Current Research

Cocoa Flavanol-Enriched Snack Bars Containing Phytosterols Effectively Lower Total and Low-Density Lipoprotein Cholesterol Levels

JOHN A. POLAGRUTO, PhD; JANICE F. WANG-POLAGRUTO, PhD; MARLIA M. BRAUN; LUKE LEE; CATHERINE KWIK-URIBE, PhD; CARL L. KEEN, PhD

ABSTRACT

Background Dietary intervention studies incorporating phytosterol-enriched margarine spreads have reported significant decreases in total and low-density lipoprotein (LDL) cholesterol in populations with both normal lipid levels and those with hypercholesterolemia. There is emerging support for more diverse and lower-fat phytosterol-enriched matrixes. Controversy exists, however, over whether phytosterol-enriched foods affect serum fat-soluble vitamins.

Objective We investigated whether a flavanol-rich cocoa snack food containing phytosterols would decrease total and LDL cholesterol levels in subjects with hypercholesterolemia and significantly affect serum fat-soluble vitamins and carotenoids.

Design A randomized, double-blind parallel arm study design was used. Subjects were randomized to one of two dietary treatments: a cocoa flavanol-enriched snack bar containing 1.5 g phytosterol (n=32), or a control product containing no phytosterols (n=35). Subjects consumed two servings per day.

Results Consumption of the phytosterol-enriched snack bars but not control bars for 6 weeks was associated with significant reductions in plasma total (4.7%; $P<0.01$) and LDL cholesterol (6%; $P<0.01$), and the ratio of total to high-density lipoprotein cholesterol (7.4%; $P<0.001$). There were no changes in high-density lipoprotein cholesterol, triglycerides, or lipid-adjusted lycopene, β-cryptoxanthin, lutein/zeaxanthin, α-carotene levels, or levels of serum vitamins A or E. A significant reduction in lipid-adjusted serum β-carotene was observed in the phytosterol but not the no–phytosterol-added group ($P<0.05$).

Conclusions This study supports the use of a novel phytosterol-enriched snack bar to effectively reduce plasma total and LDL cholesterol levels in a population with hypercholesterolemia. The data suggest that the incorporation of this snack food into a balanced diet represents a practical dietary strategy in the management of serum cholesterol levels.

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Phytosterols are plant-derived compounds structurally related to mammalian cell-derived cholesterol that function as essential constituents of plant cell membranes (1). Although a diverse group of compounds, phytosterols can be divided into two distinct classes, the sterols and stanols, with the sterols the predominate form found in nature. Both classes of phytosterol are naturally occurring and can be found in a variety of foods, including nuts, vegetable oils, seeds, and cereals (2).

During the past decade, there has been increased interest in the functional properties of phytosterols, principally due to their well-documented ability to influence cholesterol homeostasis. After an extensive review of the scientific literature, the US Food and Drug Administration found that there was compelling evidence to support the use of plant sterols/stanols as part of a dietary strategy to reduce the risk of coronary heart disease. In 2000, the United States allowed products containing phytosterols to carry a health claim.

Although the cholesterol-lowering properties of phytosterols have been well documented, and recently summarized by Berger and colleagues (2), the mechanisms underlying these effects are still under investigation. Phytosterols are poorly absorbed in human beings, and as such are hypothesized to interfere with the solubility of cholesterol in the oil and micelle phases during digestive processes, thereby decreasing net cholesterol absorption (3). It is also possible that when absorbed by enterocytes, phytosterols may increase expression of adenosine binding cassette proteins, which can efflux both phytosterol and cholesterol back into the lumen (4). The clinical implications of these mechanisms are that when included in diets of human beings, phytosterols have repeatedly been shown to reduce total and low-density lipoprotein (LDL) cholesterol levels (4-17).

J. A. Polagruto and J. F. Wang-Polagruto are postdoctoral researchers, M. M. Braun is a doctoral candidate, L. Lee is a staff research associate, and C. L. Keen is a professor, Department of Nutrition, University of California, Davis. C. Kwik-Uribe is research manager, Analytical and Applied Sciences, Masterfoods USA, Hackettstown, NJ.

Address correspondence to: Carl L. Keen, PhD, Department of Nutrition, University of California, Davis, One Shields Ave, Davis, CA 95616. E-mail: clkeen@ucdavis.edu

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Although food products containing plant sterols are now available commercially (eg, enriched margarines, milk, and orange juice), and are considered safe for adults that would benefit from total and LDL cholesterol-lowering (18), some concern exists as to their effects on fat-soluble vitamins or antioxidant availability. Richelle and colleagues (19) reported reduced plasma appearance of β-carotene and retinyl palmitate in subjects following the consumption of milk containing either plant-free sterols or sterol esters, while the consumption of sterol esters exhibited a trend toward decreased α-tocopherol availability. These results support observations that consumption of foods containing free plant sterols (14), sterol esters (20,21), and plant stanols (22) is associated with decreased plasma carotenoids. The clinical significance of this effect remains to be determined.

It has been reported that more than 100 million Americans have total blood cholesterol values greater than 200 mg/dL (5.16 mmol/L), while as many as 37 million have values greater than 240 mg/dL (6.2 mmol/L) (23). Although high serum cholesterol is a known atherosclerosis risk factor, it has been reported that less than 42% of patients with hypercholesterolemia discharged from the hospital following an acute myocardial infarction were prescribed lipid-lowering medications (24). Despite the proven effectiveness of statin medications, many individuals remain hesitant to use lipid-lowering medications and desire an alternative dietary means to lower serum cholesterol levels. It has been argued that dietary intervention strategies, including the incorporation of plant sterols, should be considered first before prescribing pharmacologic treatment (25). Therefore, the objective of this study was to determine the effectiveness of a novel flavanol-rich chocolate product containing phytosterol in lowering serum cholesterol levels in a free-living population of men and women with elevated serum cholesterol levels.

SUBJECTS AND METHODS

Subjects

Adult men and women were recruited from the University of California, Davis, campus and surrounding Yolo and Sacramento County areas using newspaper advertisements and posted flyers. Seventy-two subjects had a fasting serum cholesterol level >200 mg/dL (5.16 mmol/L), which qualified them for the study, and were enrolled into the study. Exclusion criteria included taking cholesterol-lowering medications (eg, statin medications), niacin, or other cholesterol-lowering agents, and using phytosterol-enriched foods such as margarine spreads (eg, Benecol, McNeil Nutritionals, LLC, Ft Washington, PA, or Take Control, Unilever US Inc, Engelwood Cliffs, NJ). Subjects were instructed to refrain from using antioxidant supplements (eg, vitamin C >100 mg/day, vitamin E >100 IU/day [67 mg/day]), herbal supplements, and for 4 days before a clinic visit, to refrain from using nonsteroidal anti-inflammatory medications. Participants were allowed to continue to use a generic multivitamin/mineral supplement. Subjects were asked to not alter their schedule of physical activity, except for the mornings of the clinic visits; subjects were free to exercise following their visit. Two subjects dropped out for personal reasons and an inability to comply with the study protocol. Seventy subjects completed the study. Participants provided written, informed consent, and the Institutional Review Board at University of California, Davis, approved the study.

Study Design

The study was a randomized, double-blind, placebo-controlled, parallel-arm study with two treatment groups. Subjects were randomly assigned to consume one of two cocoa snack bar products: a phytosterol-enriched cocoa bar (+phytosterol bar), or a control bar deficient in phytosterol (−phytosterol bar). Study participants were entered on a rolling-enrollment basis, and the study was conducted across a 6-month period. At the beginning of the 6-week study, all subjects attended a 45-minute nutrition education seminar, which covered a variety of health topics, including dietary guidelines, low-fat diets, and how to lead a healthful lifestyle. Participants made a total of five visits to the clinic. A 3-day diet record was kept at three time-points throughout the study, at weeks 0, 3, and 6; subjects were instructed on how to maintain a 3-day diet record, and each subject was provided an example food diary with tips on how to determine serving size. Subjects were instructed to avoid consumption of cocoa and other chocolate products during the entire 6-week study period. Subjects otherwise maintained their regular diets, but were restricted from using herbal and dietary supplements, and commercial food products fortified with phytosterols. During the 6-week period, study participants received a weekly newsletter covering a variety of health- and nutrition-related topics.

Cocoa Products

Subjects were supplied with individually wrapped servings of a CocoaVia Crunch (+phytosterol) bar (24 g/bar) or control (−phytosterol) bar product (both provided by Masterfoods USA, Hackettstown, NJ). Phytosterol-enriched bars delivered 1.5 g sterol esters (0.9 g free sterol equivalents) and 100 mg cocoa flavanols per serving. The flavanols in cocoa have been reported to have positive cardiovascular effects (34). The phytosterols in the +phytosterol bar were derived from soybean oils, and composed of β-sitosterol (51%), campesterol (27%), and stigmasterol (22%). There was no difference in taste or product composition between phytosterol-enriched product and placebo product, other than the inclusion of sterol esters and cocoa flavanols. The CocoaVia Crunch bar and placebo bar provided 82 kcal. The compositions of the snack bars are presented in Table 1. Study participants were instructed to consume one serving of the snack product within 30 minutes of a meal, twice a day, during the 6-week intervention period. To ensure that subjects maintained, and did not add to their kilocalories per day, instructions were given such that the snack products were consumed as a replacement for another snack in the diet. To ensure blinding to both investigators and study participants, products were labeled with a numerical code. To assess compliance, empty wrappers and any unconsumed servings were collected at follow-up visits and counted.

Table 1. Nutritional composition of flavanol-enriched and control cocoa bars used in the intervention study[a]

	+ Phytosterol bar	− Phytosterol bar
Total energy (kcal)	80	80
Total fat (g)	2	2
Saturated fat (g)	1	1
Trans fat (g)	0	0
Cholesterol (mg)	0	0
Sodium (mg)	65	65
Total carbohydrates (g)	13	13
Dietary fiber (g)	1	1
Sugars (g)	6	6
Protein (g)	2	2
Calcium (% DV[b])	25	25
Iron (% DV)	2	2
Vitamin C (% DV)	10	10
Vitamin E (% DV)	15	15
Vitamin B-6 (% DV)	10	10
Folic acid (% DV)	10	10
Vitamin B-12 (% DV)	10	10
Cocoa flavanols[c] (mg)	127.9	10.4
Sterol esters (g)	1.5	0
Caffeine (mg)	2.3	4.6
Theobromine (mg)	29.9	71.3

[a] Per 23-g bar.

[b] DV=daily value. Percent daily values are based on a 2,000-kcal diet.

[c] Procyanidin monomer through decamers.

Sample Collection

Fasting (12-hour) blood samples were obtained from study participants twice at week 0 (baseline), once at week 3, and twice at week 6. To assess changes in serum lipid levels and serum vitamin A, vitamin E, and carotenoid concentrations, blood was collected in evacuated serum separator tubes. Serum lipids were analyzed on the day of collection, and samples collected for vitamin A, vitamin E, and carotenoid analysis were stored at −80°C until the end of the study. Samples from all of the study time points were analyzed concurrently to prevent interassay variation. For height and weight measurements, subjects were dressed but had their shoes removed. Height was recorded at their first visit using a wall-mounted stadiometer (Ayrton S-100, Quickmedical Inc, Snoqualmie, WA), and at this and subsequent visits, their weight was measured using an electronic scale (Scale-tronix 6002, Scale-tronix Inc, White Plains, NY), and an automated blood pressure machine (Critikon Vital Signs Monitor 1846 SX, Critikon LLC, Tampa, FL) was used to record their blood pressure and pulse (an average of two measurements).

To measure plasma bioavailability of flavanols from the +phytosterol cocoa products, 10 subjects (10 women) were recruited to participate in an acute study designed to measure metabolites of plasma flavanols following consumption of two flavanol-rich +phytosterol snack bars containing 100 mg flavanols. For 24 hours before the acute intervention, subjects refrained from consuming flavonoid-rich foods. On the day of the acute feeding, subjects donated a fasting (baseline) blood sample and then were requested to consume two of the flavanol-rich +phytosterol bars. Repeated blood sampling was conducted at 2 hours and 4 hours postconsumption. Plasma samples were stored at −80°C until analyzed.

Serum Lipids and Vitamins A and E

Lipid and chemistry panels were performed in the University of California, Davis, Medical Center clinical pathology laboratory. Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride levels were analyzed using a Synchron LX-20 System (Beckman Coulter, Inc, Brea, CA). LDL cholesterol level was calculated using the Friedewald equation. Retinol and tocopherol were analyzed simultaneously from serum combined with an internal standard, using a Hewlett Packard model 1100 high-performance liquid chromatography (HPLC) (Agilent Technologies, Palo Alto, CA) by the University of California, Davis, Clinical Nutrition Research Unit.

Serum Phytosterols

Serum concentrations of β-sitosterol, campesterol, and stigmasterol in subjects at the 6-week time point (baseline, fasting) were measured using established methodology. In brief, 1 mL serum was saponified in a solution of ethanolic potassium hydroxide for 1 hour at 70°C. An internal standard (5β-cholestan-3α-ol) was used in the extraction procedure. Saponified samples were then extracted with pentane, dried under nitrogen, and stored at −80°C until the time of derivatization and subsequent gas chromatography analysis. The pentane extract residues were reconstituted with 300 μL N,O-bis trimethylsilyl trifluoroacetamide and 700 μL pyridine. All reaction vials were then capped and sealed with silicone septa-containing screw caps. The reconstituted extracts were placed in a thermostated heating block at 75°C for 1 hour to facilitate derivatization of phytosterol to their trimethylsilyl ether derivatives. Following derivatization, the reaction vials were cooled to room temperature and samples were transferred to vials for gas chromotography analysis. Gas chromatographic analysis was performed on an Agilent 6850 gas chromatographer (Palo Alto, CA) equipped with a 30 m×0.25 mm×0.25 μm HP-5 capillary column and flame ionization detector detection. The inlet temperature was held at 32°C while detection was at 340°C. Injection volume was 2 μL with a 50:1 split ratio. Concentrations of β-sitosterol, stigmasterol, and campesterol were determined with 5β-cholestan-3α-ol as an internal standard. Separate calibration curves were constructed for each of the sterol species measured. Typical correlations for calibration curves were 0.999.

Serum Carotenoids

The carotenoid analysis was carried out at the University of Illinois, Urbana-Champaign.

In brief, thawed serum samples were mixed with 0.1% butylated hydroxytoluene in ethanol and an internal standard, and were maintained on ice under yellow lights throughout the preparation and extraction processes. Carotenoid extraction was conducted using hexane (three repetitions). Extracts were pooled and dried in a speed vacuum, flushed under argon, and then stored at –20°C for no longer than 2 days before HPLC analysis. The HPLC system consisted of a Rainin Dynamax (Woburn, MA) gradient pump system model SD-200, a Varian Prostar model 410 (Walnut Creek, CA) autosampler (tray cool at 4°C), a C_{30}, 4.6×150 mm column (YMC, Wilmington, NC), a Cera column cooler (16°C), and a Rainin Dynamax absorbance detector model UV-DII (450 nm).

Plasma Epicatechin Concentrations

Sample Preparation. Plasma samples were drawn into ethylenediaminetetraacetic acid-containing vials, supplemented with ascorbate (1 mg/mL), snap-frozen in liquid nitrogen, and stored at −80°C. For HPLC analysis, samples were defrosted on ice, and prepared using a three-step procedure. Step 1: Samples were mixed with twice their volume of acidified methanol (100% by volume, −20°C, internal standard A=3 3′-*O*-ethyl-epicatechin) and centrifuged at 17,000*g* for 15 minutes at 4°C. Step 2: The supernate was collected and the pellet was resuspended in methanol as detailed for step 1. This resuspension was centrifuged (as above), the supernate was collected and the pellet was washed (step 3) in methanol (50% by volume, internal standard A). Following centrifugation the supernate was collected and combined with the supernates from steps 1 and 2. This mixture was centrifuged (as above) and the solvents were removed using a rotary evaporation system at 4°C.

Dried samples were then subjected to an enzymatic treatment. They were mixed with the following reagents, in order: 40 μL 100% methanol (not acidified), vortexed; 352 μL 0.1 mol/L sodium acetate buffer, vortexed; 4 μL 10% weight per volume ascorbic acid solution, vortexed; and 4 μL enzyme solution, vortex (200 U/μL glucuronidase Type L-II [Sigma G8132] in a benzenesulfonic acid solution containing 0.15% sodium chloride and 0.003% sodium azide). Samples were quickly centrifuged, purged with argon gas, and then incubated for 30 minutes at 37°C. The enzymatic reaction was stopped by adding ice-cold acidified methanol (100% methanol acidified with 0.5% acetic acid) in the amount of 1.5 volumes of enzyme mixture. Samples were kept at −20°C for 20 to 30 minutes, briefly vortexed, and centrifuged for 15 minutes at 4°C. Supernatant fractions were collected and stored in tubes rinsed with ethanol, and then redried using a rotary evaporation system at 4°C. Residues were resuspended in 200 μL methanol (25% by volume, internal standard B=catechol), which represents a fivefold concentration as compared to plasma. The average recovery for all compounds investigated was 85%±9%.

Detection

Fifty to 100 μL of extracted sample was injected onto an HPLC system (Agilent Technologies, Model 1100, Palo Alto, CA) and separated on a reversed-phase column (Phenomenex Luna C18-2, 150×4.6 mm, 3 μm) with guard column (Phenomenex C18 4×3.0 mm). Separation of analytes was accomplished using an acetonitrile gradient in 50 mmol/L methanolic (4% by volume) sodium acetate, pH 4.4, with a flow rate of 0.8 mL/min. Acetonitrile concentrations were linearly increased from 0% to 10% between minutes 5 and 20. Thereafter, acetonitrile concentrations were further increased in linear segments (20 to 28 minutes, 10% to 12%; 28 to 34 minutes, 12% to 20%; 34 to 41 minutes, 20% to 30%; 41 to 45 minutes, 30% to 71%) and held at 71% for 10 minutes. Peaks were detected and quantified using a fluorescence detection system, and were identified by comparing their retention time with the retention time of authentic standards and on-top spiking of the samples with authentic standards.

Table 2. Baseline characteristics of subjects with elevated serum cholesterol levels recruited to study the effectiveness of a flavanol-enriched chocolate product in lowering cholesterol levels[a]

	+ Phytosterol bar group	− Phytosterol bar group
	⟵ *mean*±*SEM*[b] ⟶	
Age (y)	49±2	56±2
Weight (kg)	76.9±3.2	71.0±2.7
Body mass index	27.2±1.0	25.6±1.0
Systolic blood pressure (mm Hg)	120±3	120±4
Diastolic blood pressure (mm Hg)	67±2	67±2
Heart rate (bpm)	68±2	68±2
Males/Females	10/22	10/25
	⟵ % ⟶	
Compliance[c]	97	98
	⟵ *n* ⟶	
Multivitamin use (daily)	19	17

[a] n=32 in the phytosterol-enriched bar group and n=35 in the no-added-phytosterol bar group.

[b] SEM=standard error of the mean.

[c] Subjects who returned opened and unopened packages: n=31 in the phytosterol-enriched group and n=33 in the no-phytosterol-added group.

DATA ANALYSIS

Throughout these investigations, the analytical chemists were blinded with regard to sample information. Peak/area ratios of individual authentic standards and internal standards were plotted against concentrations to achieve a multilevel external calibration curve using linear regression. Peak/area ratios (analyte/internal standards) were compared to standard curves in order to quantify the analytes.

Dietary Analysis

Study participants were required to record daily food intake for 3 days at three time points throughout the study: week 0 (baseline), week 3, and week 6 (end of study). Food intake records were analyzed using Nutritionist Pro software (version 1.0, First DataBank Inc, San Bruno, CA). Dietary data were entered by student interns, and then checked for accuracy by a second research assistant. Afterward, the study researchers made a final quality control check to ensure that food records were entered in their entirety into the diet database.

Statistical Analysis

Data are presented as the mean±standard error of the mean. Statistical analysis was performed using Sigma-

Table 3. Reported dietary intakes for each treatment group during the 6-week intervention trial[a]

	+ Phytosterol Bar Group		− Phytosterol Bar Group	
	Week 0	**Week 6**	**Week 0**	**Week 6**
	←——— *mean± SEM*[b]			———→
Energy (MJ)	8.1±0.4	8.5±0.4	7.8±0.3	7.5±0.3
Protein (g)	76±4	83±5	78±3	74±4
Carbohydrate (g)	255±15	253±14	258±11	243±11
Fat (g)	68±3	77±5	61±3	62±3
Saturated fatty acid (g)	21±1	25±2	19±1	19±1
Monounsaturated fatty acid (g)	19±1	20±2	18±1	17±1
Polyunsaturated fatty acid (g)	11±1	12±1	10±1	10±1
Cholesterol (mg)	235±27	239±23	183±18	179±16
Vitamin A (μg RAE[c])	2,166±310	1,826±188	2,603±333	2,762±491
Beta carotene (IU)[d]	1,592±34	946±191	2,119±332	1,912±356
Vitamin E (mg)	6.3±0.6	8.0±0.6	8.4±1.3	7.4±1.3
Vitamin C (mg)	164±24^{y**}	105±13^{z**}	161±16	142±15
Dietary fiber (g)	21±1	20±1	24±2	21±1
Soluble fiber (g)	0.6±0.1	0.4±0.1	1.0±0.3	0.9±0.3
Insoluble fiber (g)	2.2±0.4	1.8±0.3	2.9±0.4	2.1±0.3
Caffeine (mg)	176±35	162±32	150±25	141±25
Alcohol (g)	9.8±3.4	9.5±2.6	6.6±2.0	5.7±1.9
Fruit (servings/d)	3.2±0.5^{y**}	2.1±0.3^{z**}	3.1±0.4	2.8±0.4

[a] n=30 in the phytosterol-enriched bar group and n=34 in the no-phytosterol-added bar group; three subjects who did not complete food diaries at week 6 were excluded from statistical analyses. Values reflect average daily intakes over each 3-day record period, weeks 0 and 6.

[b] SEM=standard error of the mean.

[c] RAE=retinol activity equivalents.

[d] Significant reduction between week 0 and 6, regardless of treatment; $P<0.05$.

[yz] Values in the same row with different superscript letters are significantly different.

**$P<0.01$.

Table 4. Serum lipid concentrations in the subjects before and after 6 weeks of consumption of control and phytosterol-enriched bars[a]

	+ Phytosterol Bar Group			− Phytosterol Bar Group		
	Week 0	**Week 6**	**% change**[b]	**Week 0**	**Week 6**	**% change**
	←— *mean± SEM*[c] —→			←— *mean± SEM* —→		
Total cholesterol (mg/dL)[d]	247.4±5.4^{y**}	234.9±4.9^{z**}	−4.7	241.4±5.8	241.0±4.9	0.4
HDL[e] (mg/dL)[d]	52.1±2.4	54.0±0.1	+3.3	58.6±2.9	58.3±3.0	−0.1
LDL[f] (mg/dL)[d]	167.4±5.1^{y***}	156.1±4.3^{z***}	−6.0	158.6±5.3	158.3±4.7	0.4
Triacylglycerol (mg/dL)[g]	143.1±12.3	124.4±10.0	−8.5	119.1±87.7	122.4±8.8	3.9
Ratio of total to HDL cholesterol	5.0±0.2^{y***}	4.6±0.2^{z***}	−7.4	4.4±0.2	4.4±0.2	1.2

[a] n=32 in the phytosterol-enriched bar group and n=35 in the no-phytosterol-added bar group.

[b] Percent change=average of individual changes: [(wk 6−wk 0)/wk 0]×100%.

[c] SEM=standard error of the mean.

[d] To convert mg/dL cholesterol to mmol/L, multiply mg/dL by 0.026. To convert mmol/L cholesterol to mg/dL, multiply mmol/L by 38.7. Cholesterol of 5.00 mmol/L=193 mg/dL.

[e] HDL=high-density lipoprotein cholesterol.

[f] LDL=low-density lipoprotein cholesterol.

[g] To convert mg/dL triglycerides to mmol/L, multiply mg/dL by 0.0113. To convert mmol/L triglycerides to mg/dL, multiply mmol/L by 88.6. Triglycerides of 1.80 mmol/L=159 mg/dL.

[yz] Values in the same row with different superscript are significantly different, within treatment group.

**$P<0.01$.

***$P<0.001$.

Stat for Windows (version 2.0, 1992-1997, SPSS Inc, Chicago, IL). Three subjects were removed from all analyses due to the appearance of hypertriglyceridemia (>300 mg/dL [3.38 mmol/L]) that limited the accuracy of cholesterol measurements. Data were analyzed by two-way repeated measures analysis of variance. Data that were not normally distributed were transformed using appropriate transformations (ln, Log). General linear models were used to examine differences in post-intervention values compared to baseline values using Tukey's procedure for

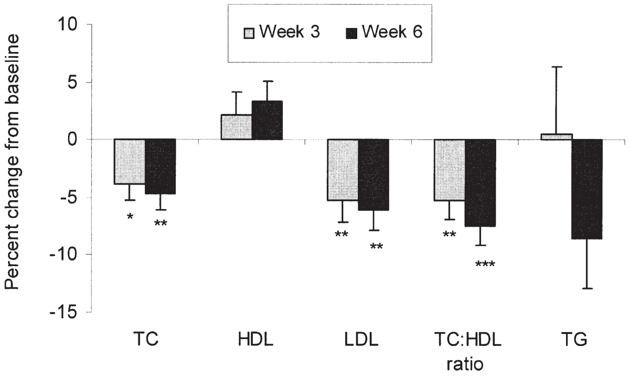

Figure. Percent change in serum lipids from baseline following consumption of phytosterol-enriched bars, at weeks 3 and 6 (mean±standard error of the mean). TC=total cholesterol. HDL=high-density lipoprotein cholesterol. LDL=low-density lipoprotein cholesterol. TG=triglycerides. *$P<0.05$. **$P<0.01$. ***$P<0.001$.

comparison of means. Comparison of serum phytosterol concentrations at week 6 was conducted using the Mann-Whitney Rank Sum Test. Differences were considered significant for all statistical analyses at $P\leq 0.05$.

RESULTS

Subject Characteristics

Seventy subjects (22 men, 48 women) completed the 6-week intervention study. Thirty-five subjects (12 men, 23 women) were assigned to the phytosterol-enriched cocoa bar product (+phytosterol bar), and 35 subjects (10 men, 25 women) consumed the phytosterol-free version of the bar (−phytosterol bar). Three subjects were removed from all analyses due to the appearance of hypertriglyceridemia (>300 mg/dL [3.38 mmol/L]) that limited the accuracy of serum cholesterol measurements. Baseline subject characteristics were similar between the groups (Table 2). There were no significant changes in body weight, body mass index, blood pressure, or heart rate in either treatment group following the 6-week intervention. Mean age of the subjects in the +phytosterol bar and −phytosterol bar groups was 49±2 and 56±2 years, respectively. Assessment of empty and unopened product packages indicated that subjects in the +phytosterol group (n=31) consumed 97% of assigned products, while subjects in the −phytosterol group (n=33) consumed 98% of products. Self-reported multivitamin use did not differ between groups; 47% of −phytosterol (17/36) and 59% of +phytosterol (19/32) subjects reported use of a daily multivitamin.

Plasma Epicatechin

Concentrations of free plasma epicatechin in a subset of subjects (n=10) following the consumption of two flavanol- and phytosterol-enriched bars were 119.5±24 nmol/L at 2 hours postconsumption and 61.1±7.8 nmol/L at 4 hours postconsumption. Plasma concentrations among subjects at 2 hours postconsumption ranged from 17.3 to 228.8 nmol/L, whereas plasma concentrations ranged from 26.1 to 102.0 nmol/L at 4 hours postconsumption.

Dietary Analysis

Three-day food records were analyzed for total energy intake, macronutrients, and selected micronutrients, then averaged over the 3-day period, at two time points throughout the study (weeks 0 and 6). One subject in the +phytosterol group reported eating a vegetarian diet. Four subjects in the −phytosterol and six subjects in the +phytosterol groups reported eating low-fat diets. There were no statistically significant changes in energy intake or macronutrient profiles of the diet during the 6-week intervention in either treatment group (Table 3). However, subjects in the +phytosterol treatment group consumed significantly less vitamin C and servings of fruit per day at week 6 vs week 0; this was not the case for the −phytosterol group. In addition, both groups reported significant reductions in β-carotene intake during the 6-week intervention period of the study; there were no significant differences between the groups.

Changes in Serum Lipids

Following the 6-week intervention, subjects who consumed the +phytosterol bar experienced a significant decrease of 4.7% in total cholesterol level ($P=0.002$), compared with baseline (Table 4). There was no change in total cholesterol in the −phytosterol bar group, after the intervention. LDL cholesterol level also decreased significantly by 6% in the +phytosterol bar group ($P<0.001$), after 6 weeks. The ratio of total cholesterol to HDL cholesterol also decreased significantly by 7.4% ($P<0.001$) following daily consumption of the +phytosterol bar for 6 weeks, compared with baseline. Changes in serum total cholesterol, LDL, and the ratio of total to HDL cholesterol were also significant at week 3 (Figure). There was no change in LDL cholesterol level, or the ratio of total to HDL cholesterol, following the 6-week intervention with the −phytosterol bar. There was no change in HDL or triglycerides following the 6-week intervention in either treatment group.

Changes in Serum Vitamins A and E and Carotenoids Level

There were no significant changes in either treatment group in serum levels of vitamins A and E, total lycopene, β-cryptoxanthin, lutein/zeaxanthin, and α-carotene following the 6-week intervention. There were also no significant changes in these lipid soluble antioxidants when adjusting for total cholesterol (Table 5). Although the two treatment groups differed significantly in serum α-carotene at each time point (and when adjusted for total cholesterol) ($P<0.05$), consumption of +phytosterol bars for 6 weeks was not associated with a significant change in serum α-carotene level. A significant reduction in serum beta carotene level was seen following 6 weeks of consumption of the +phytosterol bars (28.2±2.2 at baseline vs 23.9±2.3 μg/dL [0.53±0.04 vs 0.44±0.03 μmol] after 6 weeks, $P<0.01$); this difference was maintained when adjusted for total cholesterol (0.12±0.01 at baseline vs 0.10±0.01 μg/dL beta carotene per mg/dL total cholesterol after 6 weeks, $P<0.01$). No difference in serum beta carotene level was observed in the −phytosterol group after the 6-week intervention.

Table 5. Average concentrations of serum fat-soluble vitamins and carotenoids in the subjects at the onset of the trial and at week 6

	+ Phytosterol Bar Group		− Phytosterol Bar Group	
	Week 0	**Week 6**	**Week 0**	**Week 6**
	← *mean± SEM*^{a} →			
α-tocopherol (mg/dL)^{b}	2.1±0.2	2.1±0.1	2.0±0.1	2.1±0.1
Total cholesterol ratio^{c}	12.8±0.8	13.4±0.8	13.1±0.9	14.2±1.1
Retinol (μg/dL)	81.5±5.7	77.6±4.8	74.2±3.9	77.4±5.1
Total cholesterol ratio^{c}	0.49±0.04	0.50±0.40	0.49±0.03	0.50±0.04
α-carotene (μg/dL)^{de}	10.4±0.8^{y*}	9.9±0.7^{y*}	13.2±1.2^{z*}	14.5±1.6^{z*}
Total cholesterol ratio^{f}	0.043±0.003^{y*}	0.042±0.003^{y*}	0.057±0.005^{z*}	0.061±0.007^{z*}
β-carotene (μg/dL)^{de}	28.3±2.2^{w**}	23.9±2.3^{x**}	34.6±3.4	33.2±3.7
Total cholesterol ratio	0.12±0.01^{w**}	0.10±0.01^{x**}	0.15±0.01	0.14±0.01
Total^{g} lycopene (μg/dL)^{de}	55.4±3.4	50.7±3.2	58.5±4.3	58.6±3.5
Total cholesterol ratio	0.23±0.02	0.22±0.01	0.25±0.02	0.25±0.01
Lutein/zeaxanthin (μg/dL)^{h}	16.2±1.3	14.8±1.1	16.9±1.1	16.4±1.3
Total cholesterol ratio	0.067±0.005	0.065±0.005	0.074±0.006	0.069±0.005
β-cryptoxanthin (μg/dL)^{i}	14.7±1.1	13.7±0.9	17.4±1.6	16.2±1.5
Total cholesterol ratio	0.062±0.005	0.060±0.005	0.075±0.007	0.068±0.006

[a] SEM=standard error of the mean.

[b] n=32 in the phytosterol-enriched bar group and n=35 in the no-phytosterol-added bar group available for α-tocopherol and retinol measurements.

[c] Data were normalized to total serum cholesterol: [(mg/dL α-tocopherol or μg/dL retinol)/(mg/dL total cholesterol)].

[d] n=26 in the phytosterol-enriched bar group and n=28 in the no-phytosterol-added bar group available for serum carotenoid measurements.

[e] To convert μg/dL α-carotene, β-carotene, or lycopene to μmol/L, multiply μg/dL by 0.0186. To convert μmol/L α-carotene, β-carotene, or lycopene to μg/dL, multiply μmol/L by 53.8. α-carotene of 10.4 μg/dL=0.19 μmol/L. β-carotene of 28.3 μg/dL=0.53 μmol/L. Lycopene of 55.4 μg/dL=1.03 μmol/L.

[f] Data were normalized to total serum cholesterol: [(μg/mL carotenoid)/(mg/dL total cholesterol)].

[g] Sum of *cis*-1, *cis*-2, *cis*-3, *cis*-5, and all-*trans* lycopene serum concentrations.

[h] To convert μg/dL lutein/zeaxanthin to μmol/L, multiply μg/dL by 0.0176. To convert μmol/L lutein/zeaxanthin to μg/dL, multiply μmol/L by 57. Lutein/zeaxanthin of 16.2 μg/dL=0.29 μmol/L.

[i] To convert μg/dL β-cryptoxanthin to μmol/L, multiply μg/dL by 0.0181. To convert μmol/L β-cryptoxanthin to μg/dL, multiply μmol/L by 55.2. β-cryptoxanthin of 14.7 μg/dL=1.2 μmol/L.

[wx] Values in the same row with different superscript are significantly different.

[yz] Treatment groups are significantly different, independent of time.

*$P<0.05$.

**$P<0.01$.

Changes in Serum Phytosterol Levels

Subjects who consumed the +phytosterol bar for 6 weeks were associated with significantly higher serum campesterol and β-sitosterol concentrations at week 6 vs those who consumed the −phytosterol bar (Table 6). Serum campesterol concentrations were 1.4 times greater, on average, in subjects consuming the +phytosterol bar than the −phytosterol bar (0.99±0.17 vs 0.69±30.13 mg/dL [24.9±4.2 μmol vs 17.2±3.2 μmol], $P<0.01$), whereas serum β-sitosterol concentrations were 1.3 times greater (0.55±0.06 vs 0.41±0.04 mg/dL [13.2±1.3 μmol vs 9.9±0.9 μmol], $P<0.05$), on average. These relationships were maintained when adjusting for total cholesterol. No significant differences in serum stigmasterol concentrations were observed between the +phytosterol and −phytosterol groups following the 6-week intervention.

DISCUSSION

In a free-living population of men and women with elevated cholesterol levels, we observed a significant decrease in both total and LDL cholesterol levels, by 4.7% and 6%, respectively, following consumption of a novel chocolate snack-food product that delivered 1.5 g sterol esters per serving. In addition, the decrease in total and LDL cholesterol levels was achieved without any changes to serum levels of the fat-soluble vitamins retinol and α-tocopherol.

Our results are in accordance with previous studies that incorporated plant sterols-enriched foods into free-living diets (6,12,14,17,20,26,27), and are similar in magnitude to those reported elsewhere. In general, the consumption of 1.2 to 2.0 g plant sterols per day has consistently been shown to have a favorable effect on total cholesterol and LDL cholesterol levels. The use of sterol-enriched foods to reduce total and LDL cholesterol levels, as well as improve the ratio of LDL to HDL (17), has recently been recommended as an effective dietary strategy in the outpatient management of patients with hypercholesterolemia. Observations in free-living adults with hypercholesterolemia who consumed phytosterol for 8 weeks have reported significant decreases in total cholesterol level alone of 9.4% (26), and in both total cholesterol and LDL cholesterol levels of 7.2% and 12.4%, respectively (6). In children with familial hypercholesterolemia, consumption of plant sterols for 8 weeks significantly reduced both total and LDL cholesterol levels by 8.3% and 10.9%, respectively (14). A shorter 4-week intervention reported a significant decrease in total (6.7%) and LDL cholesterol (11.1%) levels in free-living men and women who

Table 6. Average serum phytosterol concentrations following consumption of control and phytosterol-enriched bars at the conclusion of the intervention period (week 6)[a]

	+ Phytosterol bar group	− Phytosterol bar group
	←——— *mean*±*SEM*[b]	———→
Campesterol[c] (mg/dL)	$0.99\pm0.44^{y**}$	$0.69\pm0.13^{z**}$
Campesterol/total cholesterol	$0.0040\pm0.0006^{y**}$	$0.0029\pm0.0005^{z**}$
Stigmasterol[d] (mg/dL)	0.09±0.02	0.08±0.01
Stigmasterol/total cholesterol	0.0003±0.0001	0.0003±0.0001
β-sitosterol[e] (mg/dL)	$0.55\pm0.06^{y*}$	$0.41\pm0.04^{z*}$
β-sitosterol/total cholesterol	$0.0022\pm0.0002^{y*}$	$0.0017\pm0.0001^{z*}$

[a] n=23 in the phytosterol-enriched bar group and n=28 in the no-phytosterol-enriched bar group; 54 samples were available for phytosterol analysis: three were removed as extreme outliers (>6 standard deviations from the mean). Data were analyzed using Mann-Whitney rank sum test.

[b] SEM=standard error of the mean.

[c] To convert mg/dL campesterol to μmol/L, multiply mg/dL by 24.96. To convert μmol/L campesterol to mg/dL, multiply by 0.04. Campesterol of 0.99 mg/dL=24.7 μmol/L.

[d] To convert mg/dL stigmasterol to μmol/L, multiply mg/dL by 24.1. To convert μmol/L stigmasterol to mg/dL, multiply μmol/L by 0.04. Stigmasterol of 0.09 mg/dL=2.17 μmol/L.

[e] To convert mg/dL β-sitosterol to μmol/L, multiply mg/dL by 24. To convert μmol/L β-sitosterol to mg/dL, multiply μmol/L by 0.04. β-sitosterol of 0.55 mg/dL=0.02 μmol/L.

[yz] Values in the same row with different superscript are significantly different, within treatment group.

*$P<0.05$.

**$P<0.01$.

consumed yogurt-based drinks containing plant sterols (1 g total/day) (12).

Although our primary outcome duration was 6 weeks, analyses of serum lipids at 3 weeks indicated that subjects consuming +phytosterol bars exhibited significant reductions in serum LDL (5.2%) and total cholesterol (3.8%) levels. These reductions appear in agreement with previous reports of decreases in total cholesterol (5.6%) and LDL cholesterol (8.6%) levels after 3 weeks of 1.6 g sterol ester consumption per day (5), and 6% and 5% reductions in LDL cholesterol level using either plant sterol ester- or plant stanol ester-enriched yogurts, respectively (20).

In defined dietary intervention studies (as opposed to free-living), ranging from 4 to 8 weeks, the addition of plant sterols has been reported to reduce both LDL and total cholesterol levels by approximately 4% to 15% and approximately 3% to 15%, respectively (8-11,13,28). In the only other published study in which a cocoa product (chocolate) was enriched with phytosterol, De Graaf and colleagues reported that consumption of phytosterol-rich chocolate three times per day for 4 weeks resulted in reductions in total cholesterol and LDL cholesterol levels of 6.4% and 10.3%, respectively (8). Whereas we provided an introductory nutrition education seminar (45 minutes), followed up by weekly nutrition- and health-related newsletters, De Graaf and colleagues asked their subjects to maintain lipid-lowering, American Heart Association Step 1 diets throughout the duration of their study. In addition, their subjects also received written and oral dietary counseling, twice, before dietary intervention. Therefore, the larger reductions in total and LDL cholesterol level reported by De Graaf and colleagues compared to ours may be due to a greater likelihood by study participants in their study to make healthful food choices. For example, when subjects with hyperlipidemia adhering to Step II diets were switched to vegetarian diets containing plant sterols and nuts, fibers, and soy protein, Jenkins and colleagues (7) reported substantial reductions in LDL cholesterol level of 28.2%. Thus, it is possible that greater reductions in total cholesterol and LDL concentrations would have been achieved had an American Heart Association Step I or Step II diet been followed.

Recently, Richelle and colleagues (19) reported that consumption of either free or esterified plant sterols for 1 week resulted in reduced acute bioavailability of β-carotene and α-tocopherol (19). These observations support the controversy that has existed in the plant sterol literature as to whether or not plant sterols influence fat-soluble vitamin and antioxidant status. For example, it has been reported that consumption of 2 to 3 g/day plant sitostanol esters in margarine for up to 1 year resulted in significant reductions in plasma or serum β-carotene levels after adjusting for reductions in cholesterol (29). Judd and colleagues (21) also reported that sterol ester consumption for 3 weeks on controlled diets (breakfast and dinner provided) significantly reduced plasma carotenoids and lycopene. Yet, when adjusted for total cholesterol, other researchers have reported that only β-carotene (carotenoid/total cholesterol) significantly decreased after 3 weeks of plant sterol ester intervention (2 g/day plant sterols) (20). Conversely, in children and adults with hypercholesterolemia, significant reductions in lipid-adjusted α- and β-carotene levels following an 8-week placebo-controlled intervention were reported (14). Thus, the literature at present is inconsistent. We report here that consumption of a chocolate product containing sterol esters (1.5 g) twice/day for 6 weeks (3.0 g/day) did not significantly alter plasma and serum lipid-adjusted concentrations of vitamins A and E, total lycopene, β-cryptoxanthin, lutein/zeaxanthin, and α-carotene. A significant reduction in serum beta carotene level was observed following consumption of the +phytosterol bars for 6 weeks.

It has been reported that just one additional serving per day of a high-carotenoid fruit or vegetable was able to ameliorate any reductions in plasma carotenoid concentrations induced by phytosterols (30). It is possible that increased fruit and vegetable consumption may not compensate for reductions in plasma β-carotene level due to very high intakes of phytosterols (6.6 g/day) (27). Subjects in our study consumed 3.0 g sterol esters (1.8 g free sterol equivalents) per day. Given that the self-reported fruit intake decreased significantly in the +phytosterol but not −phytosterol group during the 6 weeks, it is possible that an intake of carotenoid-rich fruit or vegetables in the +phytosterol group, comparable to the −phytosterol group, may have been sufficient to avoid the observed reductions in serum β-carotene concentrations.

The dietary intervention products used in this study also provided a rich source of the dietary flavonoids, flavanols, and proanthocyanidins, found in such foods as cocoa, red wine, and tea. Recent studies indicate that these nutrients exhibit promising effects in promoting cardiovascular health, such as improving endothelial function and providing an aspirin-like benefit (31-33). As such, foods that contain appreciable amounts of flavanols and phytosterols have the potential to not only improve indices of total and LDL cholesterol, but also provide a cardioprotective benefit independent of cholesterol reduction. We observed a significant increase in plasma flavanol metabolites following consumption of the cocoa snack foods used in this intervention, with a range of plasma concentrations between 17.3 to 228.8 nmol/L at 2 hours and 26.1 to 102.0 nmol/L at 4 hours postconsumption. Because previous studies have reported significant increases in antioxidant activity (34,35) and endothelial function (36,37), and reductions in platelet aggregation (33,38) following consumption of high-flavanol cocoa products, future studies should examine the potential for a synergistic health benefit from high-flavanol, phytosterol-enriched food products.

CONCLUSIONS

Moderate intakes of phytosterols and flavanols in a cocoa snack food appear to be an efficacious dietary-based method to lower plasma total and LDL cholesterol levels and boost plasma flavan-3-ol concentrations. Reductions in lipid-adjusted carotenoids reported here (β-carotene) and elsewhere may imply that subjects taking phytosterol products could benefit from recommendations to include carotenoid-rich foods in their diets (2,30). Further studies are needed to address the potential for beta carotene fortification of phytosterol-containing foods and determine more specific dietary recommendations to increase β-carotene consumption.

Consumption of a phytosterol-containing chocolate product, twice per day, by free-living adults with hypercholesterolemia resulted in a decrease in total and LDL cholesterol of 4.7% and 6.0%, respectively. In conjunction with a healthful diet and regular exercise, the inclusion of a novel food product, such as a chocolate product that contains plant sterols, can be a safe and effective means to lower both total and LDL cholesterol levels.

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