Stearate-Enriched Plant Sterol Esters Lower Serum LDL Cholesterol Concentration in Normo- and Hypercholesterolemic Adults1–3

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Abstract

Studies in our laboratory have previously demonstrated in hamsters a superior cholesterol-lowering ability of plant sterol (PS) esters enriched in stearate compared with linoleate. We therefore conducted a randomized, double-blind, 2-group parallel, placebo-controlled study to test the cholesterol-lowering properties of stearate-enriched PS esters in normo- and hypercholesterolemic adults. Thirty-two adults, 16 per group with equal number of males and females in each group, participated in the 4-wk study. Participants consumed 3 g/d (1 g three times per day with meals) of either PS esters or placebo delivered in capsules. Serum LDL cholesterol concentration significantly decreased 0.42 mmol/L (11%) and the LDL:HDL cholesterol ratio decreased 10% with PS ester supplementation, whereas LDL particle size and lipoprotein subclass particle concentrations (as measured by NMR) were not affected. The percent change in LDL cholesterol was positively correlated with baseline lathosterol concentration ($r = 0.729; P = 0.0014$), indicating an association between the magnitude of LDL change and the rate of whole-body cholesterol synthesis. Serum campesterol (but not sitosterol) concentration significantly increased in the PS ester group. Serum tocopherol, retinol, and $b$-carotene concentrations were not affected by PS ester supplementation. Thus, our findings demonstrate the usefulness of a novel stearate-enriched PS ester compound in decreasing LDL cholesterol in both normo- and hypercholesterolemic adults. The extent to which PS ester fatty acid composition affects intestinal micelle formation and cholesterol absorption in humans requires further study.


Introduction

The direct association between serum LDL cholesterol concentration and risk of atherosclerotic diseases is well established. Moreover, the mean serum LDL cholesterol level of U.S. adults is $>3$ mmol/L (1), exceeding the current recommendation of $<2.6$ mmol/L (2). Statin drugs have been used for 2 decades as an effective cholesterol-lowering therapy, but they can cause severe adverse effects in a considerable proportion of patients (3,4). Therefore, lowering serum LDL cholesterol through nondrug means would be most desirable.

Consumption of phytosterols and their fatty acyl esters (the term “phytosterol” refers to plant sterols and stanols, abbreviated PS) can lower serum LDL cholesterol concentration 10–15% by reducing cholesterol absorption in the small intestine (5). The National Cholesterol Education Program and the AHA recommend 2 g/d of PS esters as a therapeutic option for reducing serum LDL cholesterol concentration (6,7). Although ingestion of unesterified (free) PS can also decrease cholesterol absorption, esterified PS are more soluble in oils and therefore incorporate more easily into food products (8,9). The maximum solubility of free PS in oil is ~2% (wt:wt), whereas the solubility of PS esters is at least 20% (10).

Esterification of PS is achieved commercially using common vegetable oils (e.g. canola, sunflower seed, or soybean) comprised largely of mono- and polyunsaturated fatty acids. In an extensive review of randomized controlled trials testing dietary PS, 39 of the 41 trials examined PS esters rather than free PS and, where indicated, most of these studies reported that the PS esters contained mainly unsaturated fatty acids derived from vegetable oils (11). In contrast, the metabolic impact of PS esters made with SFA is vastly understudied.

We recently reported that hamsters fed stearate-enriched PS esters, i.e. PS esters made with purified stearic acid (97% stearate) or beef tallow fatty acids (19% stearate), had significantly lower plasma non-HDL cholesterol concentrations and lower cholesterol absorption compared with hamsters fed...
linoleate-enriched PS esters that contained only 3% stearate (12,13). In view of the superior ability of stearate-enriched PS esters to inhibit cholesterol absorption and lower plasma cholesterol in hamsters, we conducted the present study to test the efficacy of PS esters made with beef tallow fatty acids in normo- and hypercholesterolemic male and female adults.

Materials and Methods

Experimental design. The study design was a randomized, double-blind, 2-group parallel, placebo-controlled trial lasting 4 wk. All participants gave written informed consent to participate and the study was approved by the Institutional Review Board of the University of Nebraska. Participants were free-living males and females recruited from the community and were selected based on the following criteria: aged 19–70 y, BMI 18.5–30.0 kg/m², serum LDL cholesterol 2.59–4.91 mmol/L (100–190 mg/dL), avoidance of lipid-lowering medication, nonsmoker, not pregnant or lactating, and absence of chronic metabolic diseases. Thirty-two adults, 16 per group with an equal number of males and females in each group, participated in the study. Participants consumed 3 g/d (1 g three times per day with meals) of either PS esters or placebo delivered in capsules. PS esters were prepared by IRIX Pharmaceuticals as previously described using beef tallow fatty acids (12). Placebo was cellulose powder (International Fiber). Participants were instructed to check in to the clinic (MDS Pharma Services) each week to have blood drawn and blood pressure measured and to receive their weekly allotment of capsules. Compliance was very good; 7 missed doses were reported in the placebo group during the study and only 4 missed doses in the PS ester group.

Blood samples were collected on d 1, 8, 15, 22, and 29 for analysis of several biomarkers of metabolic function and to monitor overall health of the participants. Samples collected on d 1 and 29 were used to document the effect of PS ester treatment. Three-day food records (completed on 2 weekdays and 1 weekend day) were collected during the first and last week of the study to monitor nutrient and energy intake. Prior to the study, participants were trained by a registered diettitian to keep accurate food records and all records were validated and analyzed by trained personnel who were blinded to the treatment assignments. The nutrient content of foods eaten was analyzed using the Nutrition Data System for Research (version 5.0, University of Minnesota, Minneapolis, MN). Participants were instructed to maintain their normal eating and exercise habits throughout the study. An exercise questionnaire administered during the first and last weeks indicated no changes in exercise habits in either group.

Analytical procedures. Serum isolated from weekly blood samples was analyzed for hemoglobin, blood urea nitrogen, creatinine, alkaline phosphatase, lactate dehydrogenase, and liver alanine aminotransferase and aspartate aminotransferase. In addition, samples collected on d 1 and 29 were analyzed for glucose, triglycerides, and LDL and HDL cholesterol. All analytes (except hemoglobin) were quantified on a Cobas Integra 800 (Roche Diagnostics); hemoglobin was quantified on a Coulter Gen-S hematology analyzer (Beckman Coulter).

Lipoprotein subclass particle concentration was determined by 1H NMR (LipoScience) as previously described (14). Briefly, lipoprotein subclasses of different sizes produce a distinct lipid methyl signal whose amplitude is directly proportional to lipoprotein particle concentration. NMR simultaneously quantifies >30 lipoprotein subclasses that are empirically grouped into fewer subclasses based on particle diameters: total VLDL (>23 nm), large LDL (21.2–23 nm), medium LDL (19.8–21.2 nm), small LDL (18–19.8 nm), large HDL (8.8–13 nm), medium HDL (8.2–8.8 nm), and small HDL (7.3–8.2 nm). Weighted mean lipoprotein particle sizes in diameters were calculated based on the diameter of each lipoprotein subclass multiplied by its respective relative concentration.

LDL atherogenic particle size pattern was determined using the Lipoprint LDL system (Quantimetrix) via nongradient, high-resolution PAGE (15). LDL with a mean particle size ≥ 25.5 nm is characterized as pattern A, whereas pattern B is the atherogenic phenotype and has a mean particle size < 25.5 nm (16).

Serum vitamin E (tocopherols), vitamin A (retinol), and β-carotene were measured by HPLC (17). Serum was combined with an equal volume of absolute ethanol containing an internal standard (tocopherol acetate) and antioxidant (butylhydroxytoluene). After mixing, the samples were combined with hexane to recover the lipids. The solvent was evaporated under nitrogen and the lipids resuspended in methanol. The samples were injected onto a Zorbax 300SB C-18 column (Agilent) interfaced to a Waters 600 S HPLC system. Tocopherols, retinol, and β-carotene were resolved using a mobile phase of methanol, acetonitrile, and triethanolamine (90:10:1) under isocratic conditions (flow rate of 1 mL/min) and detected with a Waters 996 photodiode array detector at 295, 313, and 450 nm, respectively.

Serum noncholesterol sterols were quantified by GC. Briefly, serum lipids were extracted and silylated in 1.0 mol/L methanolic KOH, then nonsaponifiables were reextracted and silylated using Sylon BTZ (Supelco). The sterols were quantified on a 0.32-mm × 30-m AT-5 capillary column (Alltech) under the following conditions: injector temperature, 270°C; flame ionization detector, 300°C; initial oven temperature, 190°C for 1 min, increased to 220°C at 3°C/min; and helium carrier gas.

Statistical analysis. Data were analyzed by repeated-measures ANOVA using the mixed procedure of SAS (version 9.1) to test for treatment effects over time. Treatment assignment (placebo vs. PS ester) and gender were initially included in the model, although no significant differences due to gender were detected; therefore, analyses herein include the combined data for males and females. When the interaction of time × treatment was significant (P < 0.05), we included a contrast procedure for mean comparisons between groups (at same time period) and between time periods (within a group). Data are presented as means ± SEM. Pearson correlation was used to test associations between change in LDL cholesterol and serum concentration of PS. Significance was set at P < 0.05.

Results

Characteristics of the participants at baseline were within the expected normal ranges, indicating that they were healthy and met the inclusion criteria (Supplemental Table 1). All measurements were repeated weekly to monitor for potential changes in metabolic function, although no significant changes were observed during the study (data not shown). Four participants (2 in the placebo group and 2 in the PS ester group) reported a single event of gastrointestinal discomfort lasting 1 or 2 d. However, the short duration of the discomfort and the equal distribution between treatment groups indicate these adverse events were unrelated to experimental treatment.

The nutrient and energy intakes of participants were determined from 3-d food records completed during the first and last weeks of the study (Supplemental Table 2). None of the dietary variables changed from wk 1 to 4 in either group, confirming that participants maintained a consistent eating pattern during the study. In addition, the placebo and PS ester groups did not differ.

Serum LDL cholesterol was significantly decreased 0.42 mmol/L in the PS ester group on d 29 compared with baseline, indicating an 11.0% reduction (Table 1). Two participants in the PS ester group were normocholesterolemic at baseline (≥3.35 mmol/L), whereas 7 participants finished in the normocholesterolemic range on d 29 (Fig. 1). HDL cholesterol concentration was not affected by treatment, although the LDL:HDL cholesterol ratio in the PS ester group was significantly lower compared with baseline (Table 1). Serum triglycerides, glucose, and fat-soluble vitamins were not affected by treatment.

Serum lathosterol and desmosterol, indicators of cholesterol synthesis, were not affected by treatment whether the data were
TABLE 1 Serum cholesterol, triglyceride, glucose, and fat-soluble vitamins in adults consuming placebo or PS esters for 4 wk

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>PS ester</th>
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<tbody>
<tr>
<td><strong>LDL cholesterol, mmol/L</strong></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>3.36 ± 0.12</td>
<td>3.98 ± 0.17*</td>
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<tr>
<td>End</td>
<td>3.23 ± 0.15</td>
<td>3.56 ± 0.19*</td>
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<tr>
<td>Change</td>
<td>−0.07 ± 0.10</td>
<td>−0.42 ± 0.06</td>
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<td><strong>HDL cholesterol, mmol/L</strong></td>
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<tr>
<td>Baseline</td>
<td>1.82 ± 0.08</td>
<td>1.59 ± 0.10</td>
</tr>
<tr>
<td>End</td>
<td>1.50 ± 0.08</td>
<td>1.58 ± 0.09</td>
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<tr>
<td>Change</td>
<td>−0.12 ± 0.04</td>
<td>−0.01 ± 0.03</td>
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<tr>
<td><strong>LDL:HDL cholesterol ratio, mol/mol</strong></td>
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<tr>
<td>Baseline</td>
<td>2.18 ± 0.16</td>
<td>2.62 ± 0.17</td>
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<tr>
<td>End</td>
<td>2.35 ± 0.22</td>
<td>2.36 ± 0.17*</td>
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<tr>
<td>Change</td>
<td>0.16 ± 0.14</td>
<td>−0.25 ± 0.06</td>
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<tr>
<td><strong>Triglyceride, mmol/L</strong></td>
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<tr>
<td>Baseline</td>
<td>1.04 ± 0.14</td>
<td>1.44 ± 0.17</td>
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<tr>
<td>End</td>
<td>1.26 ± 0.20</td>
<td>1.49 ± 0.21</td>
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<tr>
<td>Change</td>
<td>0.22 ± 0.19</td>
<td>0.04 ± 0.10</td>
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<tr>
<td><strong>Glucose, mmol/L</strong></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>5.32 ± 0.09</td>
<td>5.16 ± 0.09</td>
</tr>
<tr>
<td>End</td>
<td>5.25 ± 0.10</td>
<td>5.32 ± 0.13</td>
</tr>
<tr>
<td>Change</td>
<td>−0.07 ± 0.09</td>
<td>0.16 ± 0.08</td>
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<tr>
<td><strong>Retinol, μmol/L</strong></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>85.6 ± 13.2</td>
<td>85.8 ± 13.5</td>
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<tr>
<td>End</td>
<td>71.1 ± 15.2</td>
<td>85.9 ± 14.5</td>
</tr>
<tr>
<td>Change</td>
<td>−11.5 ± 22.4</td>
<td>4.0 ± 20.6</td>
</tr>
<tr>
<td><strong>β-Carotene, μmol/L</strong></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>17.2 ± 2.8</td>
<td>14.9 ± 3.0</td>
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<tr>
<td>End</td>
<td>12.5 ± 2.4</td>
<td>12.9 ± 1.7</td>
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<tr>
<td>Change</td>
<td>−3.6 ± 2.4</td>
<td>0.6 ± 4.5</td>
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<tr>
<td><strong>α-Tocopherol, μmol/L</strong></td>
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<tr>
<td>Baseline</td>
<td>43.4 ± 3.5</td>
<td>38.4 ± 3.8</td>
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<tr>
<td>End</td>
<td>44.8 ± 3.8</td>
<td>40.9 ± 3.2</td>
</tr>
<tr>
<td>Change</td>
<td>1.3 ± 5.3</td>
<td>2.1 ± 5.8</td>
</tr>
<tr>
<td><strong>γ-Tocopherol, μmol/L</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>10.4 ± 1.2</td>
<td>14.3 ± 4.7</td>
</tr>
<tr>
<td>End</td>
<td>10.9 ± 1.6</td>
<td>9.5 ± 1.4</td>
</tr>
<tr>
<td>Change</td>
<td>1.4 ± 2.0</td>
<td>−4.5 ± 4.5</td>
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</table>

1 Values are means ± SEM, n = 16. *Different from placebo, P < 0.05. #Different from baseline, P < 0.05 (when the interaction of time × treatment was significant, groups at a time and time points for a group were compared using a contrast procedure).

expressed as concentration or normalized to serum cholesterol (Table 2). However, there was a strong correlation (r = 0.729; P = 0.0014) between the percent change in LDL cholesterol and baseline lathosterol concentration in participants consuming PS ester (Fig. 2), indicating an association between the magnitude of LDL change and the rate of whole-body cholesterol synthesis. The percent change in LDL cholesterol was also positively correlated with the lathosterol:cholesterol ratio (r = 0.540; P = 0.031) and with the lathosterol:campesterol ratio (r = 0.673; P = 0.004). The serum campesterol concentration and campesterol: cholesterol ratio were significantly higher in the PS ester group on d 29 compared with baseline (Table 2). Furthermore, there was a significant correlation between the percent change in LDL cholesterol and the baseline campesterol:cholesterol ratio (r = 0.589; P = 0.0164). Serum sitosterol was not affected by treatment.

As measured by NMR, the lipoprotein particle concentration and size of VLDL, LDL, and HDL were not affected by treatment (Supplemental Table 3). Similarly, when measured by gel electrophoresis, LDL size pattern did not change in PS ester participants during the 4-wk study (11 of 16 participants exhibited pattern B at baseline and at d 29). However, 3 participants in the placebo group changed from pattern A to pattern B during the study (8 of 16 participants exhibited pattern B at d 29). LDL particle diameter measured by gel electrophoresis showed good correlation (r = 0.613; P < 0.001) with LDL particle diameter measured by NMR, all participants included (n = 32). NMR-measured LDL size was negatively correlated with serum lathosterol (r = −0.447; P = 0.0103) and desmosterol (r = −0.442; P = 0.0113), indicating that high rates of whole-body cholesterol synthesis are associated with small dense LDL particles.

**Discussion**

The major finding in this study was the ability of stearate-enriched PS esters to significantly lower serum LDL cholesterol in adult males and females consuming their reported typical diets. To the best of our knowledge, this is the first clinical trial demonstrating the cholesterol-lowering properties of stearate-enriched PS ester. Another important finding was the high correlation between serum lathosterol concentration, an indicator of whole-body cholesterol synthesis, and the magnitude of LDL cholesterol lowering. Finally, this study demonstrated the cholesterol-lowering properties of stearate-enriched PS esters in both normo- and hypercholesterolemic adults.

A novel aspect of the study was the use of PS esters made with beef tallow fatty acids for the purpose of increasing the proportion of stearate esters several times higher than commercially available PS esters, which are made with stearate-deficient vegetable oils (18–20). We previously reported in hamsters that stearate-enriched PS esters had superior cholesterol-lowering properties compared with linoate-enriched PS esters (12). In that study, we utilized the same PS esters made with beef tallow fatty acids as reported herein in which stearate esters comprised ~19% of the total PS esters. It was further demonstrated in hamsters that the enhanced cholesterol lowering was specifically due to stearic acid enrichment in PS esters (12) and that intake of PS esters caused significantly greater cholesterol lowering than intake of unesterified PS and stearic acid added to the diet as separate ingredients (13). The precise mechanism for enhanced Phytosterol-stearate lowers LDL cholesterol 1447
cholesterol lowering by stearate-enriched PS esters has not been elucidated. However, we speculate that PS-stearate may resist hydrolysis in the digestive tract and that intact PS-stearate may cause greater disruption of cholesterol solubilization in micelles (and, thus, greater inhibition of cholesterol absorption). In vitro studies currently underway in our laboratory have revealed that the preferred PS ester substrates for pancreatic cholesterol esterase are oleate esters, whereas hydrolysis of stearate esters is significantly less (A.W. Brown and T.P. Carr, unpublished data). Similar results can be inferred from a recent study in rats fed either PS-oleate or PS-stearate (21); based on the nonhydrolyzed PS esters remaining in feces, it appeared that 99.5% of oleate esters were hydrolyzed compared with only 19.2% stearate esters hydrolyzed. In humans, PS or stanol esters containing unsaturated fatty acids were hydrolyzed 88% and 86%, respectively, following passage through the small intestine, indicating that sterol and stanol esters were hydrolyzed to the same extent (22). Taken together, these observations suggest that the extent of hydrolysis is more strongly influenced by the fatty acid moiety compared with the sterol/stanol moiety, although further study is needed to elucidate the mechanisms.

To our knowledge, direct comparison of PS esters with different fatty acid compositions has not been previously reported in humans. However, Katan et al. (11) summarized the results of 50 trial arms from 41 studies in which most of trials utilized PS esters made with PUFA derived from vegetable oils. The results indicated an 8–10% reduction in LDL cholesterol concentration, consistent with our results showing an 11% decrease in LDL cholesterol. Although it is possible that longer term use (>4 wk) of stearate-enriched PS esters may provide greater reductions in LDL cholesterol, additional studies are needed to confirm this possibility.

Lathosterol is a cholesterol precursor and its concentration in serum is directly correlated with whole-body cholesterol synthesis (23,24). The extent to which dietary PS esters influence lathosterol levels (and, presumably, cholesterol synthesis) has been reported in several studies but with variable results. Some studies report an increased lathosterol:cholesterol ratio in humans consuming either PS or stanol esters (25–29), whereas other studies reported no change (30–32). And in studies directly comparing PS and stanol esters, the lathosterol:cholesterol ratio significantly increased with intake of PS esters, but not stanol esters (33,34). In the present study, we observed no change in serum lathosterol as a result of PS ester supplementation. The inconsistent results are likely due to the large variation in the participants’ capacity to synthesize cholesterol, as estimated by their baseline lathosterol:cholesterol ratio. In response to PS ester supplementation, Gylling et al. (35,36) observed that the greatest decrease in LDL cholesterol occurred in participants with the lowest baseline lathosterol:cholesterol ratio. Thuluvu et al. (29) reported a strong correlation \( r = 0.799 \) between the percent change in LDL cholesterol and the lathosterol:campesterol ratio over a wide range of lathosterol concentrations in participants consuming PS esters. In the present study of PS esters, we observed a positive correlation between the percent change in LDL cholesterol and serum lathosterol concentration, consistent with our results showing an 11% decrease in LDL cholesterol. Although it is possible that longer term use (>4 wk) of stearate-enriched PS esters may provide greater reductions in LDL cholesterol, additional studies are needed to confirm this possibility.


corr = 0.729; \( P = 0.0014 \).

**FIGURE 2** Correlation between baseline serum lathosterol concentration and change in LDL cholesterol in adults consuming stearate-enriched PS esters for 4 wk \( (y = -20.3 + 1.53x; r = 0.729; P = 0.0014) \).
sterol composition of PS preparations used in clinical studies was in the range of 46–75% sitosterol and 8–30% campesterol; in contrast, their intake caused a 44–107% increase in serum campesterol, but only 23–70% increase in sitosterol (26,30,33, 37–40). In the present study, we observed a significant increase in serum campesterol, but not sitosterol, consistent with previous reports showing a disproportionate increase in serum campesterol. Using stable isotope tracers, Ostlund et al. (41) found the absorption efficiency of campesterol was 3.7 times higher than sitosterol (absorption efficiency was 1.89 vs. 0.51%, respectively), which could contribute to the preferential increase in serum campesterol. Also, the serum clearance rate of sitosterol appears to be greater than campesterol (41,42). One report indicated that biliary secretion and hepatic clearance of sitosterol was higher than campesterol and was probably related to the higher serum clearance rate of sitosterol (43). The mechanisms that mediate the differential transport of PS in liver and intestine are unknown. While it is possible that the ATP-binding cassette transporters G5/G8 might discriminate among sterols (44), intestinal absorption of campesterol was still comparatively higher than sitosterol in the absence of ABCG5/G8 (45,46).

In summary, our findings demonstrated significant LDL cholesterol lowering in male and female adults consuming a novel PS ester preparation enriched in stearic acid. We observed LDL cholesterol decreases in both normo- and hypercholesterolemic (LDL cholesterol >3.36 mmol/L) participants who followed their normal dietary and lifestyle habits. Despite stearic acid being a saturated, long-chain fatty acid, its increased presence in PS esters may beneficially alter the cholesterol-lowering function of PS esters. Further study is needed to elucidate the relationships among PS ester composition, intestinal micelle formation, and cholesterol absorption.

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Literature Cited

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