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Effects of a Water-Soluble Phytostanol Ester on Plasma Cholesterol Levels and Red Blood Cell Fragility in Hamsters

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ABSTRACT: The aim of this study was to assess the efficacy of a novel water-soluble phytostanol analog, disodium ascorbyl phytostanyl phosphates (DAPP), on plasma lipid levels and red blood cell fragility in hamsters fed atherogenic diets. For 5 wk, 50 male Golden Syrian hamsters were fed a semipurified diet without added cholesterol (noncholesterol, group 1), or a semipurified diet with 0.25% cholesterol (cholesterol-control, group 2). Groups 3–5 were fed the cholesterol-control diet with an addition of 1% phytostanols (diet 3), 0.71% DAPP (DAPP 0.7%, diet 4), or 1.43% DAPP (DAPP 1.4%, diet 5). Diets 4 and 5 provided 0.5 and 1% phytostanols, respectively. Supplementation of 0.71 and 1.43% DAPP decreased plasma total cholesterol concentrations by 34 ($P < 0.001$) and 46% ($P < 0.001$), respectively, in comparison with the cholesterol-control group, whereas free stanols reduced ($P = 0.007$) plasma cholesterol concentrations by 14%. Similarly, non-HDL-cholesterol concentrations were reduced by 39 ($P < 0.001$) and 54% ($P < 0.001$) in hamsters supplemented with DAPP 0.7% and DAPP 1.4%, respectively, relative to the cholesterol-control group. The hypocholesterolemic effect of DAPP 1.4% was threefold stronger than that of free stanols. In hamsters supplemented with DAPP 1.4%, plasma TG concentrations were 45% lower ($P = 0.018$) than in cholesterol-control-fed hamsters, whereas no such beneficial effect was observed in the free stanol group. Erythrocyte fragility was unaffected by DAPP or free phytostanols. Results of the current study demonstrate that DAPP lowers cholesterol more efficiently than free stanols, without an adverse effect on erythrocyte fragility in hamsters.

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The cholesterol-lowering effects of plant sterols have been well established (1–4). Meta-analyses have shown that dietary supplementation of plant sterols can reduce plasma LDL cholesterol levels by 8–12% in humans (4,5). The cholesterol-lowering effects of plant sterols reportedly result from the inhibition of dietary cholesterol absorption and biliary cholesterol reabsorption by inhibiting cholesterol incorporation into micelles in the intestine (2,6–8).

Through the process of hydrogenation, plant sterols are converted to plant stanols (also called phytostanols), which are absorbed at a negligible level (9,10). Based on previous data in animal and human studies, free plant stanols are believed to be more effective than free sterols in lowering plasma cholesterol (9–17). Esterification of free plant sterols and stanols with fat-soluble compounds, such as FA, increases their fat solubility. Several studies have demonstrated that the esterification of plant sterols with long-chain FA increases fat solubility, resulting in an increased suspension of plant sterols in the dietary fats or oils (5,8,18). In addition, some human trials comparing plant sterol esters with plant stanol esters have demonstrated that sterol esters and stanol esters appear to be roughly equivalent in their cholesterol-lowering efficacy (6,7,19–21). Progressively, the solubility and physical state of plant sterols and stanols appear to be determining factors in their cholesterol-lowering efficiency (22).

More recently, a water-soluble analog of phytostanols, disodium ascorbyl phytostanyl phosphates (DAPP), was developed through chemical modifications (23,24). DAPP were produced by covalently linking ascorbate to the *sn*-3 position of the phytostanol *via* a phosphodiester bond. The two major components of DAPP are disodium ascorbyl campestanyl phosphate and disodium ascorbyl sitostanyl phosphate. Results from a study in apolipoprotein E knockout mice showed that DAPP was more efficient in lowering cholesterol than the unesterified stanols (24). However, there have been no comparative studies on the effects of DAPP and unesterified stanols in wild-type animal models in which circulating cholesterol levels are increased by feeding atherogenic diets.

The ingestion of plant sterols is associated with increased plant sterol levels in red blood cell (RBC) membranes (25). Studies in hyperlipidemic, otherwise healthy individuals have shown no detrimental effect of plant sterols on RBC fragility in humans (25–27). However, a recent animal study suggested that plant sterols may decrease RBC deformability and life span in spontaneously hypertensive rats (28), raising the question of whether dietary supplementation with plant sterols or stanols is safe. Therefore, the present study was undertaken to compare the cholesterol-lowering efficacy of a water-soluble stanol analog (DAPP) and its parent compound, free stanols, and to determine their impacts on RBC fragility in hamsters fed atherogenic diets.

MATERIALS AND METHODS

Animals and diets. Fifty male Golden Syrian hamsters weighing 90–110 g (Charles River Laboratories, Montréal, Québec,

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Abbreviations: ABC, ATP-binding cassette; DAPP, disodium ascorbyl phytostanyl phosphates; RBC, red blood cell; SHRSP, stroke-prone spontaneously hypertensive rat.

Canada) were randomly divided into 5 groups. Animals were housed individually in stainless-steel colony cages with a 12 h (light)/12 h (dark) light cycle. The room temperature was set at 20 ± 1°C. After a 2-wk acclimatization period on the regular rodent chow (Charles River Laboratories), animals were randomly assigned into 5 groups and fed experimental diets for 5 wk. Groups 1 and 2 were fed a semipurified diet without cholesterol (noncholesterol) and with 0.25% cholesterol (cholesterol-control), respectively. Groups 3–5 were fed the cholesterol-control diet, with an addition of 1% (w/w) of unesterified phytostanol (stanol), 0.71% of DAPP (DAPP 0.7%), or 1.43% of DAPP (DAPP 1.4%), respectively. Diets DAPP 0.7% and DAPP 1.4% were designed to provide 0.5% and 1% of free phytostanols (parent phytostanols), respectively, based on the M.W. of the phytostanols and DAPP. DAPP contained 65% (w/w) sitostanol and 35% campestanol. Unesterified phytostanols and DAPP were provided by Forbes Medi-Tech Inc. (Vancouver, British Columbia, Canada). All diets contained 5% fat provided in the form of a mix of beef tallow and safflower oil to yield a polyunsaturated to saturated FA ratio of 0.4.

The compositions of the diets are presented in Table 1. The experiment was reviewed and approved by the Animal Care and Research Ethics Committee of McGill University and was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Blood sample collection and plasma cholesterol analyses. After 5 wk of feeding on the experimental diets, hamsters were fasted overnight prior to anesthetization with halothane. Blood samples were collected by decapitation into tubes containing EDTA and placed on ice. Plasma and RBC were separated by centrifugation at 350 × *g* rpm for 15 min. Plasma samples were stored at −80°C until analyses. Plasma total, HDL, and non-HDL cholesterol, as well as TG concentrations, were measured in duplicate by enzymatic methods (Roche Diagnostics, Laval, Québec, Canada) (29,30). HDL cholesterol was measured after precipitation of apolipoprotein B containing lipoproteins with dextran sulfate and magnesium chloride (31). Since the Friedewald equation (32) may not be applicable to hamsters, non-HDL cholesterol was used instead of LDL cholesterol; this was calculated by subtracting HDL cholesterol from total cholesterol.

Measurement of RBC fragility. RBC fragility was measured using fresh RBC collected in vacutainer tubes containing EDTA. An aliquot of 0.2 mL RBC was added to 2 mL unbuffered saline solution with a sodium chloride concentration ranging from 0.20 to 0.60%. After 1 h, the cells were centrifuged at 210 × *g* for 5 min at room temperature. The supernatants were collected and measured for absorbance at 520 nm (33). The saline concentration for 50% hemolysis was calculated and considered as the median osmotic fragility. The concentration required to obtain 50% hemolysis was determined by assuming that a linear response existed between consecutive observations.

Data analysis and statistics. Results are expressed as means ± SEM, and a P value of <0.05 was considered significant. Data were subjected to an ANOVA using the general linear model procedure of the Statistical Analysis System (SAS Institute, Cary, NC) to determine the main treatment effects. When a significant effect was detected, Duncan's New Multiple Range test was performed to identify differences among treatment groups. The relationships between plasma cholesterol concentrations and the median osmotic RBC fragility were tested using Pearson's correlation coefficients.

RESULTS

Body weight and food consumption. No significant difference was observed in final body weight among the treatment groups after 5 wk of feeding. However, hamsters fed DAPP 1.4% had relatively lower final body weights compared with other groups, although the difference was not statistically significant. Similarly, there were no significant differences among the groups in average or total food consumption during the 5-wk period.

Plasma lipid concentrations. Effects of the experimental diets on plasma total, HDL, and non-HDL cholesterol levels are

TABLE 1
Composition of Experimental Diets[a]

Diet (% w/w)	Nonchol	Chol-control	Stanol	DAPP 0.7%	DAPP 1.4%
Casein	20.0	20.0	19.8	19.8	19.8
Cornstarch	28.0	28.0	27.7	27.7	27.7
Sucrose	36.3	36.0	35.6	35.6	35.6
Beef tallow/safflower oil	5.0	5.0	5.0	5.0	5.0
Cellulose	5.0	5.0	4.9	4.9	4.9
DL-Methionine	0.5	0.5	0.5	0.5	0.5
Mineral mixture	4	4	4	4	4
Vitamin mixture	1	1	1	1	1
Choline bitartrate	0.2	0.2	0.2	0.2	0.2
Butylated hydroxytoluene	0.02	0.02	0.02	0.02	0.02
Cholesterol	—	0.25	0.25	0.25	0.25
Phytostanols	—	—	1.0	—	—
DAPP	—	—	—	0.71[b]	1.43[b]

[a]Nonchol, control diet without added cholesterol; Chol-control, control diet with 0.25% added cholesterol; Stanol, chol-control with 1% added phytostanols; DAPP (disodium ascorbyl phytostanyl phosphates), chol-cholesterol with added DAPP.
[b]DAPP 0.7% and DAPP 1.4% contained 0.5 and 1.0% unesterified phytostanols, respectively.

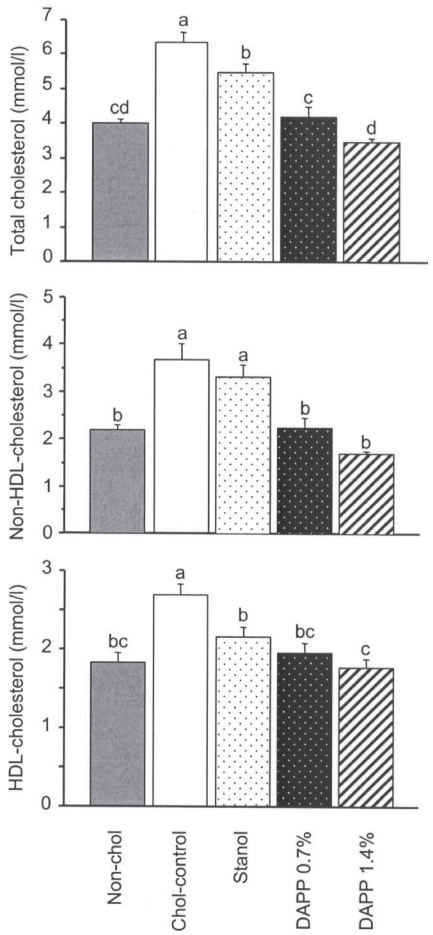

FIG. 1. Effects of phytostanols and their water-soluble analogs on plasma total cholesterol, non-HDL cholesterol, and HDL cholesterol levels in hamsters. Data are expressed as means and SEM ($n = 10$). Bars carrying different superscripts are significantly different ($P < 0.05$). Nonchol, control diet without added cholesterol; Chol-control, control diet with 0.25% added cholesterol; Stanol, chol-control with 1% added phytostanols; DAPP 0.7%, chol-cholesterol with 0.71% added disodium ascorbyl phytostanyl phosphates; DAPP 1.4%, chol-cholesterol with 1.43% added disodium ascorbyl phytostanyl phosphates.

shown in Figure 1. In hamsters fed DAPP 0.7% and DAPP 1.4%, total cholesterol was reduced by 34 ($P < 0.001$) and 47% ($P < 0.001$), respectively, in comparison with the cholesterol-control group, and these values were similar to the noncholesterol group. Similarly, non-HDL cholesterol levels in hamsters supplemented with DAPP 0.7% and DAPP 1.4% were 39 ($P < 0.001$) and 54% ($P < 0.001$) lower, respectively, than in those fed the cholesterol-control diet. The stanol diet lowered ($P = 0.007$) total cholesterol by 14% relative to the cholesterol-control diet. HDL cholesterol levels were lower in the two DAPP ($P < 0.001$) and stanol ($P = 0.005$) groups in comparison with the cholesterol-control group, and the values were similar with the noncholesterol group. However, changes in HDL cholesterol levels after feeding the DAPP and stanol diets were not reflected by changes in total cholesterol/HDL cholesterol and non-HDL cholesterol/HDL cholesterol ratios, which were decreased in the DAPP 1.4% group compared with the cholesterol-control ($P = 0.028$) and stanol groups ($P = 0.006$) (data not shown).

Effects of diet treatments on plasma TG concentrations are shown in Table 2. Although the free stanol diet did not affect plasma TG concentrations in comparison with the cholesterol-control diet, DAPP 1.4% reduced TG concentrations by 45% ($P = 0.018$) compared with the cholesterol-control group. Additionally, plasma TG concentrations were decreased by 42 ($P = 0.022$) and 49% ($P = 0.004$), respectively, in hamsters fed DAPP 0.7% and DAPP 1.4% compared with hamsters fed the free stanol diet.

RBC fragility. The saline concentration associated with the median osmotic fragility was (in %) 0.481 ± 0.004, 0.467 ± 0.007, 0.489 ± 0.007, 0.486 ± 0.005, and 0.478 ± 0.009 in DAPP 0.7%, DAPP 1.4%, stanols, cholesterol-control, and noncholesterol, respectively. There were no differences among the treatment groups. Likewise, there was no significant difference in RBC fragility across the treatment groups at any saline concentration (Fig. 2). Positive correlations were observed between the median osmotic fragility (%) and plasma total cholesterol (mmol/L) ($r = 0.44$, $P = 0.003$) and non-HDL cholesterol concentrations ($r = 0.43$, $P = 0.004$).

DISCUSSION

This is the first study to show a more potent hypocholesterolemic effect from DAPP compared with unesterified phytostanols in non-gene-treated animals with diet-induced higher circulating cholesterol levels. In hamsters fed DAPP, a strong cholesterol-lowering effect was observed, which resulted in total and non-HDL cholesterol levels similar to those observed in hamsters fed the noncholesterol diet. The DAPP diet showed a stronger hypocholesterolemic effect than the stanol diet, with a dose-dependent response being observed. Similar to the current finding, the cholesterol-lowering effect of DAPP on circulating cholesterol levels has been reported in other animal studies. For example, gerbils given 1 and 2% (w/w) DAPP in the diet, or 2 and 4% DAPP in drinking water had decreased total and LDL cholesterol levels (23,34). Recently, Lukic *et al.* (24) reported that in comparison with the control diet, a 4-wk administration of DAPP

TABLE 2
Effects of Phytostanols and Their Water-Soluble Analogs on Plasma TG Concentrations in Hamsters[a]

Diet	TG (mmol/L)	Diet[b]	TG (mmol/L)
Nonchol	4.50 ± 0.34[a,b,c]	DAPP 0.7%	3.64 ± 0.46[b,c]
Chol-control	5.88 ± 0.66[a,b]	DAPP 1.4%	3.23 ± 0.30[c]
Stanol	6.30 ± 0.96[a]		

[a]Values are mean ± SEM (n = 10 per group). Values carrying different superscripts (a–c) are significantly different (P < 0.05). For diet descriptions, see Table 1.

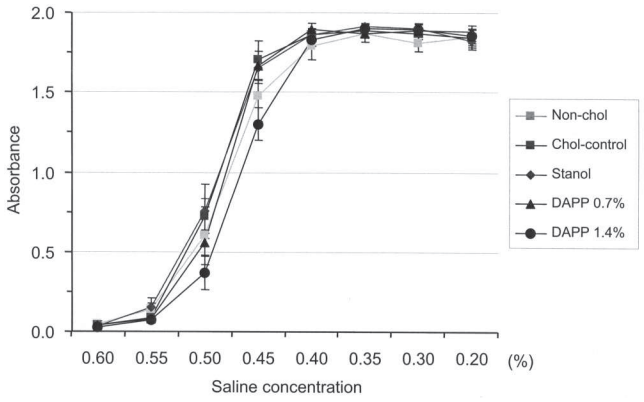

FIG. 2. Effects of phytostanols and their water-soluble analogs on red blood cell fragility in hamsters. Data are expressed as means and SEM (n = 9, except the chol-control group, where n = 7). For diet descriptions, see Figure 1.

at a dose as low as 0.5% reduced total cholesterol concentrations by approximately 75% in cholesterol-fed apolipoprotein E knockout mice.

Inhibition of cholesterol absorption from the intestine is a well-established mechanism through which plant sterols/stanols lower plasma cholesterol concentrations (2,35–39). DAPP has been shown to reduce cholesterol absorption in rats in a dose-dependent manner (40). The current study showed a stronger hypocholesterolemic effect from DAPP than from the corresponding unesterified stanols, despite the same amount of stanols being provided in the DAPP 1.4% and stanols groups. The effects of the individual components of DAPP on circulating cholesterol levels were examined previously only in apolipoprotein E-deficient mice (24). Those results indicated that DAPP resulted in an extreme lowering of total cholesterol concentrations in that model, whereas less favorable effects were observed with a mixture of ascorbic acid and unesterified stanols. Results of the present study, together with previous ones, suggest that the esterification of stanols with ascorbic acid is responsible for the increased hypocholesterolemic effect of DAPP relative to unesterified stanols. The present results also suggest that increasing the water solubility of plant stanols could be an approach to reducing the effective dose of plant sterols or stanols in lowering blood lipids.

Currently, the mechanism of action of DAPP on cholesterol absorption is not fully understood. A widely accepted explanation is that sterols and stanols compete with cholesterol in the intestine during their incorporation into micelles and thus decrease cholesterol uptake by enterocytes (40). In an *in vitro* study, DAPP decreased cholesterol accumulation in Caco-2 cells, suggesting that DAPP may modify some extracellular mechanisms of cholesterol influx and efflux (41). Most recently, Wasan *et al.* (42) observed that DAPP reduced the accumulation of cholesterol in cultured rat small intestinal crypt cells and speculated that DAPP might inhibit the interaction of cholesterol or cholesterol micelles with the receptors of enterocytes or act as an antagonist of cholesterol influx channels. Transporters of the ATP-binding cassette (ABC) family appear to play important roles in regulating cholesterol absorption and excretion (43,44). Supportive data were obtained by Berge *et al.* (45) that the increased accumulation of dietary cholesterol in subjects with autosomal recessive disorder was caused by mutations in the adjacent region of ABCG5 and ABCG8 genes. More studies are required to define the roles of ABCG5 and ABCG8 in the inhibition of cholesterol absorption by plant sterols and stanols.

A few studies have examined the potential TG-lowering effect of DAPP. When gerbils were given DAPP at concentration of 4% dissolved in the drinking water for 8 wk, TG concentrations were reduced compared with controls that did not receive DAPP (34). However, this effect was not observed in another study in which gerbils were fed 2% of DAPP for 4 wk (23). In a study conducted in apolipoprotein E knockout mice, plasma TG were reduced after feeding a 2% DAPP diet for 4 and 8 wk, respectively, in comparison with the control group, whereas no difference was observed after 12 wk (24). In the current study, we observed that DAPP was effective at lower concentrations in decreasing plasma TG levels compared with the cholesterol-control diets, as well as the unesterified phytostanol diet, whereas unesterified stanols showed no effect. In addition to the DAPP 1.4% group, although not statistically significant, the DAPP 0.7% group showed lower average TG levels in comparison with the noncholesterol-control group, implying the existence of a hypolipidemic effect of DAPP. The mechanisms through which DAPP lowers plasma TG are currently unknown. Further studies are required to verify the TG-lowering actions of DAPP and to elucidate the underlying mechanisms.

It has been suggested that plant sterols/stanols may increase the risk of hemorrhagic stroke, based on studies in stroke-prone spontaneously hypertensive rats (SHRSP) (28,46). In these hypertensive animals, when 0.2% (w/w) plant sterols were administered in their diets, decreased erythrocyte deformability was detected by using an ektacytometer, which can measure relatively minor alterations in RBC membrane rigidity, internal viscosity, and the ratio of surface area to volume (28). However, the osmotic fragility test detected no detrimental effects in the present animal model even though a higher dose of plant stanols (1%) was applied. Using the same method applied in the present study, Jenkins *et al.* (26) reported no significant changes in RBC fragility in humans after 4 wk of a low saturated fat diet enriched with plant sterols, soy protein, and viscous fibers. In addition, this research group illustrated constant results on fragility in another recent study (27). Hendriks *et al.* (25) reported that increased plant sterol concentrations in RBC membrane after long-term (1-yr) consumption of a plant sterol ester-enriched spread did not affect RBC deformability, which was measured by a method similar to that described in the study of SHRSP. Therefore, it is possible that the results obtained in SHRSP cannot be readily extrapolated to healthy animals or to other species. A significant decrease in the intestinal mRNA expressions of ABCG5 and ABCG8, which are involved in the selective transport of dietary sterols in the intestine, has been reported in SHRSP compared with Wistar rats (47). Results of this study suggest that the unfavorable effects on enterocytes observed in the SHRSP following supplementation of plant sterols are due to the increased absorption of plant sterols in this species. The present data, obtained in hypercholes-

terolemic, otherwise healthy animals, and the observations previously done in humans suggest that the use of plant stanols as food-based cholesterol-lowering agents may not be associated with an increased risk of hemorrhagic stroke for the general population. However, more effort is warranted to evaluate the risk of hemorrhagic sequelae with plant sterols or stanols used at the levels provided in functional foods. The incubated fragility test is one of the most sensitive measurements for detecting subtle changes in fragility (48,49). Since the adverse effects of plant sterols and stanols on RBC fragility are small, the incubated fragility test might be beneficial for detecting minor changes induced by dietary manipulation.

In the present study, positive correlations have been observed between the median osmotic fragility and plasma total cholesterol and non-HDL cholesterol concentrations, indicating that lowered plasma cholesterols have the potential to fortify RBC membranes against osmotic stimulation. Replacement of a part of the cholesterol with plant sterols in erythrocyte membranes has been shown to cause adverse events on RBC deformability and results in a decreased life span in spontaneously hypertensive rats (28). In contrast, a recent study that provided 0.5% cholesterol diets to Wistar rats suggested that erythrocytes become more fragile in animals with high plasma cholesterol concentrations; when cholesterol-lowering agents were added to the diet, the fragility of the RBC was partially reversed (50). Correlations observed in the present study between osmotic fragility and plasma cholesterol levels appear to support the latter observation. Plant sterols have been reported to alter plasma cholesterols as well as RBC cholesterol simultaneously in spontaneously hypertensive rats (28,46). High plasma cholesterol has been demonstrated to increase RBC fragility; therefore, plant sterols may have positive effects on RBC fragility, since sterols lower plasma cholesterol. However, plant sterols may also have unfavorable effects on RBC deformability, since sterols lower RBC cholesterol, which is essential for membrane integrity. In view of the fact that plant sterols may have either beneficial or detrimental effects on RBC fragility, this may account for the differing results seen in previous studies (25–28).

In conclusion, the results of the present study demonstrate that the esterification of plant stanols with a water-soluble compound, such as ascorbic acid, increases their cholesterol-lowering efficacy in hamsters fed atherogenic diets. DAPP could therefore offer a more efficient treatment alternative to hypercholesterolemic subjects than the other existing forms such as stanol esters with FA and free stanols. The findings of the current study also suggest that ascorbyl phytostanyl phosphate has potential for treating hypertriglyceridemia. The absence of a detrimental effect of DAPP on RBC fragility indicates that dietary supplementation with DAPP at the dose used in the current study may not be a trigger of plant sterol-induced adverse events on erythrocyte homeostasis.

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