

Inhibition of cholesterol absorption by phytosterol-replete wheat germ compared with phytosterol-depleted wheat germ¹⁻³

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ABSTRACT

Background: Low-fat vegetable foods contain phytosterols, but it is not known whether they are in biologically active forms or whether their concentrations are high enough to reduce cholesterol absorption and favorably affect lipid metabolism.

Objective: The objective was to establish whether the selective removal of phytosterols from wheat germ would increase the cholesterol absorption measured from test meals composed of wheat germ muffins.

Design: Wheat germ, which has a high content of phytosterols relative to total fat, was chosen as a low-fat test food. Cholesterol absorption was measured 3 times in 10 subjects. Each test meal was a muffin containing 30 mg heptadeuterated cholesterol tracer and, in random order, 80 g original wheat germ containing 328 mg phytosterols, wheat germ from which phytosterols had been selectively extracted, or extracted wheat germ reconstituted with purified phytosterols. Changes in cholesterol absorption were monitored by the measurement of tracer enrichment of plasma cholesterol 4 and 5 d after each meal with the use of negative ion mass spectrometry.

Results: Tracer enrichment of plasma cholesterol was 42.8% higher after consumption of phytosterol-free wheat germ than after that of the original wheat germ (0.415 ± 0.035 compared with 0.291 ± 0.024 μmol tracer/mmol cholesterol; $P < 0.01$). Tracer enrichment of plasma cholesterol was not significantly different between the wheat germ with extracted-and-reconstituted phytosterol (0.305 ± 0.022 μmol tracer/mmol cholesterol) and the original wheat germ.

Conclusion: The efficiency of cholesterol absorption from test meals was substantially lower after consumption of original wheat germ than after consumption of phytosterol-free wheat germ, which suggests that endogenous phytosterols in wheat germ and possibly in other low-fat vegetable foods may have important effects on cholesterol absorption and metabolism that are independent of major nutrients. *Am J Clin Nutr* 2003;77:1385-9.

KEY WORDS Diet, human studies, phytosterols, sitosterol, mass spectrum analysis, deuterium

INTRODUCTION

The US National Cholesterol Education Program (NCEP) recommends that, for the reduction of cardiovascular disease risk in selected persons, phytosterols be added to the diet as a supplement in a daily dose of 2000 mg to lower LDL cholesterol (1). This consensus is based on many controlled trials in which LDL

concentrations declined by $\approx 10\%$ after phytosterol supplementation (2). However, none of those studies included a phytosterol-free control diet, which would be difficult to provide, given the presence of phytosterols in almost all unprocessed vegetable foods (3). Instead, the supplemental phytosterols were simply added to those already present in the foods of the control diets. Therefore, whereas we know that supplementation is an effective clinical treatment, it is not yet clear whether the dietary phytosterols naturally present in common foods are nutritionally important or bioactive with respect to cholesterol metabolism (4). Because the consumption of phytosterols has been estimated to be only 167-437 mg/d (4), far less than the 2000-mg dose that leads to maximum cholesterol lowering, it has been presumed that dietary phytosterols are not present in amounts high enough to appreciably influence cholesterol metabolism. However, statistically significant reduction of LDL cholesterol has been reported with supplemental daily doses as low as 800 mg (5-7), and we reported a reduction in cholesterol absorption with phytosterol doses as low as 150 mg during sterol-free meals (8). Moreover, if the relation of cholesterol lowering to phytosterol dose is sigmoidal, it is possible that natural dietary phytosterols in low concentrations might have a substantial effect on cholesterol metabolism that is currently unrecognized because the steep portion of the supplement dose-response curve is hidden by uncontrolled phytosterols present in the background diet. The evidence for and against potential effects of naturally occurring dietary phytosterols was reviewed elsewhere (9).

To test the hypothesis that the selective removal of phytosterols from test foods would increase cholesterol absorption, it is necessary to establish a phytosterol-free experimental system. We previously reported that single-meal cholesterol-absorption tests—which require only small amounts of materials and are amenable

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TABLE 1
Characteristics of the subjects¹

Race-ethnicity	
White	5
African American	3
Asian	1
Hispanic	1
Age (y)	39 ± 12 ²
BMI (kg/m ²)	26.3 ± 3.1
Cholesterol (mg/dL)	
Total	208 ± 37
LDL	134 ± 33
HDL	53 ± 18
Triacylglycerol (mg/dL)	100 ± 83

¹*n* = 5 men, 5 women.

² \bar{x} ± SD.

to precise control—can be used to measure the reduction in cholesterol absorption caused by phytosterols (10). We chose wheat germ as a test food because it is low in fat but high in phytosterols and is reported to reduce lipid absorption and cholesterol concentrations in animals (11, 12). We developed a new method to selectively extract phytosterols from wheat germ and measured the ability of intrinsic wheat germ phytosterols to reduce cholesterol absorption. On 3 occasions, normal subjects were fed test meals containing [25,26,26,26,27,27,27-²H₇]cholesterol tracer incorporated into muffins made with original wheat germ or wheat germ from which phytosterols had been selectively extracted or extracted and reconstituted. Cholesterol absorption was measured by using negative ion mass spectrometry to determine tracer enrichment of plasma cholesterol after each test.

SUBJECTS AND METHODS

Subjects

Ten healthy subjects were recruited from the staff of Washington University (Table 1). They were normolipidemic, did not have active medical or surgical illness, and did not take prescription medications (except for nonsteroidal antiinflammatory drugs or antihypertensives). Written informed consent was obtained on forms approved by the Washington University Human Studies Committee.

Materials

We purchased [25,26,26,26,27,27,27-²H₇]cholesterol from CDN Isotopes (Quebec, Canada). Soybean oil and a single lot of Kretschmer Original Toasted Wheat Germ (Quaker Oats, Chicago) were purchased in a supermarket. Wheat germ oil was obtained from Columbus Foods (Chicago). Food-grade activated carbon, SX2, was obtained from Norit Americas (Atlanta), and USP ethanol was obtained from Aaper Alcohol and Chemical Co (Shelbyville, KY).

Extraction of wheat germ

To a 4000-mL beaker we added 362 g wheat germ and 1700 mL ethanol, and the suspension was stirred at the boiling point for 30 min and then filtered through a 3500-mL coarse sintered glass funnel. The process was repeated for a total of 4 extractions, and the extracted wheat germ was dried and lyophilized. Warm ethanol treatment removes phytosterols, ethanol-soluble carbohydrates, and triacylglycerol. Ethanol-soluble carbohydrates were recovered

by distillation of the ethanol until precipitation from the concentrated oil and ethanol solution occurred. The precipitate was solubilized in water, the residual lipids were extracted with diethyl ether, and the aqueous phase was lyophilized and reconstituted in water for addition to the muffin recipe.

Purification of triacylglycerol

Phytosterols were removed from commercial wheat germ and soybean oils by a new process consisting of reversed-phase bulk adsorption to activated carbon in the presence of ethanol. Unesterified sterols were removed by solubilization in warm ethanol and adsorption to the general binding sites of activated carbon. Sterol esters (the principal form of phytosterol in wheat germ oil) were removed by hydrophobic interaction with carbon (an inexpensive, disposable, stationary phase) driven by the polar solvent ethanol. Removal of both free and esterified phytosterols proceeded simultaneously in a warm mixture of activated carbon, oil, and ethanol. In a typical preparation, 400 g activated carbon was added to 1500 mL ethanol in a 4-L beaker and warmed to boiling on a thermoregulated hotplate. Then 200 g oil was added, the contents of the beaker were mixed manually for 2 min with a spatula, and then they were filtered through a 3000-mL coarse sintered glass funnel. The carbon was removed from the funnel and thoroughly mixed with 1000 mL hot ethanol; then 200 g oil was added, and the materials were mixed for 2 min and filtered. The carbon was washed twice with 1000 mL warm ethanol with manual resuspension of the carbon each time. The ethanol phases were combined, and oil was recovered by freezing at -20 °C overnight, thawing without mixing, and pouring into a separatory funnel. Residual ethanol in the oil phase was removed at room temperature by degassing for 30 min under an increasing vacuum and then exposing the oil to a reduced pressure of 1 × 10⁻⁴ bar overnight. The yield was 54% of the original amount of the oil. Ethanol was repurified and recycled in aliquots by passing 3300 mL ethanol over 400 g fresh activated carbon in a coarse sintered glass funnel. Because of a high content of sterol esters, wheat germ oil was purified twice by this technique before it was used.

For fatty acid analysis, lipids were extracted from wheat germ with 1:2 (by vol) chloroform:methanol, and the solvent was removed. We saponified 10 mg of each type of oil in 0.4 mL of a 0.5-mol NaOH/L solution in methanol for 10 min at 65 °C and added 0.5 mL 14% (by wt) BF₃ in methanol for 5 min. The solution was cooled, 2 mL heptane and then 1 mL saturated NaCl in water were added, and the fatty acid methyl esters of the heptane phase were analyzed by gas chromatography with flame ionization detection.

Wheat germ sterols

Ethanol extracts from wheat germ were concentrated by distillation, and 50 mL of the resulting oil phase was dispersed in 500 mL ethanol and saponified with 35 mL 45% (by wt) KOH for 2 h at reflux. Water (1000 mL) was added and the sterols were extracted 3 times with 400 mL isopropyl ether. The ethereal phases were combined and washed 5 times with 350 mL water, the solvent was evaporated, and the resulting sterols were crystallized from hot 96% (by vol) ethanol and water and lyophilized.

Wheat germ muffins

Each serving contained either 80 g wheat germ (with 328 mg intrinsic phytosterols) or the following equivalent amounts of the

TABLE 2Phytosterol content of original and extracted wheat germ¹

	Original wheat germ	Extracted wheat germ
	<i>mg/g</i>	
Sitosterol	3.04 ± 0.15	0.03 ± 0.00
Campesterol	1.02 ± 0.04	0.01 ± 0.00
Stigmasterol	0.04 ± 0.00	0.01 ± 0.00
Total phytosterol	4.10 ± 0.19	0.05 ± 0.01

¹ $\bar{x} \pm \text{SEM}$ for triplicate samples.

components of extracted wheat germ: 62.6 g dry extracted wheat germ, 2.1 g water, 6.1 g ethanol-soluble carbohydrate, and 8.3 g purified wheat germ oil. Deuterated cholesterol tracer (30 mg) was added to all recipes in 11.8 g soybean oil. In some experiments, purified phytosterols were added back to the muffins by inclusion of 164 mg in the wheat germ oil and 164 mg in the soybean oil. The muffins contained 80 g wheat germ (or an equivalent amount), 30.6 g white sugar, 12.9 g brown sugar, 1.6 g salt, 2.4 g baking powder, 1.4 g vanilla extract, 108.7 g skim milk, 8.5 g egg white, and 11.8 g purified soybean oil; the muffins were baked in individual serving pans at 191 °C for 40 min. The meal contained 74 g carbohydrate (50% of energy), 20 g fat (30%), and 117 g protein (20%) with 2473 kJ energy, 12.3 g fiber, <2 mg cholesterol (exclusive of the cholesterol tracer), and <3 mg phytosterols (exclusive of that in wheat germ and its components).

Clinical protocol

The 10 subjects underwent 3 cholesterol-absorption tests in random order at intervals of 2 wk. For each test, they reported to the General Clinical Research Center after an overnight fast, and a baseline plasma sample was drawn. A breakfast consisting of a wheat germ muffin was consumed, and plasma samples were drawn 4 and 5 d later and combined for analysis.

Analyses

The plasma samples were saponified, and cholesterol was extracted and derivatized as described previously (8, 13). Pentafluorobenzoyl esters of cholesterol underwent chromatography on a trifluoropropylmethyl polysiloxane column (RTX-200, 15 m × 0.25 mm inside diameter, 0.5-mm film thickness; Restek, Bellefonte, PA) and were analyzed on a quadrupole mass spectrometer (HP 5973; Hewlett-Packard, obtained from Agilent Technologies, Palo Alto, CA) in negative ion chemical ionization mode with an ion source temperature of 160 °C and methane reagent gas flow at 0.75 mL/min. Plasma enrichment was computed by reference to a standard curve and was expressed as μmol tracer/mmol cholesterol (8). The overall significance of differences between treatment groups was computed with repeated-measures analysis of variance with the use of SAS software (SAS Institute Inc, Cary, NC). The significance of comparisons between individual pairs of conditions was determined with the use of paired *t* tests with Bonferroni correction.

RESULTS

Wheat germ contained 10.3% fat by wt and 4.1 mg phytosterols/g (Table 2). After 4 extractions with warm ethanol, the phytosterol content of wheat germ was <2% of the original value.

TABLE 3Fatty acid content of wheat germ and wheat germ oils¹

Fatty acid	Original wheat germ	Original wheat germ oil	Purified wheat germ oil
	<i>mol%</i>		
14:0	0.12 ± 0.00	0.11 ± 0.00	0.11 ± 0.00
16:0	19.52 ± 0.05	18.27 ± 0.06	18.08 ± 0.02
16:1	0.23 ± 0.01	0.20 ± 0.00	0.21 ± 0.00
18:0	0.77 ± 0.01	0.79 ± 0.00	0.70 ± 0.00
18:1	17.38 ± 0.02	16.58 ± 0.04	18.14 ± 0.03
18:2	55.71 ± 0.07	57.50 ± 0.11	57.08 ± 0.05
18:3	6.27 ± 0.01	6.57 ± 0.01	5.69 ± 0.02

¹ $\bar{x} \pm \text{SEM}$ for triplicate samples.

To investigate the effect of endogenous wheat germ phytosterols on cholesterol absorption, we reconstituted the extracted wheat germ with the carbohydrates and triacylglycerol that had been removed in the extraction process. However, before reconstitution, both free and esterified phytosterols were removed from the triacylglycerol by bulk reversed-phase adsorption to activated carbon in the presence of ethanol, as described above. The original wheat germ, the wheat germ oil used as a source of wheat germ triacylglycerol, and the purified wheat germ oil used for reconstitution had very similar fatty acid compositions, as shown in Table 3. Purification was efficient, with removal of >99% of the total phytosterols from wheat germ oil, as shown in Table 4. This degree of purity reflects 2 charcoal-adsorption steps.

The effect of wheat germ phytosterols on the efficiency of cholesterol absorption was determined in 10 subjects undergoing 3 cholesterol-absorption tests, each one consisting of the consumption of a muffin made with either original wheat germ (328 mg intrinsic phytosterols), phytosterol-free wheat germ (<6 mg phytosterols), or extracted wheat germ reconstituted with purified phytosterols (328 mg phytosterols). The cholesterol tracer concentrations achieved in plasma from the 30 mg heptadeuterated cholesterol included in each muffin are shown in Figure 1. Repeated-measures analysis of variance showed a strong overall effect of wheat germ type on cholesterol absorption within the subjects ($P = 0.0002$). The efficiency of cholesterol absorption was 42.8% greater with phytosterol-free wheat germ than with original wheat germ (0.415 ± 0.035 compared with 0.291 ± 0.024 μmol tracer/mmol cholesterol; $P < 0.01$). There was no significant difference in the efficiency of cholesterol absorption between the original wheat germ and the extracted wheat germ reconstituted with phytosterols (0.291 ± 0.024 compared with 0.305 ± 0.022 μmol tracer/mmol cholesterol; $P = 0.54$ uncorrected).

TABLE 4Phytosterol content of original and purified wheat germ oil¹

	Original wheat germ oil	Purified wheat germ oil
	<i>mg/g</i>	
Sitosterol	54.56 ± 1.46	0.32 ± 0.01
Campesterol	11.02 ± 0.33	0.06 ± 0.00
Stigmasterol	0.33 ± 0.01	0.00 ± 0.00
Total phytosterol	65.91 ± 1.80	0.38 ± 0.01

¹ $\bar{x} \pm \text{SEM}$ for triplicate samples.

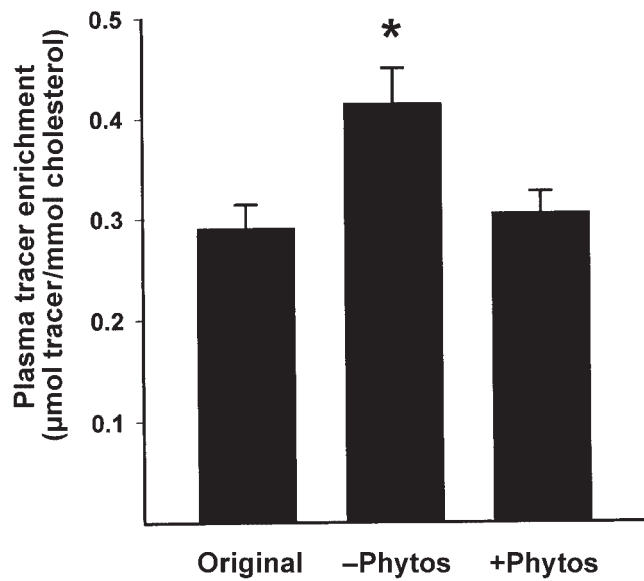


FIGURE 1. Mean (\pm SEM) tracer enrichment in plasma cholesterol after consumption of muffins made with original wheat germ, wheat germ from which phytosterols had been selectively extracted (-Phytos), or extracted wheat germ reconstituted with purified phytosterols (+Phytos) ($n = 10$). *Significantly different from original or +Phytos, $P < 0.01$ (paired t test with Bonferroni correction).

DISCUSSION

The present study shows that phytosterols intrinsic to wheat germ are biologically active and have a prominent role in reducing cholesterol absorption. In general, phytosterols do not completely block cholesterol absorption. Maximum doses of sterol or stanol supplements reduce cholesterol absorption in healthy subjects by 30–43% (10, 14, 15). The 42.8% reduction observed here when phytosterols were present in a wheat germ muffin breakfast is therefore within the range of maximum responses given by supplements. The phytosterol dose used, 328 mg, is also low compared with recommended supplement doses of 2000 mg. Although our dose was given at a single time rather than being divided for ingestion with each meal, recent work has shown that phytosterol effects appear to be long-lasting and that once-daily dosing is as effective as is dosing with each meal (16). This suggests that phytosterols in natural wheat germ may be at least as effective as phytosterols in supplements. We have not studied lower doses of wheat germ, but it is possible that such doses might also have some effect on cholesterol absorption: previous work showed that amounts as low as 150 mg in corn oil have a measurable effect in single-meal tests (8). Taken together with our present work, these data strongly suggest that phytosterols in the normal diet can be nutritionally important with respect to cholesterol absorption. Because most of the cholesterol absorbed by humans is recirculating endogenous biliary cholesterol, an effect of natural food phytosterols of reducing cholesterol absorption would be expected to lower serum cholesterol regardless of the amount of dietary cholesterol taken in (17).


Although the information available is sparse, previous work established that wheat germ feeding improves plasma lipoproteins in animals and possibly in humans. In rats, the addition of 7% wheat germ to a high-fat, high-cholesterol diet reduced VLDL

cholesterol (a major apolipoprotein B-containing lipoprotein fraction in the rat) by 37.9% (12). The inclusion of wheat germ in a test meal reduced plasma chylomicron cholesterol concentrations by 27.1% over several hours in 6 normolipidemic subjects (18). These data suggest that wheat germ may lower circulating cholesterol or at least delay the absorption of cholesterol.

The mechanism of cholesterol lowering by wheat germ is not known. Because wheat germ contains fiber, it has been thought that some effects on cholesterol metabolism might be mediated by dietary fiber (18). However, several other mechanisms have been proposed. In rats, the absorption of labeled triacylglycerol and cholesterol was both delayed and reduced by wheat germ and other wheat fractions in part as a result of the inhibition of pancreatic lipase and the reduction in triacylglycerol lipolysis (11). Soluble protein components of wheat germ are known to inhibit pancreatic lipase activity (19), and partially defatted wheat germ increases the fecal excretion of bile acids in rats (20). Therefore, the mechanism of reducing cholesterol by the use of wheat germ is likely to be complex. Our work extends these studies by showing that bioactive phytosterols are an additional mechanism by which wheat germ could lower cholesterol absorption and potentially improve cholesterol concentrations. Whether the ethanol extraction process used here alters nonlipid components of wheat germ is not known.

Further testing of the effectiveness of natural dietary phytosterols will require comparison diets that are deficient in phytosterols. New methods are needed for removing phytosterols from foods and oils on a kilogram scale so that the effects of the phytosterols can be cleanly separated from those of other components. We show here that phytosterols can be extracted efficiently from solid foods with warm ethanol and that the resulting vegetable oils can be purified in bulk by reversed-phase adsorption to activated carbon in the presence of ethanol. Both free and esterified cholesterol are quantitatively removed. The most difficult aspect of oil purification is the removal of long-chain sterol esters because their volatility and polarity are similar to those of triacylglycerol. We previously reported that this separation can be done on a kilogram scale with the use of hexane and silica (8). The present ethanol-and-charcoal reversed-phase adsorption method is easier and cheaper to use and has less potential for toxicity. Reversed-phase chromatography is generally performed with the use of an expensive solid phase consisting of hydrophobic octadecyl groups chemically bound to silica. Lipophilic molecules such as phytosterol esters are forced to bind to the solid phase by inclusion of polar solvents in the liquid phase. We report here that activated carbon, which is cheap and disposable, can be substituted for octadecyl silica and that carbon is sufficiently hydrophobic to allow phytosterol ester binding with only ethanol as a polar solvent. The expense and time needed to recycle spent octadecyl silica are not needed, and the least toxic organic solvent, USP ethanol, can be used. This method shows promise for the purification of vegetable oils for use in dietary studies.

The principal finding of this study is that endogenous phytosterols that are present in an intact, low-fat vegetable food may favorably alter cholesterol absorption. Further work is needed to determine whether this effect has clinical utility and can lower LDL-cholesterol concentrations. If similar results are obtained for other low-fat foods, it will be necessary to investigate the possibility that small amounts of naturally occurring phytosterols in the diet might have important effects on cholesterol metabolism that have been overlooked. In that case, natural dietary phytosterols

would be an additional and badly needed tool for reducing LDL-cholesterol concentrations in the population with minimal side effects and risk. 

Rita Telken and Marilyn Tanner developed the wheat germ muffins, and Laura Kobayashi and Stephen Black provided valuable technical assistance.

All authors participated in the design of the study, the analysis of the data, and the writing of the manuscript. REO was responsible for data collection. None of the authors had a financial interest in wheat germ or in companies that sell wheat products.

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