	< 0.05

[1] Values are the means ± SE. Control group, n=18; DAG group, n=21; PS/DAG group, n=18. Means in a row without a common letter differed significantly among groups using ANOVA followed with Fisher's protected least significant difference (LSD) test at each point (baseline, week 4, 8, 12 and 16). [2] Measured values and percentage changes in each variable among groups during 16 weeks were analyzed using two way repeated-measures ANOVA followed with the Fisher's protected LSD test; [A)] significant effect of treatment, $P < 0.05$; [B)] significant effect of time, $P < 0.05$; [C)] significant effect of interaction, $P < 0.05$; [NS)] no significant effect of treatment or interaction. [3] At the end-point, measured values and percentage changes in each variable among groups were analyzed using one way ANOVA followed with the Fisher's protected LSD test; [D)] significant effect of treatment, $P < 0.05$; [NS)] no significant effect of treatment. [†, ‡, §] Significantly different from the baseline value using the paired t-test: † $P < 0.05$, ‡ $P < 0.001$. TFA: total fat area, VFA: visceral fat area, SFA: subcutaneous fat area.

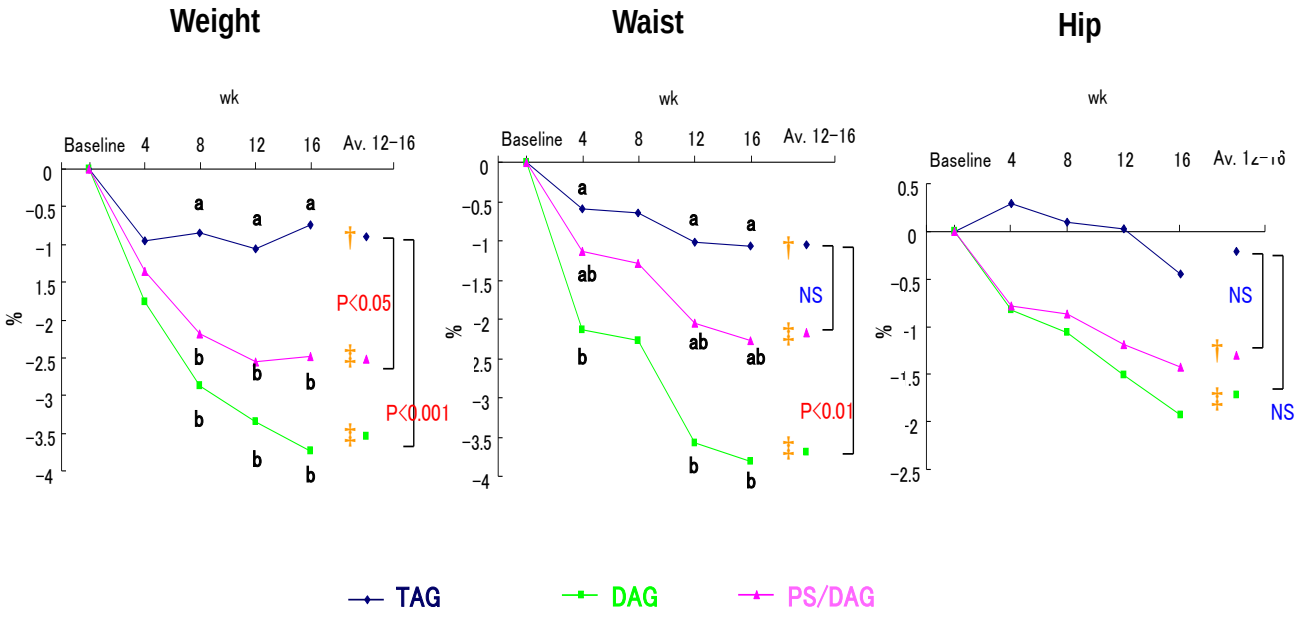


Fig. 4-3 Changes in anthropometric parameters throughout the test periods

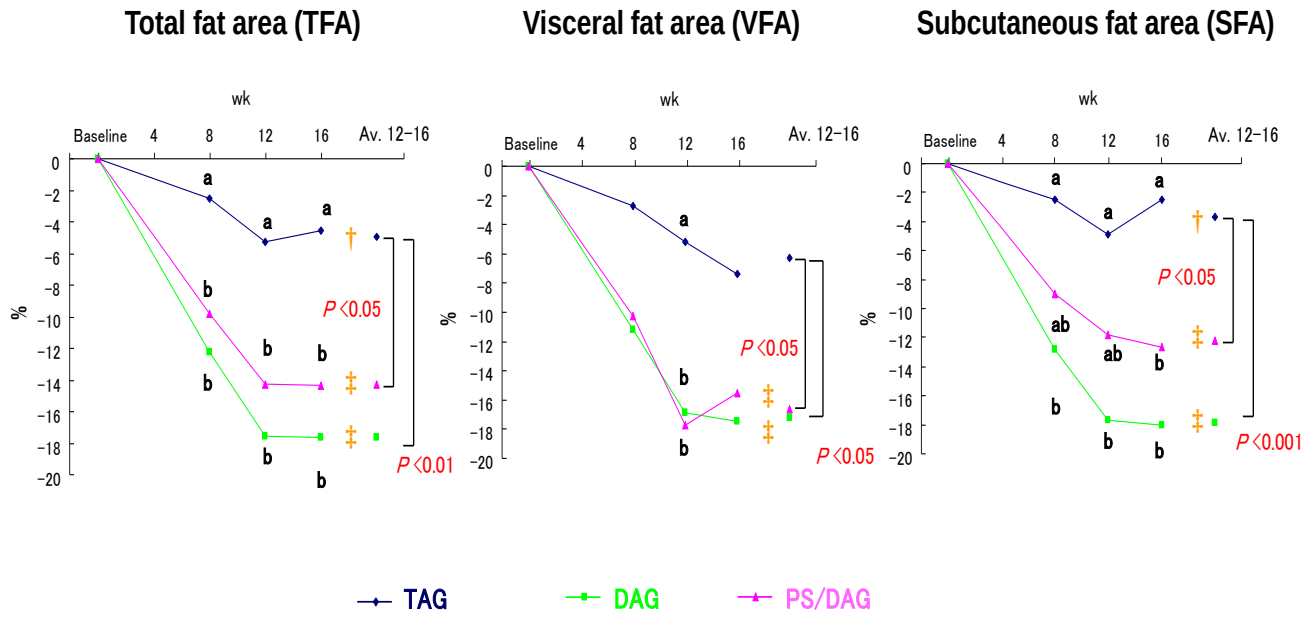


Fig. 4-4 Changes in abdominal fat parameters throughout the test periods

Table 4-5 Changes in serum lipids and apoB [1]

	Treatment	Test period					End-point (Avg. of Wks 12 and 16)		Statistical significance	
		Baseline	Week 4	Week 8	Week 12	Week 16	Mean (95% CI)	Difference (95% CI) vs. Control	Test period [2] vs. Control	End-point [3] vs. Control
Triacylglycerol (mmol/L)	TAG	1.17 ± 0.12	1.08 ± 0.11	1.19 ± 0.14	1.24 ± 0.14	1.32 ± 0.15	1.28 (1.00 to 1.56)			
	DAG	1.35 ± 0.14	1.30 ± 0.14	1.21 ± 0.13	1.39 ± 0.13	1.42 ± 0.13	1.41 (1.15 to 1.66)	0.13 (-0.25 to 0.51)	NS)	NS)
	PS/DAG	1.22 ± 0.16	1.31 ± 0.16	1.16 ± 0.13	1.31 ± 0.14	1.28 ± 0.19	1.29 (0.96 to 1.62)	0.02 (-0.38 to 0.41)		
% Δ from baseline [B)]	TAG		-2.2 ± 8.5	7.2 ± 11.4	10.3 ± 9.3	15.5 ± 9.3	12.9 (-4.1 to 29.9)			
	DAG		-1.4 ± 6.2	-8.4 ± 4.4	9.0 ± 6.0	11.6 ± 7.5	10.3 (-1.6 to 22.2)	-2.6 (-21.5 to 16.3)	NS)	NS)
	PS/DAG		18.0 ± 12.1	4.1 ± 8.1	18.5 ± 8.5	6.6 ± 6.7	12.6 (-1.1 to 26.3)	-0.3 (-19.9 to 19.3)		
Total cholesterol (mmol/L) [B)]	TAG	5.36 ± 0.23	5.38 ± 0.19	5.50 ± 0.20	5.34 ± 0.22	5.39 ± 0.21	5.37 (4.93 to 5.80)			
	DAG	5.23 ± 0.15	5.35 ± 0.21	5.19 ± 0.17	5.06 ± 0.16	5.21 ± 0.14	5.13 (4.84 to 5.43)	-0.23 (-0.71 to 0.25)	NS)	NS)
	PS/DAG	5.21 ± 0.20	5.04 ± 0.17	5.04 ± 0.17	4.78 ± 0.15	4.92 ± 0.19	4.85 (4.51 to 5.20) †	-0.51 (-1.01 to -0.02)		
% Δ from baseline [B)]	TAG		1.2 ± 2.1	3.9 ± 3.3	0.4 ± 2.8	1.8 ± 3.5	1.1 (-5.2 to 7.4)			
	DAG		2.4 ± 2.8	-0.7 ± 2.0	-3.2 ± 1.6	0.1 ± 2.0	-1.6 (-4.7 to 1.5)	-2.7 (-8.5 to 3.2)	NS)	NS)
	PS/DAG		-2.7 ± 1.6	-2.4 ± 2.2	-7.4 ± 2.1	-5.2 ± 1.8	-6.3 (-9.7 to -2.8)	-7.4 (-13.5 to -1.3)		
LDL cholesterol (mmol/L) [B)]	TAG	3.33 ± 0.16	3.35 ± 0.15	3.39 ± 0.17	3.33 ± 0.20	3.42 ± 0.16	3.37 (3.00 to 3.74)			
	DAG	3.31 ± 0.14	3.22 ± 0.16	3.20 ± 0.14	3.07 ± 0.14	3.22 ± 0.12	3.15 (2.89 to 3.41) †	-0.22 (-0.65 to 0.20)	NS)	NS)
	PS/DAG	3.34 ± 0.16	3.15 ± 0.14	3.15 ± 0.13	2.96 ± 0.15	3.10 ± 0.17	3.03 (2.70 to 3.35) ‡	-0.35 (-0.78 to 0.09)		
% Δ from baseline [A), B), D)]	TAG		1.5 ± 2.9	2.9 ± 3.4	-0.1 ± 3.2 [a]	4.0 ± 3.5 [a]	1.9 (-4.6 to 8.5)			
	DAG		-2.7 ± 2.4	-3.2 ± 2.2	-7.4 ± 1.7 [b]	-1.8 ± 2.3 [ab]	-4.6 (-7.7 to -1.5)	-6.5 (-12.5 to -0.4)	< 0.05	< 0.05
	PS/DAG		-5.1 ± 1.9	-4.8 ± 2.6	-11.0 ± 2.3 [b]	-7.6 ± 1.7 [b]	-9.3 (-12.9 to -5.7)	-11.2 (-17.5 to -4.9)	< 0.01	< 0.001
HDL cholesterol (mmol/L)	TAG	1.45 ± 0.08	1.48 ± 0.08	1.49 ± 0.07	1.44 ± 0.07	1.43 ± 0.08	1.43 (1.28 to 1.58)			
	DAG	1.33 ± 0.05	1.41 ± 0.07	1.36 ± 0.06	1.34 ± 0.05	1.40 ± 0.07	1.37 (1.25 to 1.49)	-0.06 (-0.24 to 0.11)	NS)	NS)
	PS/DAG	1.34 ± 0.06	1.32 ± 0.06	1.34 ± 0.07	1.28 ± 0.06	1.30 ± 0.07	1.29 (1.17 to 1.41)	-0.14 (-0.32 to 0.04)		
% Δ from baseline	TAG		3.3 ± 2.3	4.9 ± 3.1	1.1 ± 2.9	0.5 ± 3.7	0.8 (-5.8 to 7.4)			
	DAG		6.0 ± 3.1	2.4 ± 2.5	1.0 ± 1.8	4.9 ± 3.0	3.0 (-1.4 to 7.3)	2.1 (-4.9 to 9.2)	NS)	NS)
	PS/DAG		-0.5 ± 2.8	0.5 ± 2.4	-3.5 ± 2.6	-2.4 ± 2.9	-3.0 (-7.9 to 1.9)	-3.8 (-11.1 to 3.5)		
ApoB (mg/dL) [B)]	TAG	98.3 ± 5.4	100.6 ± 4.7	100.9 ± 5.0	98.2 ± 5.8	99.4 ± 5.2	98.8 (87.4 to 110.1)			
	DAG	97.0 ± 3.9	99.5 ± 5.1	95.5 ± 4.4	93.5 ± 4.5	95.0 ± 3.9	94.3 (85.8 to 102.7)	-4.5 (-17.8 to 8.8)	NS)	NS)
	PS/DAG	98.8 ± 5.1	97.1 ± 4.7	94.0 ± 3.9	90.8 ± 4.5	92.7 ± 5.5	91.7 (81.4 to 102.1) ‡	-7.1 (-20.9 to 6.8)		
% Δ from baseline [A), B), D)]	TAG		3.4 ± 2.3	4.1 ± 3.3	0.2 ± 2.7 [a]	2.4 ± 3.4 [a]	1.3 (-4.7 to 7.3)			
	DAG		2.4 ± 2.6	-1.7 ± 1.8	-3.9 ± 1.8 [ab]	-1.7 ± 2.2 [ab]	-2.8 (-6.0 to 0.3)	-4.1 (-9.7 to 1.4)	0.150	0.144
	PS/DAG		-1.2 ± 1.5	-3.7 ± 2.1	-7.6 ± 1.9 [b]	-6.7 ± 1.4 [b]	-7.1 (-10.1 to -4.2)	-8.4 (-14.2 to -2.6)	< 0.01	< 0.01

[1] Values are the means ± SE. Control group, n=18; DAG group, n=21; PS/DAG group, n=18. Means in a row without a common letter differed significantly among groups using ANOVA followed with Fisher's protected least significant difference (LSD) test at each point (baseline, week 4, 8, 12 and 16). [2] Measured values and percentage changes in each variable among groups during 16 weeks were analyzed using two way repeated-measures ANOVA followed with the Fisher's protected LSD test; [A)] significant effect of treatment, $P < 0.05$; [B)] significant effect of time, $P < 0.05$; [C)] significant effect of interaction, $P < 0.05$; [NS)] no significant effect of treatment or interaction. [3] At the end-point, measured values and percentage changes in each variable among groups were analyzed using one way ANOVA followed with the Fisher's protected LSD test; [D)] significant effect of treatment, $P < 0.05$; [NS)] no significant effect of treatment. [†, ‡] Significantly different from the baseline value using the paired t-test: [†] $P < 0.01$, [‡] $P < 0.001$. ApoB: apolipoprotein B.

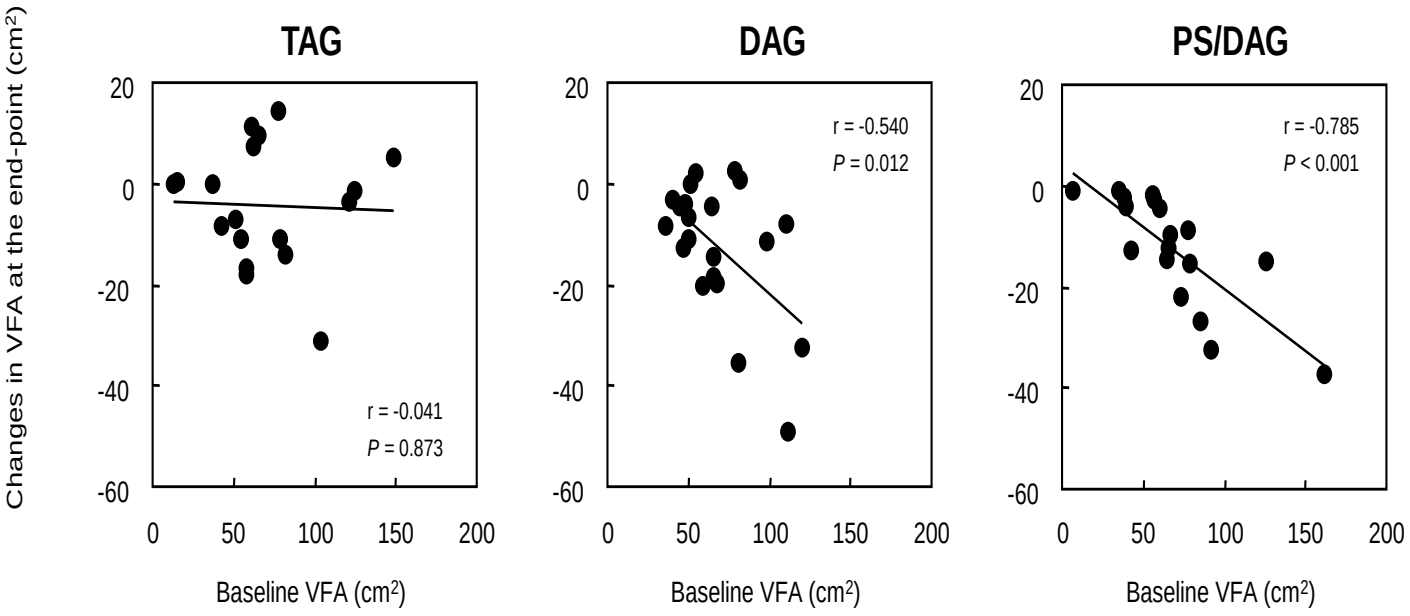


Fig. 4-5 Correlation between baseline visceral fat area and variations in visceral fat area in the three groups

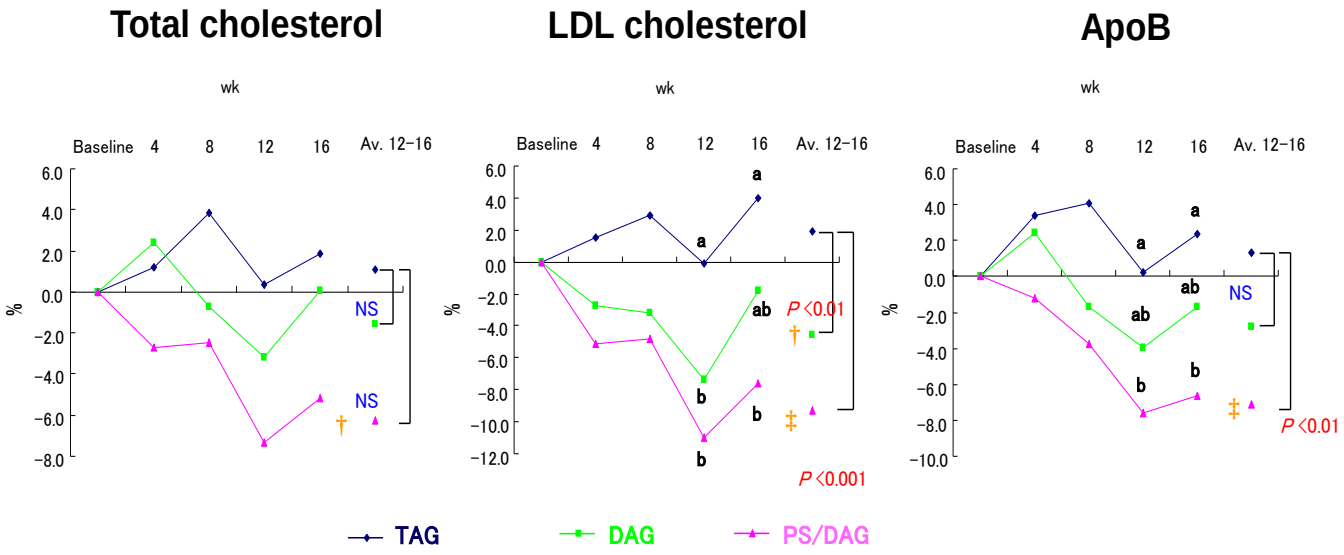


Fig. 4-6 Changes in serum cholesterols and apoB throughout the test periods

Table 4-6 Relationship between blood cholesterol and abdominal parameters

Group	Cholesterol variables	Baseline campesterol		Baseline lathosterol		Baseline weight		ΔWeight (end-point)		ΔVFA (end-point)	
		r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
PS/DAG	ΔTC end-point	-0.811	0.000	-0.324	0.189	0.076	0.764	-0.453	0.059	-0.340	0.168
PS/DAG	ΔApoB end-point	-0.600	0.008	-0.115	0.649	0.004	0.988	-0.325	0.188	-0.338	0.170
DAG	ΔTC end-point	-0.202	0.381	-0.290	0.203	-0.397	0.075	0.501	0.021	0.473	0.030
DAG	ΔApoB end-point	0.121	0.603	-0.182	0.430	-0.483	0.027	0.427	0.054	0.484	0.026

TC, total cholesterol; VFA, visceral fat area.

Table 4-7 Changes in other variables [1]

	Treatment	Test period					End-point (Avg. of Wks 12 and 16)		Statistical significance	
		Baseline	Week 4	Week 8	Week 12	Week 16	Mean (95% CI)	Difference (95% CI) vs. Control	Test period [2] vs. Control	End-point [3] vs. Control
AST (IU/L) [B)]	TAG	23.3 ± 1.7	23.5 ± 1.7	21.8 ± 1.3	20.9 ± 1.2	20.9 ± 1.7	20.9 (17.9 to 23.9)			
	DAG	19.4 ± 0.9	20.4 ± 1.1	18.2 ± 0.9	16.9 ± 1.1	16.8 ± 1.2	16.8 (14.6 to 19.1) [§]	-4.1 (-7.4 to -0.8)	NS)	NS)
	PS/DAG	21.3 ± 1.5	22.3 ± 1.7	19.3 ± 1.2	19.3 ± 1.3	17.8 ± 0.8	18.6 (16.5 to 20.6) [†]	-2.4 (-5.8 to 1.0)		
ALT (IU/L) [B)]	TAG	27.2 ± 2.6	27.2 ± 2.3	26.2 ± 2.2	24.3 ± 2.8	25.3 ± 3.5	24.8 (18.3 to 31.3)			
	DAG	22.3 ± 2.1	24.1 ± 2.3	21.0 ± 2.0	19.0 ± 2.3	19.2 ± 2.6	19.1 (14.2 to 24) [†]	-5.7 (-13.0 to 1.6)	NS)	NS)
	PS/DAG	27.3 ± 4.5	28.8 ± 4.5	22.9 ± 3.0	21.9 ± 3.0	21.2 ± 2.0	21.5 (16.5 to 26.6)	-3.3 (-10.8 to 4.3)		
γ-GTP (IU/L) [B)]	TAG	42.1 ± 8.6	38.8 ± 5.8	38.4 ± 4.8	36.9 ± 5.3	37.8 ± 4.9	37.3 (26.7 to 48)			
	DAG	40.0 ± 5.1	36.9 ± 4.9	35.0 ± 5.0	36.2 ± 6.0	40.9 ± 8.3	38.5 (23.9 to 53.2)	1.2 (-16.2 to 18.6)	NS)	NS)
	PS/DAG	37.9 ± 4.8	33.4 ± 4.4	30.4 ± 3.5	28.8 ± 3.4	41.9 ± 9.8	35.4 (22.9 to 47.8)	-2.0 (-20.0 to 16.1)		
Creatinine [B)] (mg/dL)	TAG	1.06 ± 0.03	1.06 ± 0.03	1.03 ± 0.03	1.08 ± 0.03	1.06 ± 0.03	1.07 (1.01 to 1.13)			
	DAG	1.04 ± 0.03	1.02 ± 0.03	0.99 ± 0.03	1.02 ± 0.03	1.04 ± 0.03	1.03 (0.97 to 1.09)	-0.04 (-0.12 to 0.04)	NS)	NS)
	PS/DAG	1.08 ± 0.02	1.09 ± 0.02	1.06 ± 0.02	1.10 ± 0.03	1.10 ± 0.03	1.10 (1.04 to 1.16)	0.03 (-0.05 to 0.11)		
Blood urea nitrogen (mg/dL)	TAG	13.72 ± 0.56	14.37 ± 0.87	14.08 ± 0.68	13.74 ± 0.68	13.09 ± 0.51	13.42 (12.32 to 14.51)			
	DAG	13.38 ± 0.57	12.47 ± 0.51	13.24 ± 0.64	12.68 ± 0.61	13.31 ± 0.67	13.00 (11.81 to 14.19)	-0.42 (-2.06 to 1.22)	NS)	NS)
	PS/DAG	13.95 ± 0.56	13.24 ± 0.65	14.27 ± 0.83	14.03 ± 0.78	13.16 ± 0.67	13.59 (12.22 to 14.97)	0.18 (-1.52 to 1.88)		
Retinol (μmol/L) [B)]	TAG	3.32 ± 0.18	3.29 ± 0.20	3.44 ± 0.17	3.15 ± 0.19	3.14 ± 0.17	3.15 (2.77 to 3.52)			
	DAG	3.11 ± 0.12	3.22 ± 0.16	3.21 ± 0.16	3.08 ± 0.13	3.07 ± 0.15	3.07 (2.80 to 3.34)	-0.07 (-0.50 to 0.36)	NS)	NS)
	PS/DAG	3.27 ± 0.15	3.08 ± 0.17	3.08 ± 0.19	2.93 ± 0.15	2.90 ± 0.16	2.92 (74.4 to 92.7) [‡]	-0.23 (-0.67 to 0.22)		
α-tocopherol (μmol/L) [B)]	TAG	29.21 ± 1.17	28.57 ± 1.20	29.93 ± 1.21	28.02 ± 1.27	28.15 ± 1.38	28.09 (25.43 to 30.74)			
	DAG	30.31 ± 1.33	29.01 ± 1.55	28.17 ± 1.26	26.49 ± 1.00	27.90 ± 1.16	27.20 (25.19 to 29.21) [‡]	-0.89 (-4.01 to 2.23)	NS)	NS)
	PS/DAG	30.31 ± 1.71	28.64 ± 1.60	27.21 ± 1.24	25.85 ± 1.00	26.02 ± 1.33	25.94 (23.55 to 28.33) [§]	-2.15 (-5.39 to 1.09)		
(α + β) carotene (μmol/L) [C)]	TAG	0.22 ± 0.04	0.25 ± 0.04	0.31 ± 0.07 [a]	0.28 ± 0.06	0.25 ± 0.05	0.27 (0.15 to 0.39)			
	DAG	0.22 ± 0.03	0.20 ± 0.02	0.21 ± 0.02 [ab]	0.21 ± 0.03	0.23 ± 0.03	0.22 (0.17 to 0.27)	-0.05 (-0.15 to 0.05)	0.316	NS)
	PS/DAG	0.17 ± 0.02	0.20 ± 0.03	0.17 ± 0.03 [b]	0.13 ± 0.02	0.16 ± 0.02	0.14 (0.11 to 0.18)	-0.12 (-0.23 to -0.02)	0.059	
Campesterol (μmol/L) [B), C)]	TAG	15.26 ± 1.03	15.29 ± 0.99	16.48 ± 1.00	16.16 ± 0.91	15.83 ± 0.97	16.00 (14.05 to 17.94)			
	DAG	16.19 ± 1.17	16.43 ± 1.34	16.15 ± 1.32	15.62 ± 1.15	17.19 ± 1.01	16.40 (14.22 to 18.59)	0.40 (-2.25 to 3.07)	0.714	NS)
	PS/DAG	13.51 ± 0.70	17.36 ± 1.01	18.23 ± 1.05	18.25 ± 0.89	18.94 ± 0.84	18.59 (16.87 to 20.31) [§]	2.60 (-0.17 to 5.37)	0.317	
β-sitosterol (μmol/L) [B), C)]	TAG	6.71 ± 0.46	6.99 ± 0.49	7.76 ± 0.53	7.51 ± 0.42	7.26 ± 0.46	7.39 (6.51 to 8.27) [†]			
	DAG	7.23 ± 0.57	7.48 ± 0.60	7.58 ± 0.62	7.35 ± 0.53	7.94 ± 0.46	7.65 (6.65 to 8.63)	0.27 (-0.94 to 1.47)	0.675	NS)
	PS/DAG	6.06 ± 0.38	7.31 ± 0.45	7.62 ± 0.46	7.62 ± 0.42	7.74 ± 0.39	7.68 (6.90 to 8.49) [§]	0.29 (-0.96 to 1.54)	0.970	
Lathosterol (μmol/L)	TAG	9.78 ± 1.01	9.10 ± 0.89	10.04 ± 0.76	9.57 ± 0.83	11.04 ± 0.79	10.31 (10.03 to 11.87)			
	DAG	7.64 ± 0.50	8.93 ± 0.83	7.85 ± 0.58	7.71 ± 0.52	8.99 ± 0.68	8.35 (7.34 to 9.36)	-1.97 (-3.75 to -0.16)	NS)	NS)
	PS/DAG	9.10 ± 0.74	9.95 ± 0.74	9.14 ± 0.68	8.63 ± 0.74	8.91 ± 0.73	8.77 (7.24 to 10.29)	-1.55 (-3.41 to 0.34)		

[1] Values are the means ± SE. Control group, n=18; DAG group, n=21; PS/DAG group, n=18. Means in a row without a common letter differed significantly among groups using ANOVA followed with Fisher's protected least significant difference (LSD) test at each point (baseline, week 4, 8, 12 and 16). [2] Measured values and percentage changes in each variable among groups during 16 weeks were analyzed using two way repeated-measures ANOVA followed with the Fisher's protected LSD test; [A)] significant effect of treatment, $P < 0.05$; [B)] significant effect of time, $P < 0.05$; [C)] significant effect of interaction, $P < 0.05$; [NS)] no significant effect of treatment or interaction. [3] At the end-point, measured values and percentage changes in each variable among groups were analyzed using one way ANOVA followed with the Fisher's protected LSD test; [D)] significant effect of treatment, $P < 0.05$; [NS)] no significant effect of treatment. [†, ‡, §] Significantly different from the baseline value using the paired t-test: [†] $P < 0.05$, [‡] $P < 0.01$, [§] $P < 0.001$.

4.4 Discussion

In the present study, the combined treatment of DAG oil and PS as a mayonnaise under on the Japanese recommended dietary guideline are very beneficial in the management of abdominal fat and atherogenic blood parameters such as LDL cholesterol and apoB in Japanese overweight or obese middle aged men. No serious adverse events were observed throughout the test periods.

Both the DAG and PS/DAG groups showed significant reductions in TFA (-17.6% and -14.3%) and VFA (-17.2% and 16.6%), respectively, compared to the TAG group (**Table 4**). These results were quite similar to the previous results of DAG oil conducted by Nagao et al. (2000). In the both DAG and PS/DAG groups, a significant negative correlation was also observed between changes in VFA and baseline values, respectively (**Fig. 4-5**), suggesting that the VFA-lowering effect attributable to DAG oil depends on the baseline visceral fat stored. Thus, the VFA-lowering effect of the DAG and PS/DAG groups would be expected in abdominal obesity with increased visceral fat accumulation.

National Cholesterol Education Program (NCEP) in the U.S. (2002) has recommended the use of PS consisting of Δ5-PS (‘sterols’) and 5α-reduced PS (‘stanols’), to lower blood LDL cholesterol concentrations by ≈10%. The PS/DAG oil showed substantial cholesterol-lowering effect (LDL cholesterol: -11.2% and apoB: -8.4) than TAG, closely associated with the cholesterol absorption index, which is a reasonable response and consist with previous reports with sitostanol ester margarine (Gylling et al., 1997) and previous studies using PS/DAG oil (free form PS 400 to 500 mg/day) as a cooking oil (Goto et al., 1999; Takeshita et al., 2001; Takeshita et al., 2007a) or mayonnaise (Meguro et al., 2001; Saito et al., 2006a 174; Takeshita et al.,

2007b) (**Table 4-5**).

Because PS generally could block the cholesterol absorption in the small intestine, the cholesterol-lowering response of PS may be stronger in cholesterol high absorber. Thus, the impact of PS on the obese population were considered to be less likely effective than that of non-obese population, because cholesterol absorption efficiency is lower and cholesterol synthesis is higher in obese population. On the other hand, the DAG oil exerted a mild, but significant reduction in serum apoB concentration by ~4%, depending on reducing visceral fat area ($R = 0.484$, $P = 0.026$) (**Table 4-6**). These results imply that the DAG oil could be partly involved in the lowering of VLDL production in the liver via a visceral fat reduction. This hypothesis is largely consistent with the report of Riches et al. (2007) and Simonen et al. (2002). It has been reported in visceral obesity that apoB-containing lipoproteins concentrations are elevated, and apoB may independently predict coronary risk (Lamarche et al., 1998; Sniderman et al., 2003). Thus, DAG oil has a new potential for support dyslipidemia resulted from visceral obesity or metabolic syndrome. DAG oil and PS could separately ameliorate a balance of cholesterol metabolism due to different mechanisms. However, cholesterol-lowering effect of DAG oil is needed to examine the mutual effects in detail.

The average amount of PS intake during normal meals is from 150- 450 mg/day, and trace amounts of PS exists in the blood of normal to hyperlipidemia (Salen et al, 1992). A recent study suggests that absorption and excretion of PS in the intestinal tract and excretion from the liver to bile, is functioned via ABCG5 and ABCG8 (Berge et al., 2000). In the present study, while serum concentration PS (the total of campesterol and β-sitosterol), especially campesterol, significantly increased from the baseline in the PS/DAG group (**Table 4-7**), serum PS concentration remained less than

1% of the total cholesterol concentration throughout the entire study period, as previously reported by Saito et al. (2006a), suggesting observed changes are within the normal range (Miettinen et al., 1995).

With regard to the safety of PS, the effects on absorption of fat-soluble vitamins and carotenes within the intestinal tract have been previously discussed in the a meta-analysis of 18 clinical trials conducted by Katan et al. (2003). It was reported that a continuous consumption of 1.5 g/day or more PS or stanol had a significant reduction in serum concentrations of α-tocopherol, α-carotene and β-carotene. Meanwhile, no changes in serum fat-soluble vitamins (A, E and D) were observed during a 3-month continuous consumption of DAG oil (20 g/day) compared to that of TAG oil (Watanabe et al., 2001). In the present study, changes in serum retinol and α-tocopherol concentrations were not significant among groups and both were a normal range of fluctuations (**Table 4-7**). There was also observed no significant change in (α+β)-carotene. The above effects observed in the PS/DAG group were weaker than those reported in the United States and Europe (Katan et al., 2003), and may be attributable to the lower dose of PS. Accordingly, a long-term consumption of PS/DAG oil (PS: 400 mg/day) would have lesser impact on the intestinal absorption of fat-soluble vitamins and carotenes.

In conclusion, a continuous combined ingestion of DAG oil and active form of PS can together ameliorate both visceral fat accumulation and elevated blood LDL cholesterol concentration including particle numbers of LDL (apoB), the major risk factors leading to CVD.

CHAPTER 5

Rational benefits of combined use of a cholesterol biosynthesis inhibitor and dietary phytosterol-rich diacylglycerol oil

5.1 Introduction

Hydroxymethyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), which block the rate-limiting step of cholesterol biosynthesis (**Fig. 5-1**), greatly reduce blood low-density lipoprotein (LDL) cholesterol concentrations by 25% to 60% (Shepherd et al., 1995; Downs et al., 1998; 4S Group, 1994; Sacks et al., 1996; LIPID study Group, 1998; Nakamura et al., 2006). Statins are the first-choice of cholesterol-lowering drug worldwide. Statin monotherapy is insufficient, however, for reducing blood cholesterol concentrations to target levels in hypercholesterolemic patients with increased intestinal cholesterol absorption (Miettinen et al., 2000).

Chapters 1 through 3 showed the cholesterol-lowering effects of PS/DAG oil in healthy men and women before resorting to cholesterol-lowering drug treatments. This chapter demonstrates that the consumption of PS/DAG cooking oil reinforced low-dose pravastatin therapy initially prescribed in Japan, equivalent to doubling the dose of pravastatin, especially in persistent hypercholesterolemic outpatients. This finding suggests that these agents offset the negative feedback effects of the other (increased cholesterol absorption by statin (Miettinen et al., 2000) and increased cholesterol synthesis by PS (Gylling et al., 1997) and independently work via different mechanisms. Therefore, the use of PS/DAG oil in combination with typical statin therapy may represent a potential beneficial strategy for preventing CVD, especially in

hypercholesterolemic patients with a low response to statin due to increased cholesterol absorption, versus statin monotherapy.

5.2 Material and methods

Study design

This study was a randomized, double-blind, 3-arm intervention parallel trial, and was performed as a multicenter study (**Fig. 5-2**): Mitsukoshi Health and Welfare Foundation (Tokyo, Japan), National Defense Medical College (Saitama, Japan), Nippon Medical School Chiba Hokuso Hospital (Chiba, Japan), and Nikko Memorial Hospital (Hokkaido, Japan). The protocol was permitted by each institutional review board in all facilities. All subjects participated in the study after giving informed consent, and the study was conducted ethically based on the spirit of the Helsinki declaration. All procedures such as registration and random division were independently carried out at the Mitsukoshi Health and Welfare Foundation.

Subjects

All eligible subjects were outpatients without history of CHD or stroke who were not sufficient to attain their target goals regarding blood concentrations of total and LDL cholesterols but were maintained stable specifically on low dose of pravastatin (10 mg/day) during at least 4 weeks prior to beginning the study. Subjects were excluded if they had poor control of blood glucose concentration, hepatobiliary disorders and intolerance to treatments of pravastatin and/or PS.

Experimental methods

All subjects were randomly divided to one of three groups consuming either TAG oil (control oil), DAG oil or DAG oil containing free forms of PS (4 wt%, PS/DAG oil). They were instructed to substitute their common cooking oils used at home for the test oil while continuing their pravastatin treatment (10 mg/day) for 12 weeks.

The target dose of the test oil was assumed 10 g/day, which was the average consumption of cooking oil in Japan (JMHLW, 2002). Subjects were requested to maintain their usual isocaloric diets and physical activities at a constant level in the study. Fasting blood sampling was collected from all subjects three times, prior to the run-in period (-4 weeks), at week 0 and week 12, as shown in **Fig. 5-2**. All dietary data were delivered to the Health & Nutrition Institute, Snow Brand Milk Products Co., Ltd. (Tokyo, Japan) and a specific dietician independently analyzed the data during the study from at least two 24-hour dietary records using the computer software program "Healthy box" based on the 4th revised Japanese Food Composition Table (JST, 1998) in a blind manner.

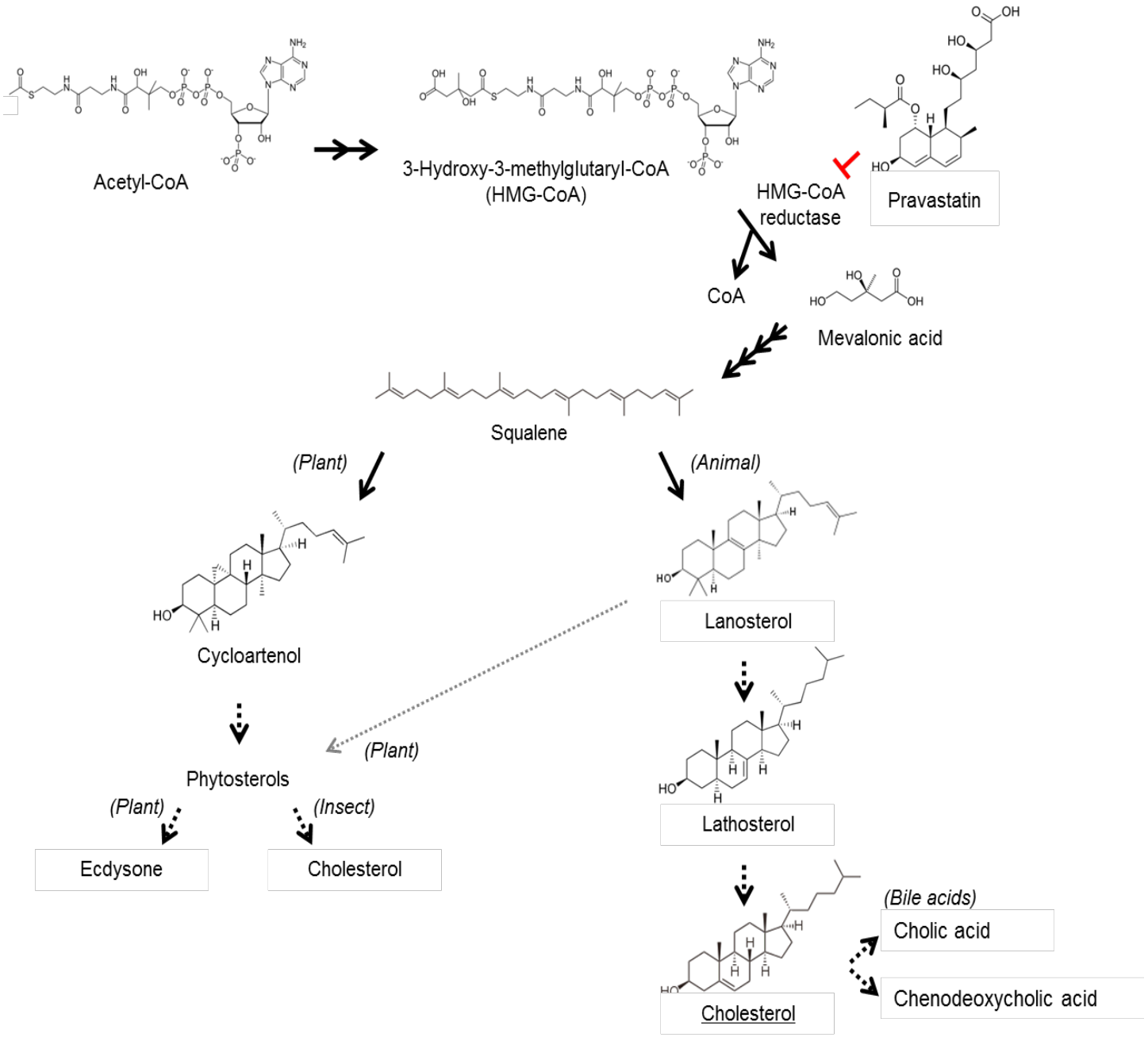


Fig. 5-1 Mechanism of cholesterol synthesis inhibition by statin

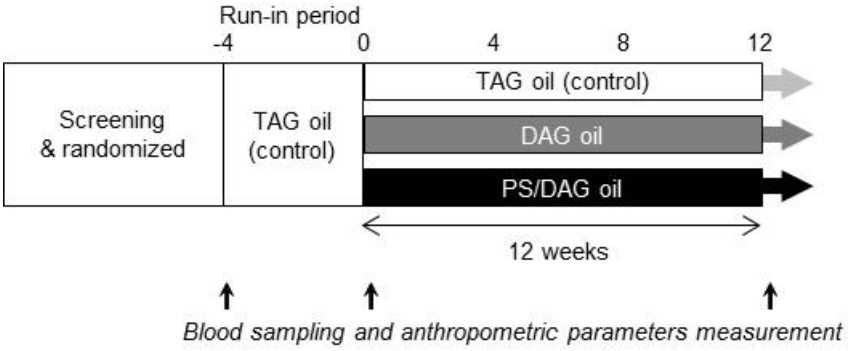


Fig. 5-2 Diagram of the study design

Test oils

PS/DAG oil (Econa H&H Cooking Oil ®, Kao Corporation, Tokyo Japan) was a functional cooking oil manufactured in Japan, permitted as a FOSHU for high blood cholesterol people by the Ministry of Health, Labour and Welfare of Japan. DAG oil (Econa Cooking Oil ®, Kao Corporation, Tokyo Japan) contained ~80 g DAG / 100 g (1,3-DAG:1(3),2-DAG = 7:3 wt/wt) and the main fatty acids of DAG oil were oleic acid and linoleic acid (C16:0, 2.4; C18:0, 0.7; C18:1, 28.0; C18:2, 61.2; C18:3, 7.4 wt%). PS/DAG oil contained 4.2 wt% of free forms of PS (β-Sitosterol, 1.87; Stigmasterol, 1.22; Campesterol, 1.02; Brassicasterol, 0.11, in wt%) on the basis of the DAG oil, while the DAG and TAG oils contained 0.28 and 0.38 wt% of PS, respectively. TAG oil was prepared from a mixture of rapeseed oil and safflower oil (Nissin Oil Tokyo, Japan). This mixture was used to match the major fatty acid composition of DAG oil and PS/DAG oil as closely as possible (C16:0, 6.2; C18:0, 2.2; C18:1, 29.9; C18:2, 54.4; C18:3, 6.4 wt%). All test oils used in the study were very similar to ordinary cooking oils (TAG oil) in taste, flavor and appearance, which were indistinguishable from each other.

Blood analyses

Blood samples were collected from fasting subjects and serum samples were gained by centrifugation at 1,500 ×g for 15 minutes at 4°C, and stored at 4°C or -80°C. All analyses except for serum noncholesterol sterols were carried out by SRL, Inc. (Tokyo, Japan). Serum triacylglycerol, total cholesterol and HDL cholesterol were measured using an enzyme assay (Pureauto S TG-N, Daiichi Pure Chemicals, Co., Ltd., Tokyo Japan; L-type Wako CHO H, Wako Pure Chemicals, Inc., Osaka Japan; Cholestest N

HDL, Daiichi Pure Chemicals, Co., Ltd., Tokyo Japan). Serum LDL cholesterol was calculated by Friedewald's formula. Serum lipoprotein (a) [Lp(a)] was measured using an anti-human Lp(a) monoclonal antibody-coated latex preparation [Lp(a) Latex "DAIICHI", Daiichi Pure Chemicals, Co., Ltd., Tokyo Japan]. Serum apoB, CII, CIII, and E were measured using an immunonephelometric kit (Auto·N KIT series, Daiichi Pure Chemicals, Co., Ltd., Tokyo Japan). Plasma fibrinogen was measured using an enzyme assay (Fibrinogen a RD, Diagnostica Stago, France). Serum AST and ALT activities were measured using an enzyme assay kit (Transaminase-HR II, Wako Pure Chemicals, Inc., Osaka Japan). Serum γ-GTP activity was measured using an enzyme assay kit (Cicaliquid Gamma-GT J, Kanto Chemical of Tokyo Japan). Serum campesterol, an index of intestinal cholesterol absorption levels (Miettinen et al., 1995; Tilvis & Miettinen, 1986), β-sitosterol, and lathosterol, an index of hepatic cholesterol synthesis levels (Miettinen et al., 1990) were analyzed at the Kao Corporation by gas-liquid chromatography on a DB-5HT column (15 m × 0.25 mm, J&W Co., USA) using saponified serum extract as trimethylsilyl derivatives in a blind manner (Saito et al., 2006a). As an internal standard for the quantitative determination of the sterols, 5α-cholestane was used.

Statistical analyses

The samples included data from all subjects whose baseline serum total cholesterol concentrations was not less than the standard borderline of the Japanese population (200 mg/dL) and who retained compliance with pravastatin administration (≥ 75%) and the test oil dose (≥ 2 g/day) despite continuous pravastatin pretreatment (10 mg/day).

Values are presented as the means ± SD. Changes in each parameter from the baseline values were analyzed using the paired t-test when the distribution of analytical values was normal or the Wilcoxon rank-sum test when the distribution was not normal. Changes in each parameter between groups were analyzed using one-way analysis of variance (ANOVA) followed with Bonferroni test. If the baseline values significantly differed between the groups, analysis of covariance (ANCOVA) was used. In the subclass analyses, the changes in lipid parameters between high serum campesterol group and low serum campesterol group on the basis of the median of the baseline serum campesterol concentration were analyzed using the Mann-Whitney U test. A type I error rate of < 0.05 was considered to be statistically significant. Correlation coefficients were assessed using the nonparametric Spearman's rank correlation test. The baseline data are the average of the value before the start of the observation period and the value before the start of the test period. Statistical analyses were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL).

5.3 Results

Subjects and profile

A total of 61 subjects participated in this study. Four subjects withdrew their consent and 57 completed 12-week intervention periods. Five subjects whose baseline serum total cholesterol concentration was < 200 mg/dL (5.17 mmol/L) were excluded. Eight subjects with a low compliance for taking pravastatin (< 75%, n=2) or consuming the test oil at home (< 2 g/day, n=6) during the test period were also excluded. Finally, data for 44 mildly to moderately hypercholesterolemic subjects (30-73 years, mean age 59 ± 9 years) were evaluated. The initial clinical characteristics for all subjects in each group are shown in **Table 5-1.**

The distribution of age (number and percentage) for the 44 subjects in each age group was 2 (4.5%) in the 30s, 2 (4.5%) in the 40s, 15 (34.1%) in the 50s, 20 (45.5%) in the 60s, and 5 (11.4%) in the 70s. The 50s-70s made up the main part (91.0%) and the average age among treatment groups did not differ significantly. The proportion of the subjects who received antihypertensive drugs was 23% (10/44). No subjects with the therapy of diabetes were observed. No changes in body weight and BMI were found in any of the groups (data not shown).

Diet survey

Total energy, fat, protein, carbohydrate, fiber, cholesterol and test oil intakes in each group did not change from the baseline to the 12 week-end point and the values at 12 weeks were not significantly different between the groups **(Table 5-2)**.

Table 5-1. Initial profiles of the 44 subjects[1] (total cholesterol ≥ 5.17 mmol/L) [1]

	TAG oil (n=15)	DAG oil (n=15)	PS/DAG oil (n=14)
Age (years)	58 ± 11 (30 to71)	58 ± 6 (50 to73)	61 ± 8 (45 to72)
Male / Female	1 / 14	3 / 12	3 / 11
Body weight (kg)	51.5 ± 5.7	53.5 ± 6.7	53.6 ± 10.7
BMI (kg/m^2)	21.8 ± 2.0	21.9 ± 2.1	21.8 ± 3.1

[1] Values are the means ± SD.

Table 5-2 Dietary composition of the 44 subjects during the test period [1]

		TAG oil	DAG oil	PS/DAG oil
Energy (MJ/day)	baseline	7.40 ± 1.34	8.10 ± 1.84	7.93 ± 0.61
	12 wk	7.57 ± 0.97	7.72 ± 1.58	7.88 ± 1.09
Fat (% of energy)	baseline	25.6 ± 6.1	25.3 ± 7.5	29.2 ± 4.7
	12 wk	27.4 ± 6.7	27.7 ± 6.0	27.7 ± 6.6
Saturated	baseline	8.2 ± 2.7	6.6 ± 2.6	9.1 ± 1.7
	12 wk	8.3 ± 3.0	7.4 ± 3.0	8.0 ± 2.4
Mono-unsaturated	baseline	10.0 ± 2.7	10.0 ± 4.0	11.9 ± 3.1
	12 wk	10.5 ± 2.9	11.0 ± 2.6	10.9 ± 2.6
Poly-unsaturated	baseline	7.5 ± 1.8	8.7 ± 2.4	8.2 ± 2.5
	12 wk	8.6 ± 1.7	9.2 ± 2.4	8.8 ± 3.1
Protein (% of energy)	baseline	16.0 ± 2.4	16.0 ± 4.3	15.5 ± 2.9
	12 wk	15.0 ± 1.4	14.8 ± 2.4	14.4 ± 2.8
Carbohydrate (% of energy)	baseline	56.8 ± 7.2	55.0 ± 9.5	53.4 ± 5.5
	12 wk	56.2 ± 7.2	54.5 ± 6.3	54.9 ± 8.5
Fiber (g/day)	baseline	15.9 ± 4.0	18.3 ± 6.4	17.1 ± 4.9
	12 wk	16.4 ± 5.6	16.6 ± 5.6	16.3 ± 5.6
Cholesterol (mg/day)	baseline	342 ± 144	222 ± 134	270 ± 126
	12 wk	312 ± 202	256 ± 130	270 ± 156
Cooking oil (g/day)	baseline	9.6 ± 7.1	13.8 ± 12.1	13.0 ± 10.9
	12 wk	12.8 ± 11.3	13.4 ± 8.5	11.9 ± 8.2
Plant sterols from a cooking oil	baseline	36 ± 30	52 ± 46	49 ± 41
(mg/day)	12 wk	49 ± 43 [a]	38 ± 24 [a]	502 ± 346 [*, b]

[1] Values are the means ± SD. [*] Significantly different from the baseline value by the paired t-test: [*] $P < 0.01$. Means in a row without a common letter differed significantly among groups by ANOVA followed by the Bonferroni test, $P < 0.05$.

In each treatment, the percentage of energy from fat was approximately 27%. The average consumption of test oil in each treatment group was approximately 12-13 g/day, similar to the average daily intake of cooking oil (10 g/day) in Japan (JMHLW, 2002). The average PS consumption from the PS/DAG oil was significantly greater (502 ± 346 mg/day, $P < 0.05$) compared to those for TAG and DAG oils (49 ± 43 and 38 ± 24 mg/day). Two smokers (< 20 cigarettes/day) participated in the study and tobacco consumption remained unchanged throughout the study. Twenty-two subjects had coffee constantly, but not more than 3 cups per day in the study.

Blood lipids analyses

In the PS/DAG oil group, serum total and LDL cholesterols, apoB and apoCIII concentrations were significantly lowered by 4.6% ($P < 0.01$), 5.2% ($P < 0.05$), 6.4% ($P < 0.01$), and 6.9% ($P < 0.01$) from the baseline values (**Table 5-3**), respectively, although no significant differences between the groups was found.

The data for females (n=11) were similar to those of the total subjects (n=14) [total and LDL cholesterols, -5.1% ($P < 0.01$) and -5.8% ($P < 0.05$); apoB, -7.6% ($P < 0.01$) from the baseline values]. The effect of age on the percentage reduction in LDL cholesterol concentration was similar between 40s-50s and 60s-70s [-4.0% (n=5) and -5.5% (n=9), not significant between groups]. No such changes were observed in the TAG and DAG oil groups. In the PS/DAG oil group, the changes in serum LDL cholesterol concentrations were inversely correlated with the baseline serum campesterol concentrations ($R = -0.560$, $P < 0.05$, **Fig. 5-3**), but not significant with baseline serum lathosterol concentrations ($R = -0.442$, $P = 0.114$).

Table 5-3 Effects on serum lipids and lipoproteins in the three groups [1]

		TAG oil	DAG oil	PS/DAG oil
Total cholesterol	Baseline	6.35 ± 0.94	6.27 ± 0.86	6.24 ± 0.64
(mmol/L)	12 wk	6.41 ± 0.85	6.25 ± 0.96	5.96 ± 0.51 **
LDL cholesterol	Baseline	3.94 ± 0.95	3.92 ± 0.83	4.04 ± 0.54
(mmol/L)	12 wk	3.88 ± 0.85	3.95 ± 0.96	3.83 ± 0.49 *
HDL cholesterol	Baseline	1.93 ± 0.44	1.80 ± 0.39	1.61 ± 0.44
(mmol/L)	12 wk	1.93 ± 0.53	1.73 ± 0.44	1.56 ± 0.37
Triacylglycerol	Baseline	1.05 ± 0.25	1.18 ± 0.41	1.30 ± 0.47
(mmol/L)	12 wk	1.32 ± 0.55	1.25 ± 0.57	1.24 ± 0.46
ApoB	Baseline	116 ± 18	121 ± 27	126 ± 18
(mg/dL)	12 wk	114 ± 18	122 ± 31	118 ± 17 **
ApoCII	Baseline	4.2± 1.6	4.3± 1.0	4.7± 1.7
(mg/dL)	12 wk	4.6± 1.6	4.2± 1.3	4.5± 1.6
ApoCIII	Baseline	10.0± 1.8	10.5± 2.1	10.4± 1.9
(mg/dL)	12 wk	11.1± 1.7 **	10.8± 2.3	9.7± 1.6 **
ApoE	Baseline	4.9± 1.7	4.2± 0.5	4.3± 0.9
(mg/dL)	12 wk	5.3± 1.4	4.3± 0.6	4.2± 0.7
Lp(a)	Baseline	37 ± 28	43 ± 51	47 ± 47
(mg/dL)	12 wk	35 ± 30	41 ± 55	42 ± 39 *

[1] Values are the means ± SD. *, ** Significantly different from the baseline value by the paired t-test: * $P < 0.05$, ** $P < 0.01$.

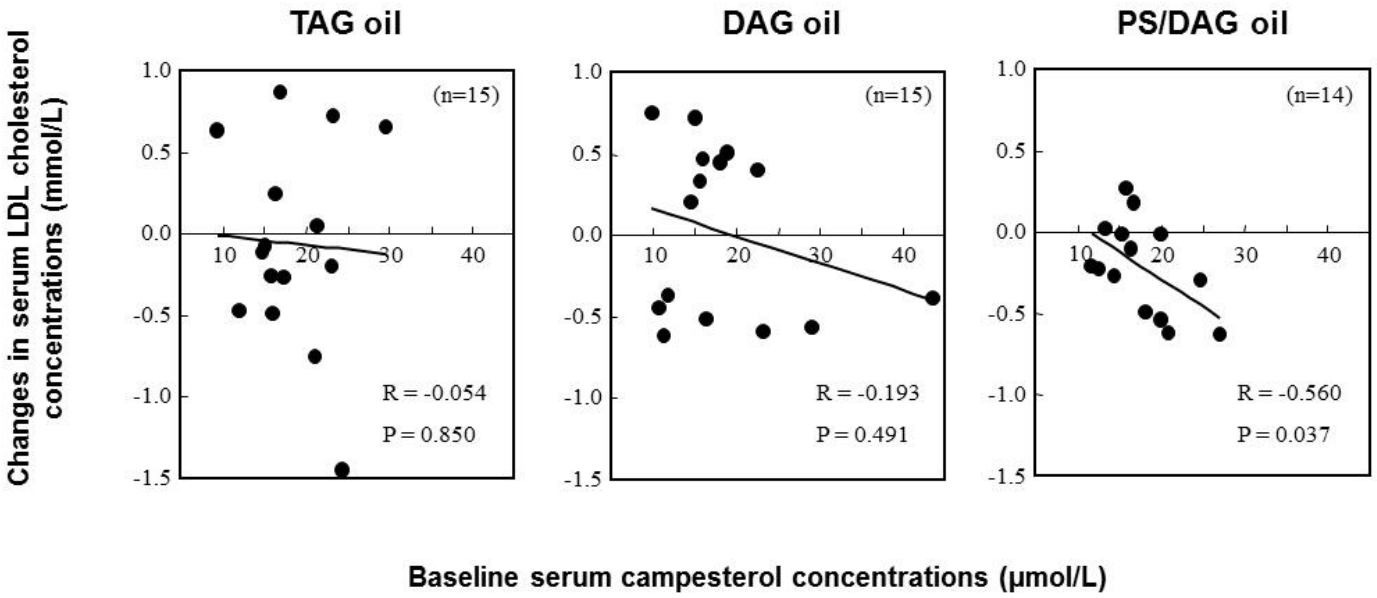


Fig. 5-3 Correlation between baseline serum campesterol concentrations and changes in serum LDL cholesterol concentration in the three groups

There were no significant correlations between the pretreatment values and changes in serum concentrations of LDL cholesterol (R = -0.415, P = 0.140) or apoB (R = -0.297, P = 0.303) in the case of PS/DAG oil treatment. No effect on serum triacylglycerol and HDL cholesterol concentrations was observed.

The effects on serum cholesterol parameters were examined in subclass analyses, in which the 44 subjects were divided into two groups, based on the median (16.47 μmol/L) of their baseline serum campesterol concentrations (**Table 5-4)**. Of the subjects with high baseline compesterol concentrations (HC: campesterol ≥16.47 μmol/L), the serum total cholesterol concentration at 12 weeks was significantly lower in the PS/DAG oil by 9.4% compared to the TAG oil groups ($P < 0.05$), whereas no significant difference in the subjects with low baseline compesterol concentrations (LC: campesterol <16.47 μmol/L). In the PS/DAG oil group, serum LDL cholesterol and apoB concentrations were reduced to a greater extent in the HC versus LC subgroups [LDL cholesterol: -0.35 mmol/L (-13.4 mg/dL) vs. -0.08 mmol/L (-3.0 mg/mL) , P = 0.064; apoB: -13.2 mg/dL vs. -3.1mg/dL, $P < 0.05$, **Fig. 5-4**]. The analysis of the female data was similar to above results (data not shown).

Table 5-4 Effects on serum cholesterol parameters between high (serum campesterol concentrations ≥ 16.47 μmol/L: HC) and low campesterol subgroups (serum concentrations < 16.47 μmol/L: LC) [1]

			TAG oil	DAG oil	PS/DAG oil
Total cholesterol	HC	baseline	6.72 ± 0.95	6.03 ± 0.42	6.31 ± 0.77
(mmol/L)		12 wk	6.86 ± 0.59 [a]	5.94 ± 0.99 [ab]	5.89 ± 0.56 [*, b]
	LC	baseline	5.80 ± 0.64	6.42 ± 1.06	6.18 ± 0.53
		12 wk	5.74 ± 0.75	6.45 ± 0.94	6.02 ± 0.49
LDL cholesterol	HC	baseline	4.19 ± 1.15	3.76 ± 0.64	4.03 ± 0.58
(mmol/L)		12 wk	4.17 ± 0.89	3.73 ± 1.04	3.68 ± 0.43 [*]
	LC	baseline	3.57 ± 0.37	4.03 ± 0.96	4.05 ± 0.54
		12 wk	3.44 ± 0.58	4.09 ± 0.94	3.98 ± 0.53
ApoB (mg/dL)	HC	baseline	122 ± 21	114 ± 26	128 ± 20
		12 wk	121 ± 18	119 ± 39	115 ± 17 [*]
	LC	baseline	107 ± 8	125 ± 28	125 ± 17
		12 wk	105 ± 12	124 ± 27	121 ± 19
Lp(a) (mg/dL)	HC	baseline	46 ± 31	58 ± 73	70 ± 55
		12 wk	45 ± 33	58 ± 83	59 ± 47 [*]
	LC	baseline	25 ± 20	34 ± 31	25 ± 24
		12 wk	20 ± 18 [*]	30 ± 25	24 ± 21

[1] Values are the means ± SD. [*] Significantly different from the baseline value by the paired t-test or the Wilcoxon rank-sum test: $P < 0.05$. Means in a row without a common letter differed significantly among groups by ANOVA or ANCOVA followed by the Bonferroni test, $P < 0.05$. HC subgroup: TAG (n=9) DAG (n=6) PS/DAG (n=7). LC subgroup: TAG (n=6) DAG (n=9) PS/DAG (n=7).

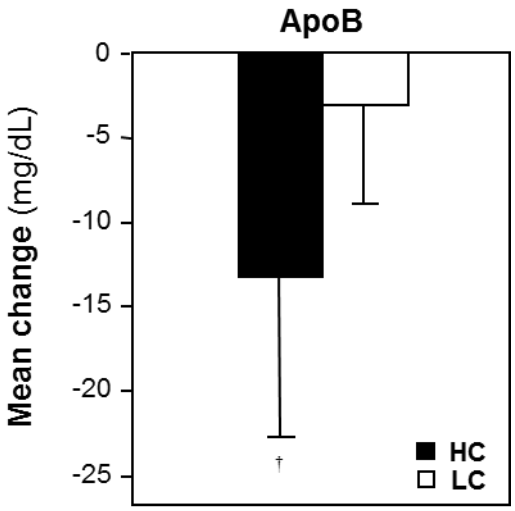


Fig. 5-4 Effect of the PS/DAG oil on serum apoB concentration between high (serum campesterol ≥ 16.47 μmol/L: HC, n=7) vs. low campesterol subgroups (serum campesterol < 16.47 μmol/L: LC, n=7)

Values are means ± SD. † Significantly different between the HC and LC subgroups by the Mann-Whitney U test: $P < 0.05$.

Serum Lp(a) concentration was mildly but significantly decreased by 12.4% ($P < 0.05$) from the baseline value in the PS/DAG oil treatment and a highly inverse correlation was found between the changes in serum Lp(a) concentrations and baseline serum Lp(a) concentrations ($R = -0.851$, $P < 0.001$). A linear reduction in serum Lp(a) concentration was observed especially in subjects with higher levels of baseline serum Lp(a) (> 20 mg/dL). A similar relationship was observed in the DAG oil group ($R = -0.541$, $P < 0.05$), but not in the TAG oil group ($R = 0.145$, $P = 0.607$). Interestingly, there was a significant positive correlation between changes in apoB and changes in Lp(a) concentrations ($R = 0.596$, $P < 0.05$, **Fig. 5-5**) in the PS/DAG oil treatment. Serum Lp(a) concentrations in the PS/DAG oil treatment group were also reduced to a greater extent in the HC versus LC subgroups (-10.8 mg/dL vs. -1.0 mg/dL, $P < 0.05$), as well as LDL cholesterol and apoB.

Other blood parameters analyses

Serum campesterol and lathosterol concentrations were increased from the baselines by 5.2 and 11.5% in the PS/DAG oil group, respectively, although these increases were not significant (**Table 5-5**). Serum PS (sum of campesterol and β-sitosterol) concentrations in the PS/DAG oil group were maintained at < 0.04 mmol/L. The ratios of serum PS to total cholesterol concentrations were also < 1% throughout the study.

In both the DAG and PS/DAG oil groups, serum AST, ALT, γ-GTP and fibrinogen concentrations showed a tendency to be lower than those of the TAG oil group by 5-14 %, but the difference between the groups was not significant (data not shown). No adverse events such as myopathy and hepatotoxity were observed for any of the subjects.

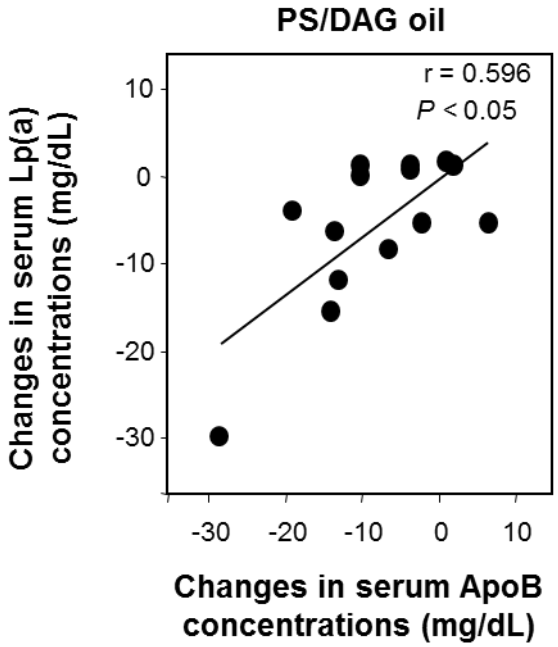


Fig. 5-5 Correlation between changes in serum apoB concentrations and changes in serum Lp(a) concentration in the PS/DAG oil group (n=14)

Table 5- 5 Effects on serum PS and lathosterol in the subjects [1]

		TAG oil	DAG oil	PS/DAG oil
Campesterol	Baseline	18.44 ± 5.27	18.47 ± 8.68	17.67 ± 4.49
(μmol/L)	12 wk	18.72 ± 7.14	17.54 ± 7.46	18.59 ± 4.42
β-Sitosterol	Baseline	9.07 ± 2.60	9.55 ± 6.22	9.31 ± 2.89
(μmol/L)	12 wk	9.28 ± 3.62	9.21 ± 5.96	8.51 ± 3.04
Lathosterol	Baseline	7.40 ± 3.18	7.01 ± 3.23	6.54 ± 2.43
(μmol/L)	12 wk	9.36 ± 4.71	7.55 ± 3.44	7.29 ± 2.25

[1] Values are the means ± SD.

5.4 Discussion

The findings of the present study show that the practical use of dietary PS/DAG oil on a 10 mg/day of pravastatin, as a low-dose combined therapy, is beneficial for further reducing, not only of blood cholesterols, but also Lp(a) concentrations in Asian hypercholesterolemic patients with a low response to pravastatin. The impact of PS/DAG oil was also closely associated with the initial serum campesterol concentrations.

Statins and PS are the most widely used agents for treating hypercholesterolemia (Shepherd et al., 1995; Downs et al., 4S Group, 1994; Sacks et al., 1996; LIPID study Group, 1998; Miettinen et al., 2000; Nakamura et al., 2006; Miettinen et al., 1995; Katan et al., 2003; Ostlund et al., 2004; NCEP Expert Panel, 2002). However, there are populations that are intolerant to a high-dose statin, such as patients with impaired renal function and patients taking a concomitant fibrate or cyclosporine (Thompson et al., 2003). Also, Asians generally achieve similar benefits to Westerners at lower statin doses (Nakamura et al., 2006; Liao, 2007). Recent pharmacokinetic studies of rosuvastatin have established a 2-fold elevation in median exposure (AUC and C_{max}) in Asian compared to Caucasian (Liao, 2007) and the U.S. Food and Drug Administration has recommend that a low dose of the drug should be considered as the start dose for Asian patients (FDA, 2005; Saito et al., 2005). PS also have potentially negative effects on carotene absorption within the recommended dosage range (Katan et al., 2003; Richelle et al., 2004). Thus, in the present study, a combined use of pravastatin and PS at low doses was examined and a further 5% reduction in serum LDL cholesterol concentration was found in the PS/DAG oil treatment group. The effect observed here was milder compared to values reported in previous studies with standard-dose

combination (Gylling H, 1997; Blair et al., 2000; Neil et al., 2001; Cater et al., 2005; Gylling & Miettinen, 2002), but was equivalent to doubling the dose of pravastatin (Law et., 2003), which clearly indicates the clinical importance.

Miettinen et al. (1995) demonstrated a substantial cholesterol-lowering effect of sitostanol-ester margarine and the reduction in total cholesterol concentrations was directly correlated with the reduction in serum campesterol concentrations, an index of intestinal cholesterol absorption. On the other hand, in the present study, the changes in serum LDL cholesterol concentrations in the PS/DAG oil treatment were inversely correlated with baseline serum campesterol concentrations ($R = -0.560$, $P < 0.05$, **Fig. 5-3**). Of the subjects in the HC subgroup, a significant reduction by 9% in serum total cholesterol concentrations in the PS/DAG oil compared to TAG oil groups was shown (**Table 4)**. Serum LDL cholesterol and apoB concentrations were also reduced to a greater extent in the HC versus LC subgroups (9-10% vs. 2-3%, **Fig. 5-4**), which is basically consistent with a previous result (Gylling & Miettinen, 2002). The agents used might reduce each other's negative feedback effects (cholesterol absorption up by statin (Miettinen et al., 2000) and synthesis up by PS (Gylling et al., 1997) and independently and effectively work. According to data from drug trials, a 10% reduction in LDL cholesterol concentrations would be expected to reduce the incidence of ischemic heart disease by 12-20% over a 5 year period (Katan et al., 2003). Therefore, the present approach with the use of low-dose combination may be an attractive option for high cholesterol absorber, especially patients with intolerance to high-dose statin.

A meta-analysis showed that supplementation with 2 g of stanols or sterols is desirable in terms of reducing LDL cholesterol concentration by ≈10% (Katan et al., 2003). However, evidence to support that theory was concentrated close to the tail of the

dose-response curve. Ostlund et al. (2002) recently showed that natural PS in commercial corn oil and sitostanol emulsified with lecithin (Ostlund et al., 1999) are bioactive at very low doses (150-300 mg) in single meal tests. Shin et al. also confirmed that water-dispersible micellar PS (300 mg) had a similar impact on cholesterol absorption (Shin et al., 2005). Meguro et al. reported that PS, when solubilized in DAG oil induced the cholesterol-lowering effect in Japanese men (500 mg/day) (Meguro et al., 2001) and the prevention of atherosclerosis in cholesterol-fed rabbits (Meguro et al., 2003). The effective minimum dosage of PS or PS esters dissolved in DAG oil has been shown to be 400 mg/day in Japanese men (Goto et al., 1999; Saito et al., 2006a). Thus, highly bioavailable formulation of such agents (stanols and sterols) would have greater possibilities for developing the potential.

The mechanism by which PS inhibit cholesterol absorption is not fully understood, but it appears that PS displace cholesterol from micelles and limit the amount of cholesterol absorption in the intestinal lumen (Ostlund et al., 2004). Recently, several studies have suggested that soluble forms of PS can interfere with the incorporation of cholesterol into micelles (Ostlund et al., 2002; Ostlund et al., 2004; Shin et al., 2005), but not insoluble forms (Ostlund et al., 1999; Denke, 1995). More recent studies using fat-free beverages containing PS indicate that the presence of a lecithin formulation and the degree of micellar PS dispersion appear to be involved in reducing blood LDL cholesterol concentrations (Spilburg et al., 2003; Jones et al., 2003). Meguro et al. (2001) also suggested that the higher solubility of PS in DAG oil versus that in TAG oil (6 vs. 1.3 wt%, respectively) may contribute to facilitating the action of PS. However, the effect of PS dissolved in 1-monoacylglycerol, a major hydrolysate of DAG (Kondo et al., 2003) that possesses a strong emulsifying activity, on biliary

micelles remains to be investigated.

Lp(a) is known to be a risk factor of CHD via promoting atherogenesis (Berglund & Ramakrishnan, 2004); however, the exact metabolism of Lp(a) is unknown. So far, there are few reports showing that the blood Lp(a) concentration is ameliorated by the consumption of dietary PS (Garoufi et al., 2014; Plat & Mensink, 2000; Thomsen et al., 2004), the treatments of statins (Sahebkar et al., 2017), or the up-regulation of hepatic LDL receptor (Plat & Mensink, 2002a). However, Teramoto et al. reported that long-term use of DAG oil reduced serum Lp(a) concentrations via lowering abdominal fat (Teramoto et al., 2004). Previous animal studies have shown that 1, 3-DAG, the main ingredient of DAG oil, might retard TAG resynthesis in small intestinal epithelial cells (Kondo et al., 2003) and suppress hepatic microsomal triglyceride transfer protein activity compared to TAG oil (Taguchi et al., 2002). The suppression of hepatic fat accumulation has also been reported in animal (Taguchi et al., 2002) and human (Nagao et al., 2000) studies, suggesting that DAG oil could decline hepatic lipoprotein synthesis. In the present study, mild, but significant reduction in serum Lp(a) concentrations was revealed mainly in the PS/DAG oil treatment, depending on the baseline values (>20 mg/dL), although body weight did not affect since the obese population was a small minority. The reduction in serum Lp(a) concentrations was closely associated with a reduction in serum apoB concentrations, a core component of Lp(a) ($R = 0.596$, $P < 0.05$, **Fig. 5-5**). Additionally, as an inhibitor of LPL, serum apoCIII concentration significantly lowered from baseline in PS/DAG oil group, consistent with the results of Chapter 2, suggesting ameliorating blood stream of lipoproteins enriched with TAG such as postprandial chylomicron and VLDL secreted from the liver, although PS did not affect blood apoCIII concentration (Plat et al., 2009). Recent meta-analysis of drugs

have reported that fibrates promote the Lp(a)-lowering effect (Sahebkar et al., 2017). Therefore, the serum Lp(a) reduction observed here may have resulted from 1) lowering the hepatic synthesis of Lp(a) by 1,3-DAG, similar to antihypertriglyceridemic agents such as nicotinic acid (Teramoto et al., 1996), fish oil (Beil et al., 1991) and fibrates (Sahebkar et al., 2017), and 2) enhancing the catabolism of Lp(a) by PS and the activated LPL in a synergistic manner. However, additional studies accompanied with DAG oil consumption will be required to address this issue.

The absorption efficiency for PS in humans is considerably less than that of cholesterol (0.04-2% vs. 60 %) (Ostlund et al., 2004). Blood levels of PS (β-sitosterol and campesterol) in humans are maintained only at less than 1 % of cholesterol concentrations (Katan et al., 2003). The individual blood concentrations of PS in the PS/DAG oil treatment did not exceed this normal range throughout the study. There were no impaired liver function and other adverse effects.

In conclusion, a low-dose combination of dietary PS/DAG and pravastatin is recommend as a beneficial treatment for further ameliorating blood cholesterols and Lp(a) concentrations especially for hypercholesterolemic patients with increased intestinal cholesterol absorption. DAG oil possibly ameliorates blood lipoproteins levels and LDL activity, but needs further clinical investigations. It is a therapeutic option to add in PS for a variety of population on statin.

CHAPTER 6

General discussion

6.1 Main findings in the present thesis

Replacements of daily foods with functional foods may be a new gateway to health promotion. To demonstrate whether dietary PS/DAG oil benefits various types of subjects with hypercholesterolemia in the prevention of CVD, four prospective clinical studies were executed with the greatest care, as below.

[1] PS/DAG oil consumption might sharply affect blood cholesterol concentrations and rebound after discontinuation, or abnormally raises blood PS concentrations, because of higher solubility for PS in DAG oil than TAG oil. Thus, a long-term practical study was examined in normal to hypercholesterolemia. As a result, long-term *ad libitum* replacement of cooking oil with PS/DAG oil brings out the robust cholesterol-lowering action in moderate to hypercholesterolemic population, not the normocholesterolemic population. In addition, PS/DAG oil possibly ameliorates blood concentrations of other atherogenic parameters including triacylglycerol, HDL cholesterol, RLP-C, apoCIII (an inhibitor of LPL), E (remnants) and PAI-1 (an inhibitor of fibrinolysis) due to an intake of DAG oil. No rebound and adverse effects including abnormal blood PS concentrations were shown in all clinical studies.

[2] Half of elderly women face an increased risk of blood atherogenic lipoproteins, because of estrogen depletion. Thus, it was precisely examined to evaluate effects of PS/DAG oil on blood lipids in postmenopausal women with elevated blood cholesterol in a crossover manner. As a result, it was found that dietary PS/DAG oil has

unique anti-atherogenic benefits for the management of high blood LDL cholesterol and Lp(a). PS dissolved in DAG oil, as well as that in TAG oil, possibly inhibit the intestinal absorption of both dietary and biliary cholesterols by displacing cholesterol from micelles, facilitating the excretion of cholesterol, but not bile acids. In increasingly aging era, PS/DAG oil may be useful for the prevention of CVD in elderly women.

[3] Long-term consumption of DAG oil possibly affects blood cholesterol concentration due to the anti-obesity effect, separately from PS, but not being addressed. Thus, to compare and analyze the interaction between DAG oil and PS, an exact intervention study was examined in middle-aged men with overweight/obesity. As a result, the consumption of DAG oil per se possibly suppresses the cholesterol synthesis in the liver by the smaller harmful visceral fat whose mechanism is different from that of PS. Simultaneous intake of dietary DAG oil and PS could efficiently ameliorate blood LDL cholesterol concentrations in obese population.

[4] Outpatients with low responder to major cholesterol-lowering drug (statin) need to increase in dose, but may produce side effects. Thus, it was examined whether additional intake of PS/DAG oil works as an adjunct to statin, or not. As a result, dietary PS/DAG oil clearly reinforced the cholesterol-lowering effect (doubling the dose of pravastatin) than stain monotherapy, in persistent hypercholesterolemic outpatients with low dose of statin. Also, PS/DAG oil consumption on the statin therapy facilitated to ameliorate other blood atherogenic parameters such as apoB, CIII and Lp(a). The combined use of functional food and drug could be a new therapeutic option.

Total reciprocal effects of PS and DAG oil on cholesterol and fat metabolism are shown in **Fig. 6-1**. DAG oil possibly ameliorates blood and hepatic lipid metabolisms mainly including cholesterol, fibrinolysis and visceral fat accumulation.

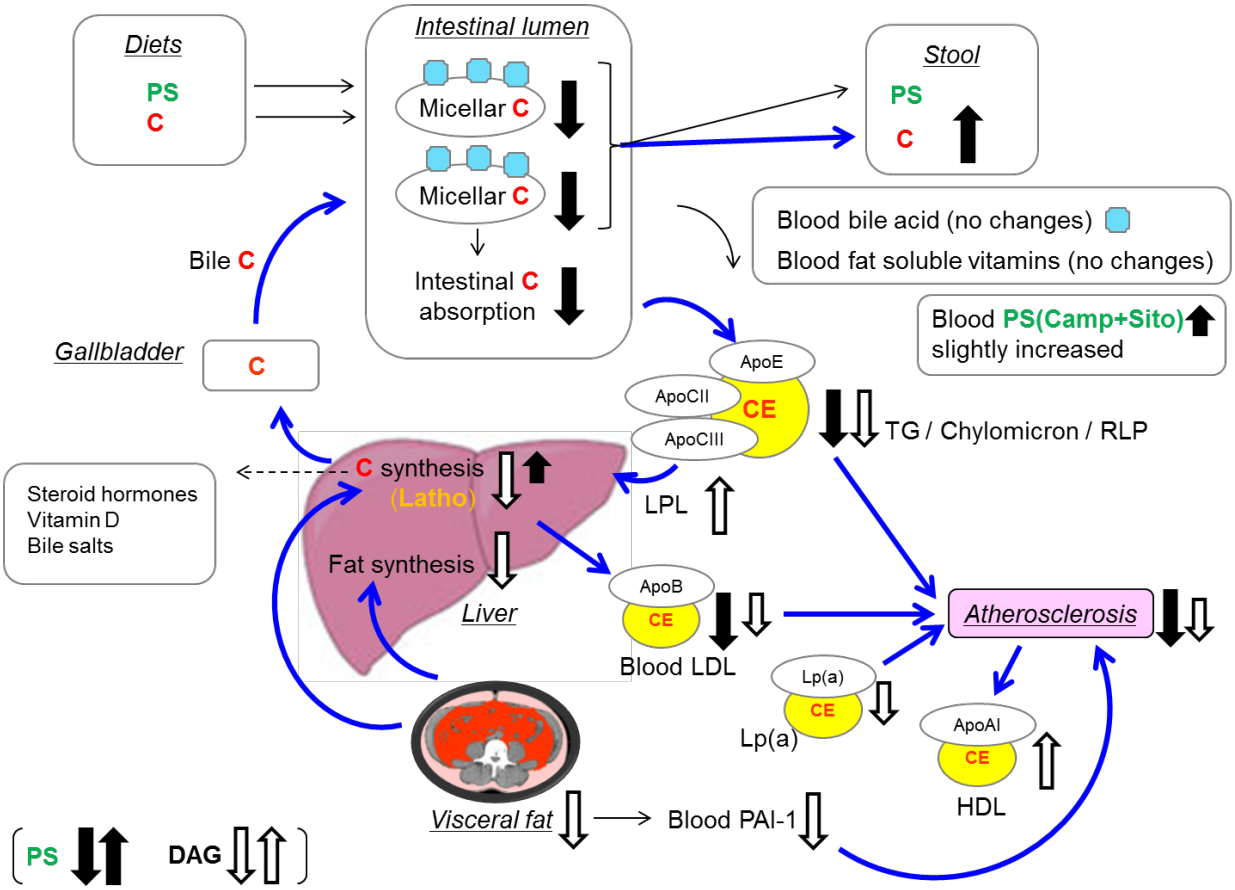


Fig. 6-1 Reciprocal effects of PS/DAG oil on cholesterol and fat metabolism

PS, phytosterols; C, cholesterol; Camp, campesterol; Sito, β-sitosterol; Latho, lathosterol; LPL, lipoprotein lipase.

White arrows illustrate effects of DAG oil, black arrows illustrate effects of PS, blue arrows illustrate the metabolism site that PS/DAG oil possibly affects.

6.2 Challenges currently facing high blood cholesterol in Japan

Based on the latest Nutrition Survey in Japan (JMHLW, 2016), it has been reported that numbers of cholesterol-lowering drug users have dramatically expanded from 2006 to 2016, especially in the 60s, 70s and over in men and women (**Fig. 6-2**). The elderly populations in both sexes have grown as a volume zone in the market of cholesterol reducers. As a result, high blood cholesterol populations whose serum total cholesterol concentrations are not less than 6.21 mmol/L (≥ 240 mg/dL) have been concentrated in the 40s and 50s in men, and 50s and 60s in women regardless of the drug therapy.

Cholesterol-lowering drugs have been developed as 3 types including statins, ezetimibe and bile acid sequestrants worldwide (**Fig. 6-3**). The cholesterol-lowering effects of statins, ezetimibe and bile acid sequestrants are by 20-60%, 20% and 10-20%, respectively. The combined uses of agents with different mechanisms (e.g. statin and ezetimibe) have been popular, because of efficiently achieving goal lipids levels with no adverse effects due to at each lower dose (Ambegaonkar et al., 2014). On the other hand, the averages of blood total cholesterol concentration in both sexes have not been changed from 2006 to 2016, respectively [5.09 mmol/L (= 197 mg/dL) to 5.09 mmol/L (= 197 mg/dL) in men; 5.25 mmol/L (= 203 mg/dL) to 5.28 mmol/L (= 204 mg/dL) in women]. Also, the rate of high blood cholesterol populations (≥ 6.21 mmol/L) at each generation is not significantly different through the decade, despite the increase in drug users year by year. In general, most drugs including cholesterol-lowering drugs will be limited in use for children and women with the chance of achieving pregnancy to avoid impermissible adverse effects. Thus, universally accepted ‘functional food’ that provides a lifetime of healthy blood cholesterol should be explored to prevent CVD.

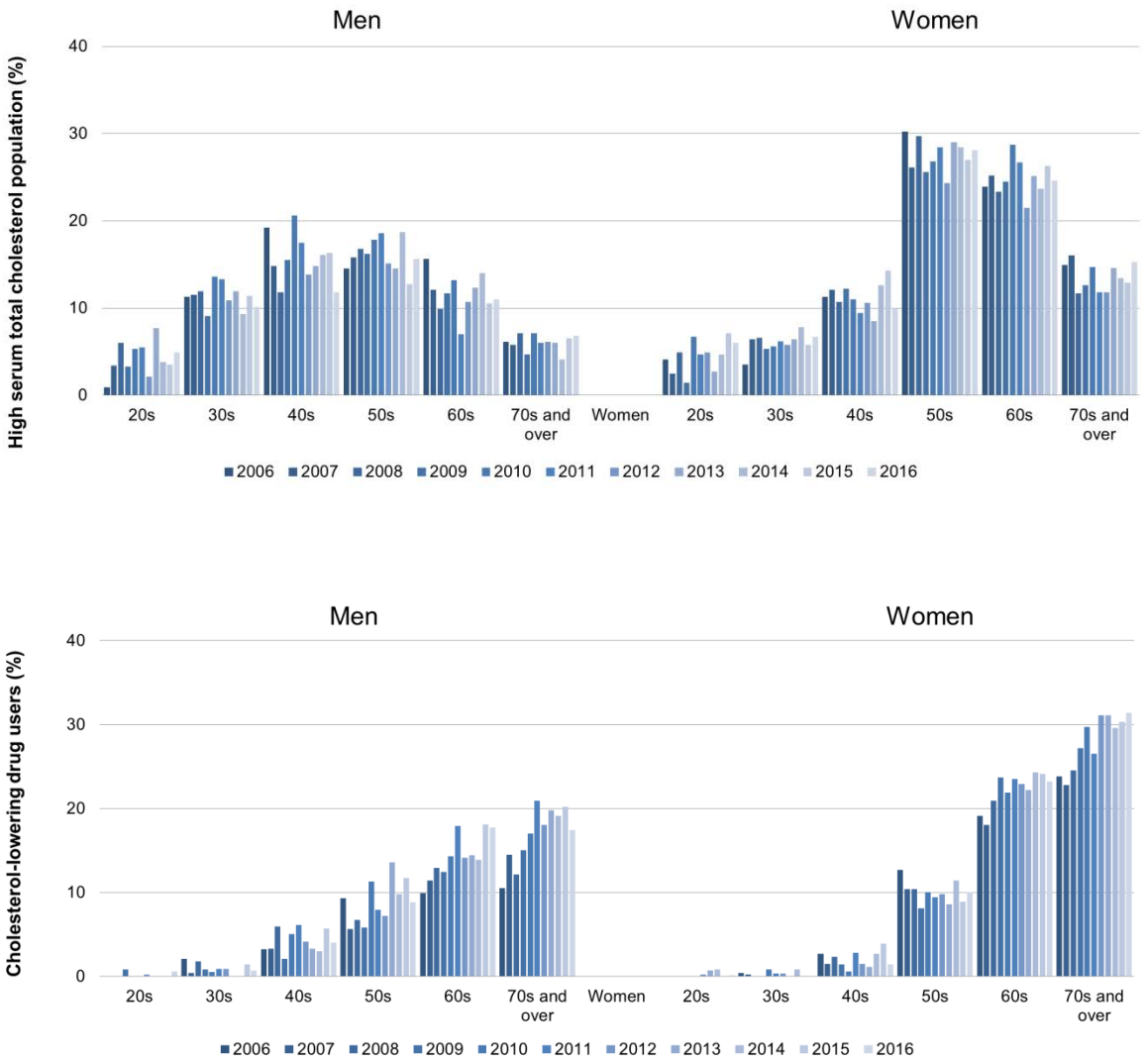


Fig 6-2 Trend in the percentage of high blood cholesterol population and cholesterol-lowering drug users from 2006 to 2016 (JMHLW, 2016)

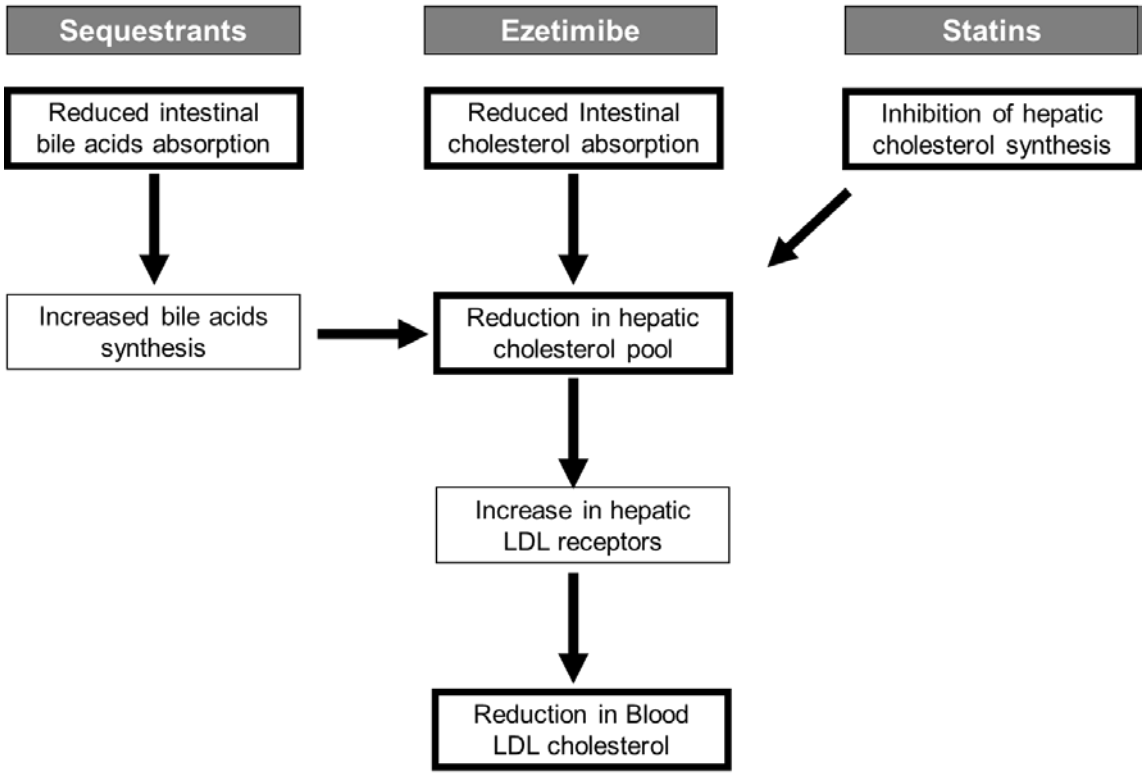


Fig 6-3 Cholesterol-lowering drugs and mechanism for LDL cholesterol reduction

6.3 Recent developments of functional foods for the prevention of CVD

In Japan, 'Foods for Specified Health Use' (FOSHU) was established 1991 by the Japanese Ministry of Health, Labour, and Welfare (MHLW) as a regulatory system to approve the health claims based on the scientific evidence of the food on the human body. With regard to FOSHUs that manage high blood cholesterol, major 6 types of ingredients are permitted, as shown in **Table 6-1**.

Major ingredients of FOSHUs in Japan that manage high blood LDL cholesterol concentration are PS, soy protein, tea catechins, fiber derives from Psyllium Seed husk, chitosan, low-molecular weight sodium alginate, and S-methylcysteine sulfoxide in Broccoli & cabbage. S-methylcysteine sulfoxide decreases blood LDL cholesterol concentration by enhancing the exchange from cholesterol to bile acids. Chitosan decreases blood LDL cholesterol concentration by the inhibition of intestinal absorption of cholesterol and bile acids. Other ingredients decrease blood LDL cholesterol concentration by the inhibition of intestinal cholesterol absorption.

Based on the numbers of PubMed searches by the term of "clinical trial" (Jan 19th, 2018), numerous clinical trials regarding PS and soy protein were executed in the world. The cholesterol-lowering action of PS seems to be significantly robust compared to other ingredients. Thus, the FDA has approved the health claim regarding PS. Plant sterol esters: foods containing at least 0.65 g per serving of plant sterol esters, eaten twice a day with meals for a daily total intake of at least 1.3 g (0.75 g/day as a free form), as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. Plant stanol esters: foods containing at least 1.7 g per serving of plant stanol esters, eaten twice a day with meals for a total daily intake of at least 3.4 g (2 g/day as a free form), as part of a diet low in saturated fat and cholesterol, may reduce the risk of

heart disease. Also, the FDA reported that a daily intake of approximately 2 g of PS (as free forms) is required to exert a relationship between PS consumption and the reduction in CVD risk.

Although the FOSHUs for PS in Japan have not permitted since 2009, the health claims for PS have been spread in other countries such as China and Canada. Nowadays, many functional foods enriched with PS are available in EU, U.S., Canada, Australia, New Zealand, Japan, China, Taiwan, South Korea, South America, and Mexico.

Meanwhile, on Oct 31th, 2017, the FDA is proposing to revoke the use of health claims on the relationship between soy protein and CHD since 1999 on the label or in the labeling of foods, because the evidence does not well support the health claim of soy protein. Thus, further scientific research for soy protein will be needed to evaluate exactly. To extend the human health span worldwide, clinical evidence on the functional foods should be verified in different populations. Also, a great deal of thought should be given to the real effectiveness of physiological function, as well as basic food features such as nutritional and sensory functions, in a daily practical field.

Table 6-1 Major ingredients that function to manage high blood cholesterol used for FOSHUs in Japan

Ingredients (permitted year)	Dose (/day) Product form	Mechanism	Cholesterol reduction (%)	Number of PubMed searches by clinical trial (Jan 19th 2018)
PS (1999-2008)	0.4-0.8 g · edible oil · margarine · mayonnaise	Inhibition of cholesterol absorption	～10-15%	401
Soy protein (1999-2011)	6-8 g · soy milk · soup	Inhibition of cholesterol absorption	～5-10%	199
Tea catechins (2011-2016)	197 mg · beverage	Inhibition of cholesterol absorption	～5%	45
Fiber derives from Psyllium Seed husk (1999-2010)	4-4.5 g · jelly beverage	Inhibition of cholesterol and bile acids absorption	～5%	41
Chitosan (2002-2015)	0.9-1 g · beverage	Inhibition of bile acids absorption	～5-10%	18
Low-molecular weight sodium alginate (1998-2015)	4 g · beverage	Inhibition of cholesterol absorption	～5%	3 (not limited by M.W.)
S-methylcysteine sulfoxide in broccoli and cabbage (2008)	26 mg · jelly beverage	Increase in bile acids synthesis	～5%	0

6.4 Further prospective research on DAG oil

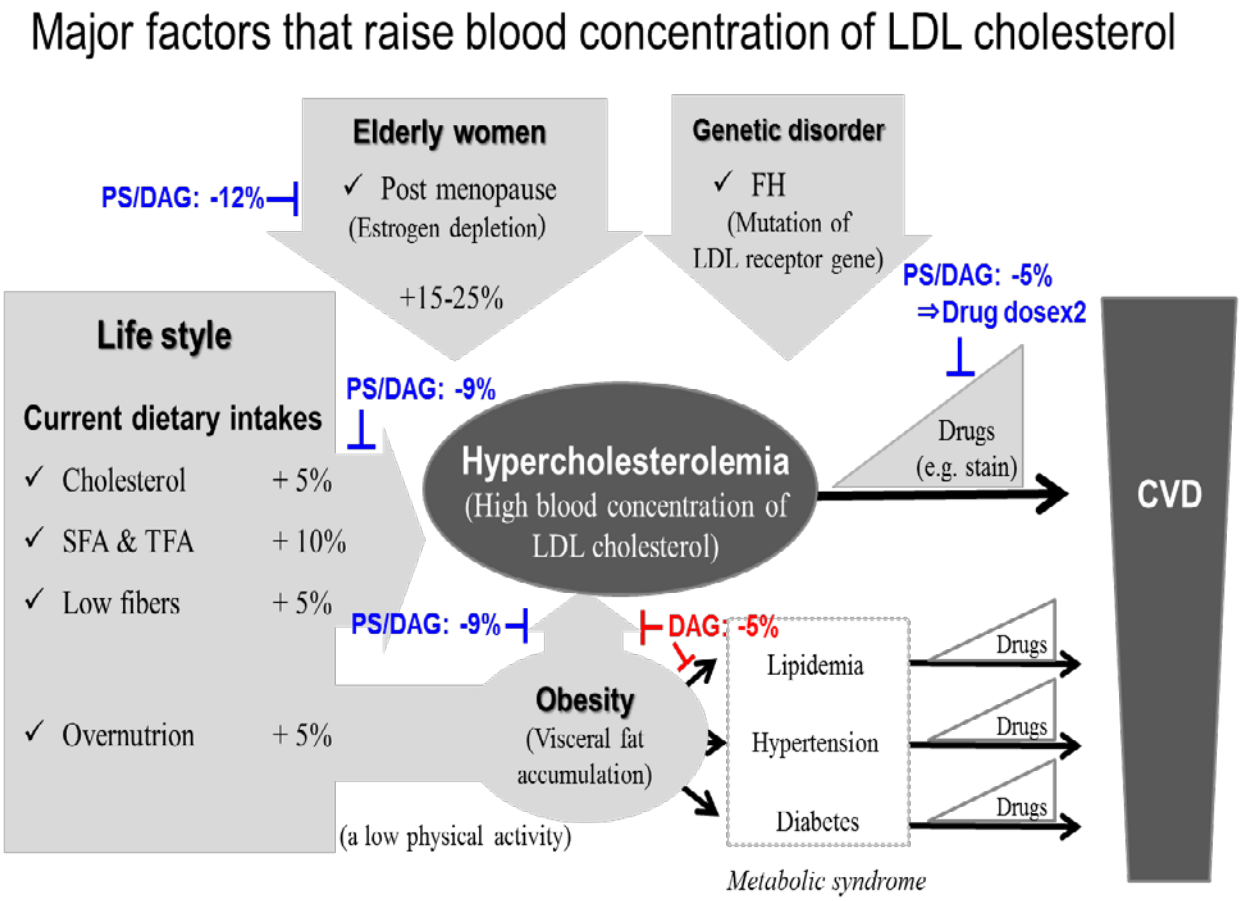
In the last decade, micro-analytical techniques have advanced. The presences of new substances which had never been found in the foods so far have been gradually identified. Although glycidol fatty acid esters (GEs) had been unknown contaminants in refined edible oils, the German Federal Institute for Risk Assessment (BfR) firstly reported in March 2009 that GEs have concern of possible exposure to glycidol (G) (2,3-epoxy-1-propanol), an animal carcinogen during digestion (BfR, 2009; Bakhiya et al., 2011). In June 2009, Econa Cooking Oil ® (DAG ≥ 80 wt%) produced by Kao Corporation in Japan was found to contain trace amounts of GEs as contaminants. Thus, although the safety of DAG oil have been previously confirmed in different studies (Morita & Soni, 2009) and no alleged safety problems were observed, the company temporarily halted the production of the product until GEs levels could be reduced, and voluntarily revoked the FOSHU status of the product. Subsequently, because adverse effects of DAG oil were not confirmed in animal studies, although DAG oil contains GE as contaminants, Food Safety Commission of Japan (FSCJ, 2015) judged that tumor promoting risk in human is negligible from the daily consumption of DAG oil in foods. The tumor promoting activity of DAG oil, once concerned, was denied.

Kao Corporation have contributed to the standardization of novel methods to quantify GEs (Masukawa et al., 2010; Masukawa et al., 2011; Shiro et al., 2011), the evaluations for the toxicity of GEs using genotoxic test (Ikeda et al., 2012) and toxicokinetic studies in animal (Wakabayashi et al., 2012) and human (Honda et al., 2011; Honda et al., 2012), respectively, demonstrating that the safety of DAG oil is almost equivalent to that of TAG oil. Also, the reduction technique of GEs has been

consistently investigated (Shimizu et al., 2012). Eventually, the GEs content of edible oils in Japan is lower level than that of products reported abroad.

More recent research brings a new insight into the mechanism by which DAG oil reduces visceral fat accumulation. Incretins are gut hormones involved with postprandial glucose and fat metabolisms. GIP that is produced and secreted by gut K cells of the duodenum and jejunum, is a key molecule linked to obesity, especially fat uptake into adipocytes. The blood concentration of GIP is elevated in obese type 2 diabetic patients (Creutzfeldt er al., 1978). It has been reported that DAG oil-containing meal possibly lowers postprandial GIP response compared to TAG oil-containing meal, suggesting that DAG oil may partly contribute to the prevention of obesity by regulating GIP secretion from the gut after meals (Shoji et al., 2012). The physiological effects of DAG oil on the gut function should be continuously examined.

In conclusion, PS/DAG oil has the potential for providing several benefits in the cholesterol metabolism (intestinal absorption and hepatic synthesis), blood lipoprotein metabolism, fibrinolysis and visceral fat accumulation (**Fig. 6-1**). PS/DAG oil will also work as a practical first-line treatment to ameliorate atherogenic lipoproteins such as LDL cholesterol and Lp(a) concentrations before resorting to drug treatments and after. PS/DAG oil may be a new lifetime of healthy edible oil for the management of high blood cholesterol and obesity at home in various populations to prevent CVD (**Fig. 6-4**). Further research should be explored to confirm the effectiveness and safety for longer-term period globally.



Saturated fatty acids: SFA, Trans fatty acids: TFA, Cardiovascular disease: CVD.
Familial hypercholesterolemia: FH
• Homozygote: 1/1,000,000,000 (LDL cholesterol: 12.9-23.3mmol/L; 500-900 mg/dL)
• Heterozygote: ~1/500 (LDL cholesterol: 3.9-10.9 mmol/L; 150-420 mg/dL)

Fig. 6-4 Effectiveness for consuming PS/DAG oil or DAG oil in different populations

Red bars illustrate preventive effects of DAG oil.

Blue bars illustrate preventive effects of PS/DAG oil.

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