Intake of a Single Morning Dose of Standard and Novel Plant Sterol Preparations for 4 Weeks Does Not Dramatically Affect Plasma Lipid Concentrations in Humans[1]

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ABSTRACT Recommendations for decreasing the risk of developing cardiovascular disease include increasing the intake of plant sterols and fish oil. The cholesterol-lowering action of plant sterols, when provided in a fish-oil fatty acids vehicle, remains to be investigated in humans. A randomized, crossover-feeding, single-blind trial was conducted in 30 subjects with mild-to-moderate hypercholesterolemia to study the effects on plasma lipids of 2 novel forms of plant sterols: those combined with, or esterified to, fish-oil fatty acids. The treatments were margarine (control), free plant sterols, plant sterols esterified to fatty acids from sunflower oil, plant sterols esterified to very long-chained fatty acids from fish oil, and plant sterols combined with the same amount of very long-chained fatty acids from fish oil. Each sterol-containing food (1.0–1.8 g plant sterols/d) was consumed for 29 d as a single dose with breakfast under staff supervision. Compared with the control treatment, none of the plant sterol preparations reduced plasma total cholesterol or LDL cholesterol, triacylglycerol, apolipoprotein A-I, apolipoprotein B, lipoprotein (a), or C-reactive protein concentration. Relative to the control phase, all plant sterols treatment increased the plasma HDL cholesterol concentration ($P < 0.05$) by ~8%. In conclusion, because standard forms of plant sterols did not reduce plasma cholesterol concentrations, the efficacy of the new formulation of plant sterols cannot be confirmed from the present study design, where plant sterols were given as a single morning dose. J. Nutr. 136: 1012–1016, 2006.

KEY WORDS: • *plant sterols* • *fish oil* • *plant sterol–fish-oil ester* • *single dose*

Cardiovascular disease (CVD)[3] remains the major cause of mortality and morbidity in developed countries (1,2). Hyperlipidemia is a significant risk factor for CVD (3). Recent recommendations to decrease CVD risk include using plant sterols (phytosterols) in hypercholesterolemic individuals (4) and increasing intake of long-chain (n-3) fatty acids of fish oil (5).

Plant sterols are compounds that have a chemical structure similar to cholesterol, with the exception of an extra methyl or ethyl group or a double bond on carbon 24 of the side chain (6). Plant sterol consumption has been reported to reduce cholesterol absorption and thus circulating cholesterol levels. Intake of plant sterols in the range of 1.5–2.5 g/d reduces LDL cholesterol levels by 8.5–10% (7). However, the efficacy of new formulations prepared to enhance plant sterol solubility and/or blood lipid–lowering effects, as well as the frequency at which plant sterols are given throughout the day, remains to be investigated.

Increasing the intake of fish-oil fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) to 0.5–1.8 g/d is recommended by many experts to reduce subsequent cardiac and all-cause mortality (5). A novel approach to increase the intake of EPA and DHA is to combine them with plant sterols. Animal studies have examined the effect of plant sterols esterified to these fatty acids. Adult guinea pigs fed a diet supplemented with plant sterol–fish-oil ester and corn oil have lower circulating concentrations of triacylglycerol, total cholesterol, and non-HDL cholesterol than controls (8). In insulin-resistant rats, supplementation with the plant sterol–fish-oil ester significantly reduced plasma triacylglycerols and improved endothelial and vascular smooth muscle cell function (9). To date, human studies that examine the effects of plant sterols on circulating lipid concentrations have been carried out using sterols esterified to fatty acids from plant oils, mainly, rapeseed (10–13), sunflower (14,15), or soybean oil (16,17). The efficacy of plant sterols as a cholesterol-lowering agent, when given in combination with fish-oil fatty acids, remains to be investigated in humans.

The objective of this study was to examine the effects of plant sterols, combined with, or esterified to, fish-oil fatty acids,

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[3] Abbreviations used: apo, apolipoprotein; CRP, C-reactive protein; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EPA, eicosapentaneoic acid; (n-3) PUFA, long-chain (n-3) polyunsaturated fatty acids.

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on blood lipid concentrations and on recent factors associated with CVD, i.e., apolipoprotein (apo) A-I and apo B, lipoprotein (a) [Lp(a)] (18–21). In addition, because plant sterols may have an anti-inflammatory action (22), we examined the effect of plant sterols on C-reactive protein (CRP), an inflammatory mediator that predicts coronary heart events (23).

Our primary hypothesis was that the effect of the new preparation of plant sterol on blood lipid levels (i.e., plant sterols combined with or esterified to fatty acids from fish oil) would be the same as traditional forms of plant sterols (i.e., free plant sterols and plant sterols esterified to fatty acids from vegetable oils). In addition, we hypothesized that the esterification of plant sterols to fatty acids from fish oil would not affect the action of plant sterols in terms of incorporating them into micelles to compete with cholesterol absorption. Thus, we expected plant sterols to have a similar effect on blood lipids when they are either combined with, or esterified to, fatty acids from fish oil. All plant sterol preparations were administered once as a single dose at a supervised breakfast to ensure compliance.

METHODS

Subjects. Male and postmenopausal female subjects were recruited through the distribution of flyers and newspaper advertisements in Montreal and surrounding areas. Criteria for being considered for the study included the following: age range of 40–85 y; body mass index between 22 and 34 kg/m^2; LDL cholesterol >2.6 mmol/L (100 mg/dL); nonsmoker; free from cardiovascular, kidney, or liver disease; nondiabetic; not consuming lipid- or glucose-lowering medications; normotensive; or hypertensive and controlled by medications within the last 3 mo. Two women who were on hormone replacement therapy maintained their current regimen for the study duration. Subjects provided a medical history and underwent a physical examination by the study physician. Eight subjects dropped out for personal reasons, including lack of time and difficulties in reaching the research clinic. The experimental protocol was approved by the Institutional Research Ethics Board for the School of Medicine at McGill University, Montreal and Tufts University-New England Medical Center, Boston. All volunteers gave their written informed consent to participate in the trial prior to the commencement of the study. Baseline characteristics of the study subjects are presented in **Table 1**.

Protocol and diet. Study subjects consumed each of the 5 experimental diets for periods of 29 d, using a randomized, single-blind, crossover design in which every subject completed every treatment. Both food and beverages were consumed in amounts sufficient to maintain a stable body weight as estimated by the Mifflin equation (24). At days 1 and 2, and then again at days 26, 27, and 28 of each diet phase, blood samples were obtained after a 12-h fast. Each diet phase was followed by a 2–4-wk washout period during which the subjects consumed their habitual diets. Diets were designed to contain 55% energy from carbohydrates, 15% from protein, and 30% from fat (**Table 2**). Foods were identical for all diet phases with the exception of the experimental component.

TABLE 1

Baseline characteristics of subjects[1]

Variable	All, n = 30
Age, *y*	59 ± 10
Weight, *kg*	79 ± 17
BMI, *kg/m^2*	28 ± 5
Plasma lipids, *mmol/L*	
Total cholesterol	5.9 ± 0.8
LDL cholesterol	3.8 ± 0.8
HDL cholesterol	1.4 ± 0.4
Triacylglycerol	1.6 ± 0.9

[1] Values are means ± SD, n = 30.

TABLE 2

Macronutrient composition of the study diet[1]

Nutrient	Composition
Protein, *% energy*	15
Carbohydrate, *% energy*	55
Total fat, *% energy*	30
Saturated fatty acids, *% energy*	8
Monounsaturated fatty acids, *% energy*	12
Polyunsaturated fatty acids, *% energy*	8
Cholesterol, *mg*	248
Fiber, *g*	37

[1] Diets provided 12.55 MJ (3000 kcal)/d.

Subjects consumed breakfast each morning at the clinical unit and under the supervision of staff. The 2 remaining meals were prepared and packed for consumption at work or at home. The experimental components of the diet were administered as a single daily dose provided with the margarine component of the breakfast meal. Subjects were instructed to consume only meals prepared by the clinical kitchen and not to consume any other food or drink, including alcohol or other beverages. Body weights were monitored daily throughout the intervention phases and maintained at baseline ± 1 kg by adjusting the amount of food ingested, if necessary. Body weights at the end of the treatment periods were 79.0 ± 16.3 kg, 78.2 ± 16.4 kg, 78.3 ± 16.5 kg, 78.6 ± 16.6 kg, and 78.9 ± 16.7 kg for control, sterol, sterol and long-chain fatty acids of fish oil, sterol ester of long-chain fatty acids from fish oil, and sterol ester of sunflower oil phases, respectively.

Subjects were randomly assigned to each of the following 5 treatments, using a Latin square design: control margarine (Unilever); free form of plant sterols given at a dose of 22 mg · kg body weight^{-1} · d^{-1} (Forbes Medi-Tech); plant sterols esterified to fatty acids from sunflower oil provided in margarine and given at a dose providing 22 mg plant sterol · kg body weight^{-1} · d^{-1} (Unilever); plant sterols esterified to long-chain (n-3) polyunsaturated fatty acids [(n-3) PUFA] from fish oil given at a dose of 35.2 mg · kg body weight^{-1} · d^{-1} (equivalent to 22 mg of plant sterols) (Forbes Medi-Tech); and free form of plant sterols (Forbes Medi-Tech) given at a dose of 22 mg · kg body weight^{-1} · d^{-1} in combination with long-chain (n-3) PUFA from fish oil (Roche) given at a dose of 13.2 mg · kg body weight^{-1} · d^{-1}. Plant sterols combinations are given below (**Table 3**). The fatty acid composition of the fish oil and the sterol fish oil ester was the same and consisted of 41.1% EPA and 19.8% DHA. To standardize the dose across subjects who ranged considerably in body mass, the plant sterol dose was administered according to body weight. The mean dose of plant sterols was 1.7 g/d, with a range of 1.0–1.8 g/d. The mean intake of long-chain (n-3) PUFA was 1.1 g/d, ranging from 0.7 to 2.1 g/d.

Analyses. Blood samples were centrifuged for 20 min at 520 × *g* at 4°C and aliquots were stored at −80°C until further analyses. Plasma total-, LDL-, and HDL cholesterol, triacylglycerol, apo A-I, apo B, Lp(a), and CRP concentrations were analyzed as previously described (25,26).

TABLE 3

Major plant sterol concentrations of the treatments[1]

	Free sterols	Sterol–sunflower oil esters	Sterol–fish-oil esters
		g/10 g	
β-Sitosterol	70.2	40.3	71.7
Campesterol	9.7	22.7	10.3
Stigmasterol	0.2	18.1	0.3
Other sterols	19.9	18.9	17.7

[1] Values are % w/w.

Plasma plant sterols, campesterol and β-sitosterol, were analyzed in duplicate by gas-liquid chromatography, as reported previously (27). Briefly, plasma samples were saponified with methanolic KOH solution and extracted twice with petroleum ether. 5-α Cholestane was used as the internal standard. Samples were analyzed by a gas-liquid chromatograph equipped with a flame ionization detector (HP 5890 Series II, Hewlett Packard) and a 30-m capillary column (SAC-5, Supelco). Detector and injector temperatures were 300°C. Sterol peaks were identified by comparing with standards (Supelco). Sitosterol and campesterol levels were analyzed to verify the bioavailability of plant sterols from the new formulations.

Statistical analyses. Data are expressed as mean ± SD. A sample size of 26 subjects was calculated as sufficient for detecting a change of 0.5 mmol/L at α (2-sided) = 0.05 and power = 0.80 and using a value for the SD of the change of 0.5 mmol/L as obtained from previous studies. Bonferroni correction was applied because comparison was of more than 2 groups. ANOVA was used to determine statistical significance. When treatment effects were identified as significant, a Tukey test was used to identify significant effects among treatments. Student's paired *t* tests were used to compare start and end point values within each treatment period. Normal distribution was tested with Shapiro-Wilks test. If a variable was not normally distributed, data were logarithmically transformed prior to analysis (11). Genders also were evaluated separately by ANOVA. Differences were considered significant at $P < 0.05$. The data were analyzed using Proc-General Linear Model SAS (version 8.0; SAS Institute).

RESULTS

The addition of free sterol, free sterol plus fish oil, sterol esterified to fish oil, or sterol esterified to sunflower oil to the diet for 4 wk did not affect percent changes (data not shown) or end point plasma concentrations of total cholesterol, LDL cholesterol, or triacylglycerol compared with the control period (**Table 4**). When genders were analyzed separately, the results were similar to those of the whole group (data not shown). Plasma HDL cholesterol levels were higher ($P < 0.05$) after subjects consumed each of the diets containing the sterols, and relative to the period of control diet. Although the trend was similar in men and women, the magnitude of difference was greater in the latter ($P < 0.05$). The increase in HDL cholesterol resulted in a lower ($P < 0.05$) total cholesterol:HDL cholesterol ratio at the end of each sterol-supplemented diet period, relative to the control diet period, although the differences were significant for all treatments only in women ($P < 0.05$). The difference in HDL cholesterol levels was not reflected in the plasma apo A-I concentrations that were not affected by the treatments. The treatments also did not affect apo B, Lp(a), or CRP concentrations.

Relative to the control, plasma campesterol levels were higher ($P < 0.05$) only after subjects consumed the diet of plant sterols esterified to sunflower oil (Table 4). The pattern for plasma β-sitosterol levels was different from that of campesterol. The levels of β-sitosterol rose ($P < 0.05$) after subjects consumed each of the plant sterol formulations relative to the control diet. However, the increase in β-sitosterol was lower ($P < 0.05$) after subjects consumed sterols esterified to fatty acids from sunflower oil than when they consumed the other sterol preparations. Over the 4-wk controlled diet period, plasma levels rose ($P < 0.05$) by 42% for the sterols esterified to fatty acids from sunflower oil, and β-sitosterol levels rose ($P < 0.05$) by 24, 49, 39, and 26% for the free sterols, free sterols plus fish oil, sterols esterified to fish oil, and sterol esterified to sunflower oil treatments, respectively.

Although the sterol preparations did not affect plasma lipid, lipoprotein, apolipoprotein, and CRP concentrations with the exception of HDL cholesterol, among diet groups, there were effects of the diet interventions on LDL and HDL cholesterol, campesterol, and β-sitosterol concentration that was attributed to shifting individuals from habitual to controlled diets. Over the 4-wk controlled diet period, plasma LDL cholesterol concentrations declined from baseline ($P < 0.05$) by 8, 11, 7, 3, and 11%, and triacylglycerol by 12, 23, 23, 11, and 13%, for control, free sterols, free sterols plus fish oil, sterols esterified to fish oil, and sterols esterified to sunflower oil periods, respectively. In contrast, HDL cholesterol declined from baseline ($P < 0.05$) by 3, 5, and 6% for control, sterols esterified to fish oil, and sterols esterified to sunflower oil periods, respectively.

DISCUSSION

The aim of this study was to determine effects of free sterols and sterols esterified to different fatty acids and mixed with very long-chain (n-3) fatty acids on plasma lipid and lipoprotein levels, CRP, and plasma plant sterols. Somewhat unexpectedly, a single daily dose of plant sterols, regardless of their physical form and taken with the breakfast meal, did not significantly lower plasma cholesterol concentrations compared with the control treatment, but was associated with an increase in HDL cholesterol. Although some previous studies reported that the lack of efficacy of plant sterols as cholesterol-lowering agents, this could be attributed to poor solubility of plant sterols in the

TABLE 4

Plasma lipid, lipoprotein, apolipoprotein, CRP, and sterol concentrations in subjects at the end of each dietary plant sterol intervention[1]

Variable	Control	Free sterols	Sterols + fish oil	Sterol esters of fish oil	Sterol esters of sunflower oil	*P*-value
Total cholesterol, *mmol/L*	5.74 ± 1.09	5.67 ± 1.11	5.78 ± 1.11	5.80 ± 1.11	5.70 ± 1.14	0.562
LDL cholesterol, *mmol/L*	3.65 ± 0.93	3.60 ± 1.01	3.70 ± 0.98	3.76 ± 1.01	3.60 ± 1.06	0.391
HDL cholesterol, *mmol/L*	1.27 ± 0.28[a]	1.37 ± 0.34[b]	1.37 ± 0.34[b]	1.35 ± 0.34[b]	1.35 ± 0.34[b]	0.0001
Triacylglycerol[2], *mmol/L*	1.51 ± 0.63	1.44 ± 0.66	1.39 ± 0.63	1.48 ± 0.71	1.56 ± 0.88	0.198
Total HDL cholesterol	4.72 ± 1.25[b]	4.34 ± 1.23[a]	4.39 ± 1.24[a,b]	4.54 ± 1.25[a,b]	4.49 ± 1.36[a,b]	0.0275
Apo B, *g/L*	0.98 ± 0.21	0.97 ± 0.21	0.98 ± 0.20	1.00 ± 0.21	0.98 ± 0.21	0.444
Apo A-I, *g/L*	1.25 ± 0.17	1.28 ± 0.17	1.29 ± 0.19	1.27 ± 0.18	1.27 ± 0.18	0.0845
Lp(a), *μmol/L*	0.96 ± 0.71	1.00 ± 0.75	1.00 ± 0.71	1.00 ± 0.75	1.00 ± 0.71	0.5746
CRP, *mg/L*	4.8 ± 7.7	2.5 ± 21	2.8 ± 3.2	3.7 ± 4.9	5.5 ± 10.1	0.106
Campesterol,[2] *μmol/L*	18.6 ± 8.3[b]	19.4 ± 9.1[b]	19.3 ± 8.5[b]	19.5 ± 7.6[b]	24.1 ± 12.6[a]	<0.0001
β-Sitosterol,[2] *μmol/L*	7.0 ± 3.7[c]	9.9 ± 5.6[b]	10.8 ± 5.9[b]	10.2 ± 4.7[b]	8.7 ± 5.5[a]	0.0002

[1] Values are means ± SD, $n = 30$. Means in a row with superscripts without a common letter differ, $P < 0.05$.
[2] Data were log transformed prior to analysis.

formulations tested (28,29). The lack of plant sterol efficacy in our study appeared not to be related to the form consumed or their bioavailability, because the data were similar for those administered as free plant sterols or as plant sterols esterified to fatty acids from either sunflower or fish oil. Plant-sterol preparations examined in our study included preparations previously shown to be bioavailable and to lower blood cholesterol, namely, free plant sterols blended with a spread (30,31) and plant sterol esters of fatty acids from vegetable oil (10,15–17,32). Thus, even if plant sterols from the new formulations are not bioavailable, this does not explain why previously tested plant sterol preparations did not reduce blood cholesterol levels in this study.

The increase in plasma HDL cholesterol concentration observed after plant sterol–supplemented diet phases has been reported in a few (33,34), but not the majority (13,14,16,30,31) of previous studies. Regardless of the significant HDL cholesterol-raising effect of the tested plant sterol preparations, no reciprocal effect on triacylglycerol concentrations occurred. The increase in HDL cholesterol concentrations observed in this study may be due to chance. Furthermore, supplementing the diet with fish oil, either mixed with plant sterols or esterified to the plant sterols, did not affect plasma triacylglycerol concentrations. The lack of an effect of fish oil on triacylglycerol levels is likely due to the quantity of fish oil fed and the absence of hypertriacylglycerolemia, by design, in the individuals studied. Differences in increases of plasma sitosterol and campesterol among the different study formulations may correspond to the differences in plant sterol contents of the formulations.

As discussed above, the lack of efficacy of different plant sterols is unlikely to be related to poor solubility. In addition, it is unlikely that the lack of efficacy of plant sterols was due to inadequate power, as other plant sterol studies have been performed with a similar number or even fewer subjects. In addition, sample-size calculation was corrected for the multiple comparisons. The lack of efficacy of different plant sterols in this study was likely due to the fact that plant sterols were administered as a single morning dose, to ensure compliance with consuming the breakfast meal under supervision. Therefore, poor compliance was not responsible for the lack of effects. Previous studies that have shown cholesterol-lowering efficacy distributed the plant sterol treatment in 2 (11,14,16,35) or 3 (10,12,13,30,31) doses per day. However, a single dose of plant sterols has also been shown to lower cholesterol levels (36,37). In a crossover study where 2.5 g/d of plant stanol esters, incorporated into margarine or shortening and consumed either once at lunch or 3 times/d, LDL cholesterol concentrations were reduced by 9 and 10%, respectively (36). In another study, a 10% reduction in LDL cholesterol occurred in subjects given a 2.7-g dose of plant sterols solely at lunch (37). Moreover, the diets were moderate in fat and cholesterol and should not have contributed to the absence of an effect of plant sterols on circulating cholesterol concentrations. The single dose of plant sterols used in this study was provided with a morning meal that contained similar cholesterol content to the other meals. Each of the 3 meals supplied ~83, 86, and 79 mg of cholesterol, respectively. Even at a low cholesterol intake, plant sterols have been shown to reduce blood cholesterol concentrations (11, 16,17,32,38,39).

Plat et al. (36) hypothesized that plant stanols remain in the intestinal lumen for extended periods, suggesting that it is not necessary to consume plant stanol ester products with every meal (36). Our results do not support this hypothesis. A single morning dose of plant sterols reduced plasma cholesterol in some individuals, but overall, there was no significant reduction in blood cholesterol compared with the control-diet phase. One of the major differences between the studies of Plat et al. (36) and Matvienko et al. (37) and our study is the size of the single dose of plant sterols. Compared with the 1.0–1.7 g/d dose of the present study, the single-dose sizes for Plat et al. (36) and Matvienko et al. (37) were 2.5 and 2.7 g /d, respectively. It is likely that a higher dose of plant sterols is needed when administered as a single dose. Plant sterols were given on a bodyweight basis to standardize the dose. In one of our previous studies (31), we also gave plant sterols at a dose of 22 mg·kg body weight^{-1} · d^{-1} and observed a 15.5% reduction in LDL cholesterol levels compared with the control. However, in that study, the plant sterol dose was distributed throughout the day.

Another major difference is that the single dose was consumed at lunch in the Plat et al. (36) and Matvienko et al. (37) studies, whereas in the present study plant sterol treatments were consumed at breakfast. The efficacy of plant sterols as a cholesterol-lowering agent may demonstrate a time-of-day variation, possibly coinciding with the circadian rhythm of cholesterol metabolism. Circadian rhythm in cholesterol synthesis has been shown in animals (40–43) and in humans (44,45,46), whereas the circadian variation in cholesterol absorption has not been studied. In rats and hamsters, maximum cholesterol synthesis occurs at midnight, whereas minimum synthesis occurs at noon (40–43). However, in these rodents, which normally eat at night, circadian rhythm of cholesterol biosynthesis depends on the time of food intake rather than on the light cycle (41,47). Similar to the data from animals, the circadian rhythm of cholesterol biosynthesis in humans is affected by food intake. Delaying the meal time by 6.5 h resulted in 8.6-h and 6.5-h delays in maximum and minimum cholesterol synthesis rate, respectively (46). Because cholesterol synthesis and absorption are inversely related, one can speculate that cholesterol absorption is low early in the morning and increases during the daytime period. Thus, further lowering of cholesterol absorption by plant sterols may not lead to a decrease in blood cholesterol concentrations. It is possible that a single dose of plant sterols taken in the morning may not lead to optimal cholesterol reduction.

In conclusion, because traditional forms of plant sterols did not reduce blood cholesterol levels, we cannot confirm the efficacy of the new formulation of plant sterols administered as a single morning dose. Future studies are needed to address whether a higher dose of plant sterols is needed when taken as a single dose or whether intake should be distributed throughout the day. Moreover, studies are needed to investigate whether the time of day affects the efficacy of a single dose of plant sterols, given that recent recommendations encourage their use in reducing the risk of CVD (4). In addition, promoting plant sterol–enriched products for consumption once a day should be based on efficacy data from well-controlled studies. Recently, a number of proteins that regulate cholesterol absorption have been identified. Niemann-pick C1 Like 1 plays a critical role in the uptake of cholesterol across the intestinal enterocytes (48). In contrast, ATP-binding cassette transporter G5 and G8 proteins pump the sterols from the enterocytes back into the intestinal lumen (49). The study of the level and/or expression of these proteins presents a promising approach for investigating the interplay among circadian rhythm, plant sterols, and cholesterol metabolism.

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