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Free phytosterols facilitate excretion of endogenous cholesterol in gerbils☆

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Abstract

To determine whether phytosterols (PST) facilitate excretion of whole body cholesterol and whether dietary fat or enhancing gallbladder contraction with curcumin might influence this process, four experiments were conducted in gerbils. In Experiment 1, naïve gerbils received cholesterol-free purified diets with 30% energy from fat and 0% or 0.75% free PST from tall oil for 4 weeks. In Experiment 2, body cholesterol pools were expanded by feeding a diet containing 0.3% cholesterol for 3 weeks. Subsequently, PST was provided in either fat-free or normal-fat diets without cholesterol for only 2 h each morning, followed by a low-fat diet for the rest of the day and food restriction overnight. In Experiment 3, gerbils were preloaded with cholesterol, followed by either PST alone or PST+curcumin to enhance gallbladder contraction. In Experiment 4, curcumin or curcumin+PST were fed with 30% as fat and 0.15% cholesterol throughout the study. Because of the small whole body cholesterol pool in Experiment 1, the impact of PST was limited. When whole body cholesterol was expanded in Experiments 2 and 3, subsequent reductions of liver esterified cholesterol by PST were significant. In the presence of dietary fat, PST caused a greater reduction (23%) than in a fat-free diet (8%) compared to respective controls. Curcumin (Experiments 3 and 4) proved ineffective in reducing liver or plasma cholesterol pools, and the 3:1 ratio between PST/diet cholesterol was less effective at blocking cholesterol absorption than a 5:1 ratio previously employed. Thus, free PST removed whole body cholesterol, which was enhanced by concomitant fat intake, but was unaffected by a gallbladder contracting agent.

Keywords: Phytosterols; Curcumin; Liver cholesterol; Plasma cholesterol; Fecal cholesterol; Gerbils

1. Introduction

Phytosterols (PST) are a potentially important dietary adjunct that can contribute to a program of plasma cholesterol reduction. Phytosterols reduce low-density lipoprotein cholesterol (LDL-C) about 10–15% in most people, or even more in some hypercholesterolemic individuals, when consumed at 1.5–2 g/day [1,2]. Previous experiments in gerbils demonstrated that 0.75% PST heat-dissolved in fat provided almost total protection against any increase in body cholesterol pools induced by a diet containing 0.15% cholesterol, that is, in a 5:1 ratio [3]. One unanswered question is how effectively PST can reduce cholesterol in hypercholesterolemic subjects who have already removed cholesterol from their diet, that is, can PST effectively remove endogenous biliary cholesterol resulting in lower plasma cholesterol, or does it mainly protect against absorption of dietary cholesterol? It is thought that esterified PST can remove considerable biliary cholesterol as it fluxes through the intestine [4], but whether free PST can achieve this is unknown. A second question is whether simultaneous intake of fat with PST enhances cholesterol removal. Most reports on the subject indicate that PST must be solubilized in fat to be effective [5], so we tested the interaction requirement for free PST in this regard. A third question is whether less than a 5:1 ratio of PST/diet cholesterol will effectively block cholesterol absorption, so we examined a 3:1 ratio. Finally, we asked whether inducing gallbladder contraction, with a contracting phytochemical like curcumin [6], might facilitate PST action by releasing more bile to increase micelle formation.

To address these points, we developed an animal model with an expanded whole body cholesterol pool. Gerbils were selected because they are sensitive to dietary choles-

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terol [7] and had previously revealed effective blockade of diet cholesterol absorption by free PST [3].

2. Materials and methods

2.1. Animals and diets

Ninety 5–6 weeks old, male, Mongolian gerbils (*Meriones unguiculatus*), Charles River Labs, Wilmington, MA) were studied in four experiments, which were approved by Brandeis Animal Care and Use Committee. In each experiment, gerbils were randomly assigned to groups (six to eight per group) and fed for 3–5 weeks a cholesterol-free, purified diet containing 0% or 0.75% of nonesterified (free) PST derived from tall oil (Reducol, provided by Novartis Consumer Health, Nyon, Switzerland) or 0.2% curcumin from *Curcuma longa* (Tumeric), obtained from Sigma (St. Louis, MO). The basal composition of the semipurified gerbil diets was (g/kg dry weight): casein, 200; dextrose, 200; cornstarch, 298; cellulose, 100; fat, 137 (a blend of 59% coconut oil or cholesterol-stripped milk fat+31% canola oil+10% soybean oil); mineral mix (Ausman-Hayes), 50; vitamin mix (Hayes-Cathcart), 12; and choline chloride, 3. Compositions of mineral and vitamin mixes have been reported previously [8]. Cholesterol, PST (Reducol, 80% sterols, 20% stanols) and curcumin were added to the diets at the expense of cornstarch. Reduction of fat was accomplished by substituting cornstarch. Cholesterol and PST were thoroughly dissolved in the fat portion of the mix by heating, whereas curcumin was added to the dry portion of the mix. The dry mix was made into a starch gel by adding starch to simmering water (800 ml/kg dry mix) and stirring into the dry mix as described previously [8].

Gerbils were housed two to three per cage and kept in a controlled environment with a 12-h light/dark cycle with free access to water. During the fourth week (in Experiment 3), feces were collected in individual wire-bottom cages, where indicated. At the end of each experiment, gerbils were food-deprived overnight (16 h), and blood was collected under O_2/CO_2 anesthesia with an EDTA-wetted syringe by cardiac puncture. After exsanguination, the liver, cecum, and adipose tissue were excised and weighed. A portion of each liver was stored at −20°C until analyzed. Plasma was separated from EDTA-treated blood by centrifugation at 12,000×*g* for 15 min and analyzed as detailed later.

2.2. Study designs

2.2.1. Experiment 1

The first experiment was designed to determine whether free PST added to a cholesterol-free diet would reduce plasma or liver cholesterol in a naïve gerbil, that is, is it possible to shrink the endogenous cholesterol pool by supplementing PST when no diet cholesterol is present? The assumption was that PST might block reabsorption of biliary cholesterol, or even bile acids. Gerbils were fed a basal cholesterol-free diet with or without 0.75% PST for 4 weeks prior to blood and tissue sampling.

2.2.2. Experiment 2

The second study used a gerbil model with an expanded whole body cholesterol pool to test the efficacy of its reduction by PST, such as might occur in a hypercholesterolemic person trying to reduce cholesterol with a cholesterol-free diet containing PST. The endogenous body pools of cholesterol were artificially expanded for 3 weeks with 0.3% cholesterol in the basal purified diet, prior to feeding specific *cholesterol-free* test diets for an additional 3-week period. In this second period, four diet groups explored the effect of dietary fat and PST on liver and plasma cholesterol pools. The design for all four groups incorporated overnight food restriction (5 p.m. to 9 a.m. each day) to insure aggressive consumption for 2 h (9–11 a.m.) of diets containing 0% or 30% energy from fat with 0% or 0.75% PST. To avoid essential fatty acid deficiency in gerbils consuming the two fat-free diets (between 9 and 11 a.m.), all 4 groups were fed a common, low-fat diet providing 3% energy from safflower oil between 11 a.m. to 5 p.m., when overnight food restriction was re-instituted. Thus, the 9–11 a.m. test diets (cholesterol-free) provided the only source of PST and a variable source of fat as follows: Group 1 (control) received a fat-free diet with neither cholesterol nor PST (fat-free, no PST). Group 2 (fat-free, +PST) received the same test diet as Group 1, but 0.75% PST was mixed directly into the dry diet (before adding water gel) to determine whether PST in a fat-free morning diet would also enhance whole body cholesterol removal. Group 3 was similar, except the morning diet contained 30% fat energy (fat, no PST) to determine whether the fat bolus alone (gallbladder flush) might reduce body reserves of cholesterol better than the fat-free diet alone. Group 4 was treated like Group 3, but received the normal-fat diet plus 0.75% PST (fat, +PST) between 9 and 11 a.m. to determine whether fat would enhance the activity of PST.

2.2.3. Experiment 3

The third experiment was designed to increase gallbladder contractions beyond those resulting from the morning fat bolus in Experiment 2. Such a stimulus (in this case, either all-day fat exposure or added curcumin) might improve PST action by enhancing secretion of bile into the gut, where bile cholesterol might be removed by PST. In order to shorten the preloading time, gerbils were fed a diet with 0.4% cholesterol for only 2 weeks. Thereafter, they consumed one of three diets for 3 weeks: (i) a cholesterol-free control (no PST, no curcumin); (ii) control with 0.75% PST; or (iii) control+0.75% PST+0.2% curcumin. Unlike Experiment 2 with its complicated diet switching, Experiment 3 continuously fed a single diet with 30% energy as fat that contained the designated supplements.

2.2.4. Experiment 4

The fourth experiment assessed the action of curcumin alone in addition to its potential interaction with PST. In contrast to the 'preload design' used in Experiment 3 that expanded endogenous cholesterol pools, 0.15% cholesterol was fed throughout the 4-week study to naïve gerbils in diets providing 30% energy from fat. Group 1 had neither PST nor curcumin. Group 2 provided PST at only 0.45%, so that the ratio of PST/cholesterol was reduced to 3:1 from the previously used 5:1 ratio. We reasoned that excess PST (i.e., at 0.75%) might provide no leeway to demonstrate improvement by a cofactor, that is, curcumin. Group 3 had curcumin alone at 0.2%, whereas Group 4 provided both PST and curcumin. Because no cholesterol preload was used, the design focused on whether curcumin might block cholesterol absorption on its own and/or contract the gallbladder to release more bile and biliary cholesterol to augment the PST blockade of cholesterol absorption.

2.3. Plasma lipid analysis

Plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were measured by enzymatic assay: TC and TG assays using Sigma kits #362 and 336, respectively (Sigma). HDL-C was assayed in the supernatant after sodium phosphotungstate-Mg^{2+} precipitation of apoE- and apoB-containing lipoproteins with reagent #543004 (Boehringer Mannheim Diagnostic, Indianapolis, IN) according to the procedure described by Weingard and Daggy [9].

2.4. Lipoprotein profiles

Plasma lipoprotein fractions were isolated from gerbil plasma by discontinuous density gradient ultracentrifugation according to the method of Goulinet and Chapman [10]. Ultracentrifugation was performed using a SW41-Ti rotor (Beckman) in a Beckman L8-55 ultracentrifuge at 36,000 rpm at 15°C for 48 h. Following the spin, lipoprotein fractions were collected at preestablished densities: very low-density lipoprotein (VLDL) $d<1.006$ g/ml, LDL $1.006<d<1.063$ g/ml and HDL $1.063<d<1.21$. Cholesterol content of each fraction was determined using Sigma kit #362.

2.5. α-Tocopherol analysis

Plasma α-tocopherol levels were assayed using high-performance liquid chromatography (HPLC) according to the method of Bieri et al. [11]. Standards (D-α-tocopherol and D-α-tocopheryl acetate) were obtained from Hoffman-La Roche (Nutley, NJ). In brief, plasma was mixed with ethanol solution and with internal standards (D-α-tocopheryl acetate), then extracted with hexane. A portion of hexane extract was evaporated under nitrogen and after dissolving in methanol injected into a C18 reverse-phase, 150×4.6 mm HPLC column (Supelcosil, LC-18, 5-μm particles; Supelco, Bellefonte, PA). The mobile phase (methanol/water, 96:4) was delivered at a flow rate of 2.0 ml/min (model 110 A pump; Beckman Instruments, Berkeley, CA). Retinol and α-tocopherol were detected at 290 nm in a Beckman UV detector and their concentrations were qualified in relation to the internal standards.

2.6. Hepatic β-carotene analysis

Liver β-carotene concentration was determined according to the HPLC method described by Pollack et al. [12].

2.7. Hepatic cholesterol analysis

Liver cholesterol was extracted with 2:1 chloroform/methanol and free cholesterol (FC) and esterified cholesterol (EC) was determined by HPLC [13]. Free cholesterol and cholesteryl esters were separated using a Waters Radial-Pack C18 column (8 mm×10 cm, 10 μm) eluted isocratically with acetonitrile/isopropanol (50:50 by volume) at 2.0 ml/min. Absorbance of the eluate was measured at 210 nm using a UV detector. Cholesterol (free and individual esters) concentrations were calculated by comparing the peak area of samples with those obtained for the standards (Sigma). To calculate EC the sum of cholesteryl esters was divided by 1.67 (calculation according to Witztum et al. [14].

2.8. Fecal sterol analysis

Sterol content of feces was determined by GLC after direct saponification with 0.5 M methanolic KOH according to the method of Ntanios and Jones [15]. Sterol analysis was performed by GLC equipped with a capillary column (XTI-5, 30-m length, 0.25 mm ID, 0.50 μm df, Baxter, IL) with helium as the carrier gas. Carrier flow was set at 2 ml/min with 20:1 split ratio. The oven temperature was set at 280°C with injector and detector temperature at 300°C and 280°C, respectively. Sample sterols were identified by comparison with authentic sterol standards (Sigma).

2.9. Statistical analysis

Statistical analysis was performed utilizing the Super ANOVA statistical software package (Abacus Concepts, Berkeley, CA). Comparisons between diets in Experiment 1 were assessed with Student's t test, while those in Experiments 2 and 4 used a two-way analysis of variance (ANOVA). Experiment 3 used one-way ANOVA. Differences were considered significant at $P<.05$.

3. Results

3.1. Experiment 1

Although plasma cholesterol (TC) was reduced 12% and liver EC by 27% in these naïve gerbils supplemented with PST for 4 weeks, neither trend was significant due to the variation and limited number of gerbils per group (Table 1).

3.2. Experiment 2

Preloading gerbils for 3 weeks with 0.3% diet cholesterol succeeded in expanding the plasma TC and hepatic EC

pools (Table 2), the latter to 207 μmol/g (basal level <10 μmol/g). Total liver weight was unaffected by PST, but was increased in the two groups of gerbils fed the fat-free meals each morning. After three additional weeks without dietary cholesterol, all groups revealed a markedly lower plasma TC. Interestingly, the smallest decline (−37%) was associated with the normal-fat, no PST diet, while the greatest plasma TC decrease (−53%) occurred with normal-fat, +PST diet. The two fat-free diets were intermediate in their effectiveness, but the two diets containing PST lowered plasma TC more than those without PST, being about 7% lower for the fat-free diet (n.s.) and 26% lower for the normal-fat diet ($P<.007$). The plasma TG and liver EC showed similar trends, with PST lowering concentrations independent of any fat effect.

3.3. Experiment 3

The gerbils in Experiment 3, receiving a normal-fat diet ad libitum, grew slightly better without the complicated diet manipulations of Experiment 2, which involved overnight diet restriction and multiple diet switching during the day. Data for growth and organ weights were comparable between groups. The preloading of the liver and plasma cholesterol pools with 0.4% dietary cholesterol for 2 weeks markedly elevated plasma TC and liver EC (Table 3).

All dietary treatments reduced these expanded pools of endogenous cholesterol over the next 3 weeks. The control diet alone (no cholesterol or PST) reduced plasma TC about 50% from the 2-week preload. Adding PST alone further enhanced the TC decline about 20%, while the addition of PST+curcumin was only slightly better (Table 3). Both treatments produced significantly lower values than the control. Also, PST+curcumin produced the best TC/HDL ratio, which was about 20% lower than the control. Both PST and PST+curcumin reduced the liver EC to about half the final control concentration. Even the control liver revealed a reduction to about one third the level observed following the 3-week preloading period. Fecal analysis revealed that PST greatly increased the output of cholesterol and plant sterols without affecting bile acid excretion (Table 4).

Table 1
Effect of free PST on plasma and liver lipids in gerbils fed with cholesterol-free diet (Experiment 1)

	Diets groups	
	0% PST	0.75% PST
Body weight (g)		
Initial	54±1	53±2
Final (after 4 weeks)	67±4	65±5
Organ weights (%BW)		
Liver	2.56±0.15	2.48±0.20
Cecum	2.02±0.36	1.94±0.38
Adipose	0.24±0.7	0.19±0.10
Plasma (mmol/L)		
TC	1.9±0.3	1.7±0.2
HDL-C	1.3±0.3	1.2±0.2
TC/HDL-C ratio	1.46±0.22	1.40±0.10
TG	0.3±0.1	0.3±0.1
Liver cholesterol (μmol/g)		
TC	22±4	20±4
FC	13±2	13±1
EC	9±2	7±3

Values are mean±S.D. ($n=6$).

Table 2
Liver and plasma lipids of gerbils preloaded with 0.3% cholesterol and subsequently fed a cholesterol-free diet with or without PST in a no-fat or high-fat bolus for 2 h daily (Experiment 2)

	Diet groups			
	No fat	No fat+ 0.75% PST	Fat	Fat+ 0.75% PST
Body weight (g)				
Initial	50±2	51±2	51±2	51±2
Mid (after 3-week cholesterol loading)[a]	63±5	62±3	62±2	62±5
Final (after 6 weeks)	64±3	62±2	64±4	63±5
Organ weights (%BW)				
Liver[b]	3.56±0.28	3.51±0.24	3.17±0.29	3.07±0.13
Adipose	0.31±0.18	0.10±0.07	0.27±0.24	0.33±0.14
Cecum	3.37±0.31	3.34±0.56	3.19±0.62	2.88±0.53
Plasma (mmol/L)				
TC				
After 3-week cholesterol loading[a]		7.5±0.7		
6 Weeks[c]	4.2 ± 1.3	3.9±0.3	4.7±0.7	3.5±0.3
TG				
After 3-week cholesterol loading[a]		1.1±0.2		
6 Weeks	1.2±0.5	0.8±0.3	1.3±0.7	0.9±0.2
Liver cholesterol (μmol/g)				
TC				
After 3-week cholesterol loading[a]		207±1		
6 Weeks[c]	119±16	111±16	129±28	101±21
FC				
After 3-week cholesterol loading[a]		16 ±0		
6 Weeks	13±2	13±1	14±1	14±2
EC				
After 3-week cholesterol loading[a]		191±1		
6 Weeks[c]	106±13	98±13	114±28	88±21

Values are means±S.D. ($n=6$, except at 3 weeks after cholesterol loading, $n=2$).

[a] Fed diet with high content of cholesterol (0.3%) for 3 weeks.

[b] $P<.05$, significant effect of fat revealed by two-way ANOVA.

[c] $P<.05$, significant effect of phytosterol revealed by two-way ANOVA.

3.4. Experiment 4

PST and PST+curcumin lowered LDL-C without decreasing HDL-C (by ultracentrifugation), so the LDL/HDL ratio was lower as well. Curcumin alone had no effect on plasma cholesterol values or liver EC, but it did cause a significant reduction in the LDL/HDL ratio (Table 5). Furthermore, LDL from curcumin groups tended to have higher vitamin E/LDL-C ratios. Curcumin alone had no effect on cholesterol

Table 3
Liver and plasma lipids of gerbils preloaded with 0.4% cholesterol and subsequently fed cholesterol-free diets with or without PST and curcumin (Experiment 3)

	Diet groups		
	Control (0% PST)	0.75% PST	0.75% PST+0.2% curcumin
Body weight (g)			
Initial	56±2	55±2	55±1
Mid (after 2-week cholesterol loading)*	66±3	67±2	63±2
Final (after 5 weeks)	69±5	70±5	70±3
Organ weights (%BW)			
Liver	3.50±0.19	3.35±0.33	3.34±0.19
Cecum	2.58±0.49	2.97±0.74	2.86±0.32
Adipose	0.56±0.22	0.59±0.14	0.50±0.10
Plasma (mmol/L)			
TC			
After 2-week cholesterol loading*		7.4±1.0	
5 Weeks	3.7±0.4^{a,b}	3.0±0.3^{a}	2.7±0.3^{b}
HDL-C			
5 Weeks	2.0±0.4	1.8±0.2	1.8±0.2
TC/HDL-C ratio			
5 Weeks	1.89±0.35^{a}	1.63±0.17	1.50±0.16^{a}
TG			
After 2-week cholesterol loading*		0.8±0.1	
5 Weeks	0.6±0.1	0.5±0.2	0.5±0.4
Liver cholesterol (μmol/g)			
TC			
After 2-week cholesterol loading*		194±54	
5 Weeks	85±21^{a,b}	39±8^{a}	49±8^{b}
FC			
After 2-week cholesterol loading*		18±1	
5 Weeks	13±1	13±1	12±1
EC			
After 2-week cholesterol loading*		176±54	
5 Weeks	70±21^{a,b}	26±8^{a}	36±8^{b}

Values are means±S.D. (n=7, except at 2 weeks after cholesterol loading, n=2). ^{a,b}Means in a row sharing a common superscript are significantly different (P<.05) using one-way ANOVA and Scheffe's F test.

* After 2 weeks on diet with high content of cholesterol (0.4%).

absorption because the liver contained the same EC content as the control (with no curcumin or PST).

4. Discussion

The objective of these studies was to determine whether PST would reduce whole body cholesterol (as opposed to simply blocking dietary cholesterol absorption), both in a naïve model fed with no diet cholesterol and under similar diet conditions after expanding the whole body cholesterol pool. The first experiment tested the naive model and was not definitive, probably because the model was wanting and the number of gerbils was small. That is, with neither dietary cholesterol nor an expanded whole body pool of cholesterol, PST had minimal opportunity to demonstrate its functional capacity for blocking absorption. Nonetheless, a tendency for PST to lower both plasma and liver cholesterol was apparent (P<.11 and P<.09, respectively), suggesting that it might reduce whole body cholesterol if conditions were right. The minimal effect of PST here may also reflect the fact that the content of biliary cholesterol in gerbils, and a potential source for removal, is much less in chow-fed gerbils compared to humans (unpublished data). By comparison, normolipemic humans with inherently higher biliary cholesterol than gerbil bile have been observed to lower LDL-C when supplemented with PST [4,16].

The second experiment tested the model of an expanded whole body cholesterol pool. It proved effective and unique because PST facilitated cholesterol removal in the absence of dietary cholesterol. The objective was to remove excess body stores of cholesterol by enhancing bile secretion, as opposed to the typical PST intervention designed to prevent cholesterol absorption primarily from dietary sources. The design attempted to modulate intestinal CCK and bile excretion by feeding either fat-free (low CCK release and bile secretion) or fat-containing (normal bile secretion) diets with exposure to PST for 2 h each morning. The fat-free diet tended to be less effective than the normal-fat diet for removing whole body cholesterol, but in these gerbils with expanded cholesterol pools, hepatic EC declined substantially (about 40%) by simply feeding the cholesterol-free diets with or without fat (i.e., the control groups). Thus, fat intake had less effect than the simple removal of diet cholesterol, demonstrating the inherent ability of the gerbil to shed excess body cholesterol when fed these purified diets, even without PST.

Furthermore, the overall response in TC and hepatic cholesterol among all four groups in Experiment 2 indicated a greater differential for PST action between the two normal-fat diets than the two fat-free diets, that is, the greatest reduction in cholesterol occurred when PST was added with

Table 4
Effect of PST alone or with curcumin on fecal excretion of sterols and bile acids (Experiment 3)

	Diet groups		
	0% PST	0.75% PST	0.75% PST+0.2% curcumin
Feces excretion			
Dry weight (g/day)	0.74±0.12	0.72±0.28	0.84±0.10
Fecal sterols (μmol/day)			
Cholesterol	0.8±0.3^{a,b}	4.8±1.5^{a}	6.1±0.8^{b}
Total neutral sterols	1.1±0.4^{a,b}	5.1±1.6^{a}	6.3±0.7^{b}
Campesterol	0.6±0.3^{a,b}	9.2±3.5^{a}	11.2±1.7^{b}
Stigmasterol	0.1±0.1^{a,b}	0.8±0.3^{a}	0.9±0.1^{b}
Beta-sitosterol	0.9±0.4^{a,b}	24.8±9.2^{a}	30.5±4.8^{b}
Campestanol	ND	2.5±1.3	3.1±0.4
Sitostanol	0.05±0.05^{a,b}	18.5±5.3^{a}	20.9±2.9^{b}
Total plant sterols	1.5±0.7^{a,b}	55.7±19.4^{a}	65.4±9.3^{b}
Bile acids (μmol/day)	3.1±0.9	3.2±1.1	2.6±0.2

Values are means±S.D. (n=7). ^{a,b}Means in a row sharing a common superscript are significantly different (P<.05) using one-way ANOVA and Scheffe's F test.

Table 5
The effect of free PST and curcumin on plasma and liver lipids in gerbils fed with cholesterol-containing (0.15%) diets for 4 weeks (Experiment 4)

	Diet groups			
	Control (0% PST, 0% curcumin)	0.45% PST	0.2% Curcumin	0.45% PST + 0.2% curcumin
PST/cholesterol ratio	0:1	3:1	0:1	3:1
Body weight (g)				
Initial (after cholesterol loading)[a]	54±1	53±2	53±2	54±1
Final (after 4 weeks)	67±7	68±5	71±4	69±4
Feces excretion				
Dry weight (g/day)	0.62±0.12	0.84±0.07	0.76±0.06	0.71±0.15
Organ weights (%BW)				
Liver[b]	3.11±0.26	2.98±0.34	3.28±0.12	3.27±0.27
Cecum	2.51±0.87	2.27±0.37	2.00±0.36	2.41±0.36
Adipose	0.39±0.15	0.50±0.12	0.51±0.09	0.57±0.09
Plasma (mmol/L)				
TC[b,c]	5.4±0.5	3.4±0.7	5.1±0.5	4.1±0.4
HDL-C[a]	2.0±0.2	1.9±0.5	1.9±0.3	2.3±0.5
TC/HDL-C ratio[a,b]	2.81±0.33	1.87±0.18	2.70±0.34	1.81±0.34
TG	0.7±0.2	0.8±0.6	1.0±0.7	1.3±0.5
VLDL-C[b,d]	2.5±0.4	1.0±0.2	2.4±0.3	1.2±0.1
LDL-C[b,c,d]	1.3±0.0	0.6±0.1	1.1±0.1	0.7±0.0
HDL-C[d]	1.7±0.2	1.9±0.4	1.7±0.1	2.2±0.0
LDL-C/HDL-C ratio[b,d,e]	0.79±0.08	0.33±0.06	0.61±0.06	0.32±0.01
LDL α-tocopherol (μmol/L)[b]	9.1±1.5	5.1±1.1	8.7±2.2	6.7±1.1
LDL α-tocopherol/cholesterol (μmol/mmol) ratio	6.7±1.2	7.7±1.0	8.2±1.1	9.7±1.5
Liver cholesterol (mg/g)				
TC[b]	114±10	59±10	121±13	52±10
FC[b]	16±2	13±1	15±2	13±1
EC[b]	98±10	47±10	106±13	39±8
Liver β-carotene (nmol/g)	26±1	22±3	25±5	23±2

Values are mean±S.D. (n=7–8).

[a] HDL-C obtained by PTA-Mg^{2+}precipitation.

[b] P<.05, significant effect of phytosterol revealed by two-way ANOVA.

[c] P<.05, significant interaction between effect of phytosterols and curcumin by two-way ANOVA.

[d] VLDL-C, LDL-C and HDL-C obtained by discontinuous gradient ultracentrifugation; n=3 (each representing two to three plasma samples combined).

[e] P<.05, significant effect of curcumin revealed by two-way ANOVA.

a normal fat load, while the least decline occurred during normal fat intake without PST. This tendency for fat to be either good or bad depending on the presence or absence of PST was interpreted as fat facilitating biliary cholesterol reabsorption when no PST was consumed, while assisting cholesterol excretion when PST was present. A plausible mechanism for the latter case would have fat forming more micelles to better disperse cholesterol for PST intervention, coupled with fat stimulation of the gallbladder to release more bile acids to enhance micelle formation. Greater gallbladder contraction by fat would also enhance secretion of biliary cholesterol for potential removal by PST. On the other hand, this same impact of fat on bile secretion and micelle formation would favor cholesterol reabsorption in the absence of PST (reflected in the highest plasma and liver cholesterol concentrations observed in that group). By contrast, the fat-free diet would be expected to slow bile secretion, enterohepatic cycling of bile acids and reabsorption of biliary cholesterol. One might even have predicted a greater differential for PST effects between the fat-free and normal-fat diets since it is generally acknowledged that PST must be dispersed in fat to be effective [1]. Possibly the 2-h per day exposure to PST was too short, as food consumption during that 2 h represented only about 35% of the daily total.

This combined impact of fat and PST mass is further demonstrated by comparing the fat+PST diet groups in Experiments 2 and 3. All-day exposure to fat+PST in Experiment 3 depleted liver reserves of EC almost twice as effectively as the same diet in Experiment 2, fed only 2 h each morning, and thrice the rate as the control group (fat, no PST). This likely reflects the fact that the amount of PST consumed in 2 h (Experiment 2) was less than half that in Experiment 3 (data not shown). In addition, previous experiments with a 5:1 ratio of PST/cholesterol [8] was substantially better at reducing TC and liver EC (−60% and −80%, respectively) than the 3:1 ratio in Experiment 4 (−40% and 50%), again emphasizing the importance of the relative mass of PST consumed in fat (Table 5).

The fact that plasma TC declined 50% in control gerbils (no PST) only 3 weeks after removing diet cholesterol in Experiment 3 reconfirmed the ability of gerbils to spontaneously normalize overloaded cholesterol pools once the diet cholesterol challenge had been removed. Nonetheless, the decline in plasma TC and liver EC was appreciably greater with PST added, but the decrease was not enhanced by adding curcumin to PST. Thus, the potent combination of diet fat and PST on cholesterol removal was demonstrated, but the hypothesis that curcumin would enhance the ability of PST to lower cholesterol pools by increasing bile secretion was not supported.

It is noteworthy that PST+curcumin reduced the TC/HDL ratio significantly compared to the control, being associated with an increase in the tocopherol content of LDL. Thus, the lower TC/HDL ratio attributed to curcumin suggests it may have orchestrated an LDL decline independent of whole body cholesterol removal, possibly linked to its antioxidant potential [17].

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