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Beneficial Effects of Plant Sterols/Stanols-Containing Milk Powder on Lipid Metabolism in Hamsters

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ABSTRACT

The effect of plant sterols/stanols-containing milk powder (phytosterol milk powder, PSMP, containing 2.78% phytosterols mixture) on lipid metabolism in hamsters was investigated. One hundred male 7-week-old Golden Syrian hamsters were given free access to regular rodent chow and water for 1 week to acclimatize. Four extremely (10%) over- and underweight hamsters were eliminated. Sixteen hamsters were killed and examined for plasma and liver lipid compositions to establish the baseline. The remaining hamster were randomly divided into 5 groups, each group of hamsters had statistically similar average body weight, but fed with different experimental diets for 4 weeks. All 5 groups of hamsters were fed with high fat, high cholesterol diet containing different ingredients. Regular rodent chow diet was supplemented with 0.5% (w/w) cholesterol and corn/coconut oil mixture (corn oil/coconut oil = 1:1) to raise the final fat content to 15% (w/w) (Group 1 - control group). Group 2 was the positive control (PC) group, which was fed with diet containing 0.72% (w/w) phytosterols (consisted of 75% β-sitosterol and 10% campesterol). Groups 3-5 were the experimental groups, of which 12.95, 25.90 or 64.75% (w/w) PSMP (1×, 2× and 5× PSMP groups) was added to the diet and the phytosterol mixture contents in these diets were 0.36, 0.72 and 1.8% (w/w), respectively. At the end of 4-week feeding period, hamsters were killed and the plasma and hepatic lipid compositions together with the fecal neutral sterol content were determined. No adverse effects of PSMP on growth and health condition in hamsters were found in this experiment. At the highest feeding dose of PSMP (64.75%, w/w), hamsters had the highest body weight gain and the lowest plasma and liver lipid contents. PSMP showed significant effects on lowering the concentrations of plasma total cholesterol and low density lipoprotein cholesterol. It could also lower the atherogenic index (LDL-C/HDL-C), hepatic lipid levels and relative liver weight while raising the fecal cholesterol and phytosterols excretion in the hyperlipidemic hamsters.

Key words: phytosterol milk, cholesterol, phytosterols, lipid metabolism, hamster, lipoprotein cholesterol, hyperlipidemia

INTRODUCTION

Atherosclerosis and its related complications, such as cardiovascular disease (CVD) and cerebrovascular disease, are the leading causes of death in the Western world and many developed countries. A consistent and strong association between plasma cholesterol level and atherosclerosis has been well documented[1]. Dyslipidemia, including elevation of the total cholesterol (TC), the low density lipoprotein (LDL) cholesterol and the triacylglycerol (TG) concentrations, and a decrease in the high density lipoprotein (HDL) cholesterol concentration in the blood[1,2], is the major risk factor of atherosclerosis. Epidemiological studies indicate that atherosclerosis is increasingly prevalence worldwide due to the adaptation of Western life-style and is likely to reach epidemic proportions in the coming decades[3].

Phytosterols (also called plant sterols) are structurally related to cholesterol, but differ in their side chain confirguration at the C24 position. Compared with cholesterol, plant sterols have an extra methyl (campesterol), ethyl (β-sitosterol) group or a double bond (stigmasterol) in the side chain[4-9]. Phytosterols occur naturally in small quantities in many plant foods, such as vegetable oils, seeds, and nuts[5,10]. Adult human intake of plant sterols is approximately 0.1% (by dry weight)[9]. The plant sterols include sitosterol (65%), campesterol (30%), and other phytosterols, mainly stigmasterol[11]. Although these plant sterols are similar to cholesterol, they are absorbed to a much lesser extent[5]. Only about 5% of ingested plant sterols are absorbed by humans and other mammals, which is significantly lower than that of cholesterol (over 40%)[12-14], thus plasma levels of phytosterols are very low in healthy subjects[12].

Phytostanols are saturated phytosterols that are produced by 5α-hydrogenation, and less than 1% of dietary phytostanols are absorbed (the amount absorbed from the gut is negligible)[15,16]. Phytostanols are less abundant in nature than phytosterols and occur naturally in tall oil (tall oil phytosterols contain 20% to 30% sitostanol), wood pulp or via the processing course[5,6]. Phytosterols and phytostanols can interfere with the intestinal cholesterol absorption by inhibiting cholesterol incorporation into micelles in the lumen of the intes-

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tine[4,6,14,16,17]. Without micellar solubilization, cholesterol is poorly absorbed, if at all. Since 1951, when Peterson[18] demonstrated the cholesterol-lowering effect of β-sitosterol in cholesterol-fed chickens, many investigators have studied the effects of these natural substances on disorders of lipid metabolism and atherogenesis in both humans and laboratory animals[6,14,16]. Now plant sterols and stanols are well known cholesterol-lowering agents and have been used to treat hypercholesterolemia successfully[19-22].

Because dietary cholesterol contributes to LDL cholesterol and lowering LDL cholesterol is proven to be "cardioprotective", the food industry is incorporating plant sterols/stanols and their fatty acid esters into a variety of foodstuffs. These phytosterol-supplemented food products include "vegetable oil sterol esters" marketed by Lipton in Take Control™ brand spread[8,23], "plant stanol esters" marketed by McNeil Consumer Healthcare in Benecol™ brand spread[8,23], and "tall oil phytosterols" marketed by Novartis Consumer Health, Inc. in Phytrol™ brand spread[23]. The United States Food and Drug Administration (FDA) has previously evaluated these ingredients to be Generally Recognized as Safe (GRAS) when consumed in spread[23,24].

Recent studies reported that dietary phytosterols could reduce proinflammatory cytokine interleukin (IL)-6 and tumor necrosis factor (TNF)-α production in apolipoprotein E-knockout (apo E-KO) mice[25], stimulate the release of prostacyclin (PGI2) from rat vascular smooth muscle cells[26], and decrease prostaglandin (PGE) release from macrophages[26]. Plant sterols can also reduce cyclosporine-induced hypercholesterolemia (cyclosporine has been used widely as an immunosuppressive agent but cyclosporine therapy is commonly associated with side effects including hyperlipidemia, hypertension and nephrotoxicity)[27] and probucol-induced atherogenesis (the pro-atherogenic effects of probucol was due to reduced HDL cholesterol and increased plasma fibrinogen levels) in apo E-KO mice[28]. These results confirm the antiatherogenic effects of phytosterols.

The commercially available phytosterols and phytostanols are esterified with long chain fatty acid to increase their fat solubility and thus easier to be incorporated into food products, such as butter, margarine, mayonnaise and vegetable oil[20-22,29-32].

Researches in recent years focused on how to further increase the utilization of phytosterols. More potent hypocholesterolemic effect of plant sterols or stanols was observed when they were combined with other cholesterol-lowering agents, such as statins[20,29,33-35] or soy protein[35]. Rudkowska and his workers[36] used a new functional oil, which consisted of 6~10% phytosterol in a mixture of medium-chain triglyceride (MCT) oil (45~47%) and high-oleic canola (HOC) oil (45~47%) to replace 75% of dietary oil in 23 overweight, hyperlipidemic men. After the 6-week study period, the subjects showed 17, 21 and 15% decrease in plasma TC, LDL-cholesterol (LDL-C) and TG concentrations, respectively. Phytostanol phosphoryl ascorbate (FM-VP4), a novel water-soluble phytostanol analog, was proved to decrease cholesterol gastrointestinal absorption and lower the plasma cholesterol and LDL-C concentrations[37-39]. In hamster model, FM-VP4 provided more hypocholesterolemic effect than sitostanol[40]. This hydrophilic phytostanol analog can be formulated into a wide range of delivery vehicles.

Milk plays an important role in human diet. It is an excellent source of protein, riboflavin, vitamin B_{12}, calcium, phosphorus and a good source of vitamin A, thiamin, niacin and magnesium[41,42]. The nutritional quality of a protein is usually a more important consideration than its quantity. Milk proteins correspond very well to human requirements and therefore are regarded as high quality proteins[42]. However, high contents of saturated fatty acids (SFA) (about 83% of total fatty acids) and cholesterol (10 mg/100g) of dairy products, and the association of dairy food intake to the risk of coronary heart disease (CHD) has been a long-standing topic of discussion(43). It is of interest to know if milk containing plant sterols and stanols could have cholesterol lowering effect. Thus, the aim of this study was to examine the effect of milk supplemented with plant sterols/stanols mixture (phytosterols:phytostanols = 4:1) on lipid metabolism (including TC, lipoprotein cholesterol and TG concentrations in plasma, liver cholesterol and TG and fecal neutral sterols) of hyperlipidemic hamster. Syrian golden hamster was chosen as the animal model because the cholesterol and bile acid metabolism in hamster were similar to human[44-46] and this model was also widely used in atherosclerosis research[47].

MATERIALS AND METHODS

I. *Materials*

The lyophilized powder of plant sterols/stanols-containing milk was supplied by Uni-President Enterprises Corp., Tainan, Taiwan. It contains 2.78% (w/w) phytosterol/phytostanol mixture which was composed of 55.3% β-sitosterol, 17.2% campesterol, 17.2% β-sitostanol and 10.3% campestanol. This milk is a mixture of fresh milk and defatted milk powder formulated with a commercial fiber, Fibersol-2™ (Matsutant, Tokyo, Japan). The cholesterol content of this milk powder is 60 mg/100 g of powder.

II. *Animals and Diets*

One hundred male 7-week-old Golden Syrian hamsters were obtained from Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan). Hamsters were housed in wire-bottomed cages (2 per

cage) and maintained in a controlled environment (22 ± 2°C) with a 12-hr light/dark cycle (lights on 07:00-19:00 hr). These animals were maintained according to the guidelines established in "Guide for the Care and Use of Laboratory Animals (NSC)". The animals were given free access to regular rodent chow (LabDiet®, 5001 Rodent diet, Purina, St. Louis, MO, USA) and water for 1 week to acclimatize. Four animals which were extremely overweight and underweight were eliminated. Sixteen hamsters were killed and examined for plasma and liver lipid compositions to establish the base line (Basal group). The remaining 80 hamster were randomly divided into 5 groups of 16 animals. Each group of hamster had statistically similar average body weight, and then fed with one of the various experimental diets for 4 weeks. All 5 groups of hamsters were fed with high fat high cholesterol diet, which was prepared from regular rodent chow supplemented with 0.5% (w/w) cholesterol and oil mixture (corn oil/coconut oil = 1:1) to raise the fat content to 15% (w/w). Group 1 was the hyperlipidemia control (C) group. Group 2 was the positive control (PC) group, in which 0.72% (w/w) phytosterol (containing 75% β-sitosterol and 10% campesterol, Merck, Darmstadt, FRG) was added to the high fat high cholesterol diet. Groups 3-5 were the experimental groups. Various dosages of lyophilized powder of plant sterols/stanols-containing milk (phytosterol milk powder, PSMP) were incorporated into the high fat high cholesterol diet. Group 3, containing 12.95% (w/w) PSMP, had 360 mg phytosterol/phytostanol mixture in each 100 g of diet and was regarded as the basic dosage (1×) group. Groups 4 and 5 were fed with the 2 and 5 folds of the basic dosage and contained 720 and 1800 mg of phytosterol/phytostanol mixture per 100 g of diet. The phytosterol/phytostanol mixture was composed of 70~75% β-sitosterol, 7~9% campesterol, 12~16% sitostanol, and 4~6% campestanol. The detailed compositions of the experimental diets are shown in Table 1. To ensure homogenous mixing of the diet, cholesterol and phytosterol (PC group) were incorporated into the mixed oil blend at 50°C in a water bath. The warmed fat was mixed with other dietary ingredients and PSMP. All diets were prepared every week and stored at -20°C. A chow-based, rather than a semi-purified, diet was used because it can result in the hamster's lipoprotein profile (predominately LDL-C) more similar to human's[48,49].

III. *Animal Experiment and Sample Collection*

During the 4-week experimental period, hamsters were housed in wire-bottomed cages (2 per cage) and given free access to experimental diets and tap water. Tap water was refreshed every 3 days and experimental diets were refreshed everyday. Body weights were

Table 1. Composition and nutrients of the experimental diets

	Feed composition (%)				
Ingredients	C[a]	PC[a]	1×[a]	2×[a]	5×[a]
Chow[b]	88.5	87.78	76.45	64.35	28.2
Oil (corn oil-coconut oil = 1/1)	11	11	9.5	8	3.3
Cholesterol	0.5	0.5	0.5	0.5	0.5
PSMP[c]	—	—	12.95	25.9	64.75
Cellulose	—	—	0.1	0.15	0.35
Sucrose	—	—	0.5	1.1	2.9
Phytosterol	—	0.72	—	—	—
Nutrients	Nutrients composition (%)				
Protein	20.71	20.54	20.70	20.69	20.67
Fat	14.98	14.95	15.02	15.06	14.99
NFE[d]	43.37	43.01	42.99	42.69	41.85
Fiber	5.13	5.09	5.13	5.08	4.99
Cholesterol	0.50	0.50	0.50	0.50	0.50
Phytosterol and phytostanol	—	0.72	0.36	0.72	1.80
Calorie (kcal/100g)	391	389	390	389	385

[a]C: high fat high cholesterol control; PC: positive control; 1×: 12.95% plant sterol/stanol-containing milk powder (PSMP); 2×: 25.9% PSMP; 5×: 64.75% PSMP.

[b]The chemical composition of rodent chow: protein, 23.4%; crude fat, 4.5%; crude fiber, 5.3%; ash, 6.9%. Please see http://www.labdiet.com/indexlabdiethome.htm for detailed composition.

[c]PSMP (100g) contained 21.73 g protein, 16.09 g fat, 38.8 g NFE, 4.46 g fiber and 2.78 g phytosterol and phytostanol.

[d]NFE: nitrogen-free extract, obtained by calculation.

recorded twice a week. Fecal samples from each cage were collected and combined for 2 successive days before animals were sacrificed and stored at -70°C for fecal neutral sterols determination. At termination of the study, hamsters were deprived of food overnight (~16 hr), then weighed and anesthetized prior to killing by carbon dioxide inhalation. Blood samples were drawn from the abdominal vein and collected in heparin-containing tube and placed on ice. Plasma was separated from the blood by centrifugation (365×g) for 10 min at 4°C then stored at -20°C for further measurement of plasma total cholesterol (TC), plasma triglyceride (TG) and lipoprotein cholesterol concentrations. After exsanguinations, liver was excised, washed in ice-cold saline, weighed, frozen in liquid nitrogen, and then stored at -70°C for liver lipids analysis.

IV. *Plasma Lipids and Lipoproteins Determination*

(I) *Plasma Total Cholesterol (TC) Determination*

Plasma total cholesterol (TC) concentration was determined by the enzymatic CHOD-PAP (cholesterol oxidase-peroxidase aminophenazone) method[50] using a total cholesterol test kit (Merck, Darmstadt, FRG). Cholesterol esters were hydrolyzed to free cholesterol by cholesterol esterase. The free cholesterol produced was oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous production of hydrogen peroxide (H_2O_2), which was oxidatively coupled with 4-aminophenazone and phenol via the presence of peroxidase to yield quinoneimine with maximum absorption at 500 nm. An aliquot of 10 μL plasma was incubated with 200 μL of enzyme reagent in a 37°C water bath for 10 min and the absorbance at 500 nm was measured. Concentrations of TC in the samples were determined from a standard curve constructed by using cholesterol standard (Merck, Darmstadt, FRG).

(II) *Plasma Triacylglycerol (TG) Determination*

Plasma triacylglycerol (TG) was analyzed according to the fully-enzymatic GPO (glycerol phosphate oxidase)-PAP method(51) using a total triglyceride test kit (Merck, Darmstadt, FRG). Triglycerides were hydrolyzed to glycerol and fatty acid by lipase. Under a successive enzymatic reactions catalyzed by glycerol kinase and glycerol-3-phosphate oxidase, H_2O_2 was produced and oxidativly coupled with 4-aminophenazone and phenol via the presence of peroxidase to yield quinoneimine with maximum absorption at 500 nm. An aliquot of 10 μL plasma was incubated with 200 μL enzyme reagent in a 37°C water bath for 15 min and the absorbance at 500 nm was measured. Concentrations of TG in the samples were determined from a standard curve constructed by using triglyceride standard (Merck, Darmstadt, FRG).

(III) *Lipoprotein Isolation and Determination*

Plasma samples from both hamsters in each cage were combined and lipoproteins were isolated by continuous density gradient ultracentrifugation. The lipoprotein fractions of very low density lipoprotein and intermediate density lipoprotein (VLDL+IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) were isolated from plasma by preparative ultracentrifugation at $d < 1.019$ g/mL, $d = 1.019 \sim 1.063$ g/mL and $d = 1.063 \sim 1.21$ g/mL, respectively, using a Beckman Optima^{TM} LE-80K ultracentrifuge (Beckman, Palo Alto, CA) and a Ti 50.4 rotor. Cholesterol concentrations of each isolated lipoprotein were determined according to the enzymatic CHOD-PAP method[50] as described previously.

V. *Hepatic Lipids Analysis*

Liver lipid was extracted by the method of Folch[52]. Liver tissue (0.2 g) was homogenized with chloroform/methanol (2/1, v/v) to a final volume of 20 times the tissue sample (1 g in 20 mL of solvent mixture) in an ice bath. The homogenate was filterted (with the Whatman No. 1 filter paper) to obtain the liquid phase and replenished the volume to 5 mL. An aliquot of 10 μL filtrate was evaporated under a nitrogen stream in an eppendroff. TC and TG concentrations in the filtrates were determined according to the enzymatic CHOD-PAP and GPO -PAP methods. Liver TC and TG were expressed as mg/g liver.

VI. *Fecal Neutral Sterols Determination*

The collected feces were lyophilized and weighed. Fecal samples were ground to a fine powder and extracted with 20-fold weight to volume ratio of Folch solution (chloroform:methanol = 2:1, v/v) in an orbital shaker at room temperature for 12 hr in a screw-capped glass tube. The extract was filtered through Whatman No. 1 filter paper and the filtrate was replenished to 5 mL in volume. Fecal neutral sterols (cholesterol and β-sitosterol) were analyzed by reverse phase high performance liquid chromatography (HPLC) (Hitachi, Tokyo) with UV-VIS detector and Hypersil HS C18 column (4.6 mm ID × 250 mm, 5 μm particle size, ThermoQuest Hypersil Division, Runcorn, UK) using a method modified from Holen[53]. The mobile phase was methanol/acetonitrile (56/44, v/v), flow rate was 1.0 mL/min and detected with short-wave UV detection (205 nm). Sterol concentrations of samples were determined from a standard curve constructed with cholesterol and phytosterols (containing 10% campesterol and 75% beta-sitosterol, Merck, Darmstadt, FRG) standards.

VII. *Statistical Analysis*

All data were expressed as mean ± standard deviation. Significant differences were determined using

Student's t test where differences were considered significant if $p < 0.05$. All of the samples were measured in triplicate. Plasma and liver lipid compositions data in each group of animals were compared with Basal or group C. Body weight and liver weight data in each group of animals were compared with that of group C.

RESULTS AND DISCUSSION

I. *Dosage in the Diets*

Previous investigation revealed that high dose of plant sterols (10-20 g/d)[19] could reduce cholesterols in liver and serum. More recent studies, however, have shown that relatively low doses of plant sterols (1.5-3.0 g/d)[22,54] could also significantly reduce serum and liver cholesterol concentrations. Normal or mildly hypercholesterolemic adults ingested 1~3.3 g/d plant sterols or stanols or their esters for 4~6 weeks, the concentrations of plasma LDL cholesterol (LDL-C) and total cholesterol (TC) reduced by 7~14% and 5~10%, respectively[30-31,55-57]. These phytosterol substances (unsaturated, saturated or their esters) were also found to be effective in hypercholesterolemic adults (serum TC ≧ 6.0 mmol/L and serum TG ≦ 3.0 mmol/L)[20-22] and in children with heterozygous familial hypercholesterolemia[58]. Subjects ingested 2.2~3.4 g/d for 6~8 weeks could have 8~14% and 7~15% reduction in serum LDL-C and TC. In the hamster model, supplementation of 0.24% (w/w) plant sterol esters in the high cholesterol high fat diet caused significant lowering of plasma total cholesterol and LDL-C[35,59], but plasma plant sterol concentration increased as dietary intake of plant sterol esters exceeded 1.92%[59]. Significantly higher plasma phytosterol concentration might be associated with increased premature cardiovascular disease, but the definite effect of high plasma phytosterols level on atherosclerosis was still under debate[59]. Thus, the dose of plant sterols seems to be important, high doses may not only be ineffective[22,54,59] but may also produce adverse effects, such as high plasma phytosterols concentrations[8]. From the reports in the literature, 1.8~2 g/d of stanols or sterols in the adult human diet seem to offer an ideal dose for lowering cholesterol[8].

In this study, we hoped to develop the phytosterols-containing milk (PSM) and evaluate its blood lipid-lowering effects. From the above discussion, we assume that the adequate content of phytosterols in a healthy adult diet is 1.8 g in 500g dry weight/day, i.e. 0.36%. We, therefore, used 0.36% phytosterol mixture in the hamster's diet as the base dose (1×). Considering the difference between men and hamsters, diets containing 0.72% (2×) and 1.8% (5×) phytosterol mixtures were also examined for the blood lipid lowering effect. The highest phytosterol level should still not affect the hamster plasma phytosterol levels[59]. Reduced cholesterol solubilitzation in bile acid micelles is proposed as the major factor in inhibiting the absorption of cholesterol by phytosterols[60]. Because more hydrophobic sterols have the higher affinity for micelles[5], free plant stanols (especially sitostanols) are believed to be more effective than free sterols in interfering with cholesterol absorption and lowering plasma cholesterol levels[5,22,54]. Recent studies[55,56,61,62], however, indicate that a mixture of plant sterols and stanols may be as effective as stanols alone. In this study we used 2.78% phytosterol mixture, which consists of sterols/stanols at about 4/1 ratio in the phytosterol milk powder (PSMP). The amounts of PSMP in the 1×, 2×, and 5× doses groups were 12.95, 25.9 and 64.75 g/100g diet, respectively.

II. *Hamsters Condition and Body Weight Gain*

Except for group 5×, there were no differences in the mean final body weights and the mean body weight gains at termination of the study (Table 2). Group 5× had more body weight gain than the hyperlipidemic control (group C) ($p < 0.05$), possibly due to milk protein. Casein and whey protein are the major proteins in milk. In general, whey protein is not only a very high-quality protein but also offers additional benefits. Both human

Table 2. Body weights and liver weights of different groups of test aminals

	C	PC	1×	2×	5×
Body weight (g)					
Initial	121.2 ± 20.4	127.1 ± 9.7	120.8 ± 10.3	119.8 ± 5.3	129.5 ± 11.5
Final	141.1 ± 21.4	153.3 ± 9.5	143.0 ± 14.0	139.9 ± 7.8	156.8 ± 15.8*
Gain	19.9 ± 5.5	26.2 ± 8.6	22.2 ± 5.6	20.1 ± 8.5	27.3 ± 6.07*
Liver weight (g)	7.87 ± 1.30	7.15 ± 0.90	7.46 ± 0.84	6.90 ± 0.74	6.17 ± 0.67**
Liver weight/body weight (g/100g)	5.6 ± 0.3	4.6 ± 0.4***	5.2 ± 0.2**	4.9 ± 0.4***	3.9 ± 0.2***

Each value represented as mean ± SD, n = 16.

*Significantly different ($p < 0.05$) when compared with C group by Student's t test.

**Significantly different ($p < 0.01$) when compared with C group by Student's t test.

***Significantly different ($p < 0.001$) when compared with C group by Student's t test.

and animal studies showed that dietary supplementation with whey protein or dairy food could promote immune functions[63]. Full-lifespan studies on Syrian hamsters showed a dramatic increase in lifespan (43~52% increase) with animals on varying levels of whey protein (10~20% in diet), in contrast to a commercial diet[64]. In addition, liver glutathione concentrations were also found to be more than twice as high in whey and casein-fed rats than in soybean-protein fed rats[65].

III. *Plasma Lipid and Lipoprotein Profiles*

Despite strong evidence that phytosterols have a hypocholesterolemic effect in populations consuming high-fat diets, it is not known if they are equally effective in subjects eating low-fat diets. Denke[66] reported that low-dose (3 g/d) sitostanol capsule in low-cholesterol American Heart Association (AHA) Step 2 diet (cholesterol in diet is less than 200 mg/d) lacks cholesterol-lowering activity in men with moderate hypercholesterolemia or normal serum cholesterol level. Above studies suggested that the hypocholesterolemic effect of phytosterols may be affected by the subject's serum cholesterol level and the cholesterol content in the diet. In addition, the decrease in cholesterol is usually higher in subjects with high baseline cholesterol level or with high baseline cholesterol absorption[8]. In the present study, hyperlipidemic hamsters induced by feeding high fat high cholesterol diet were used to evaluate the hypocholesterolemic effect of phytosterols-rich milk.

Table 3 shows the changes in hamster plasma lipids and lipoprotein profiles before and after 4 weeks of feeding period. Initial (Basal group) levels of hamster plasma total cholesterol (TC) and triacylglycerol (TG) were 149.1 and 157.0 mg/dL, respectively. At termination, plasma TC and TG in control group (C) were significantly raised to 468.9 and 328.7 mg/dL ($p < 0.001$). This indicated that 15% fat and 0.5% cholesterol diet has successfully induced hyperlipidemia in the test animals. As compared with the control group, plasma TG of all experimental groups was not significantly different. Concerning plasma TC, all three groups fed with PSMP exhibited significantly lower TC than control group and the cholesterol-lowering effect showed dose dependency. Plasma TC were reduced (compared with the control group) by 15.1%, 30.9% and 50.6% in 1×, 2× and 5× groups, respectively. In lipoprotein profile, 2× and 5× PSMP diets significantly lowered very low density lipoprotein and intermediate density lipoprotein cholesterol (VLDL+IDL)-C ($p < 0.05$) and low density lipoprotein cholesterol LDL-C ($p < 0.001$). Compared with the control group, LDL-C of 2× and 5× groups were reduced by 52.0% ($p < 0.001$) and 72.9% ($p < 0.001$), respectively. As for HDL-C levels, dietary 0.72% phytosterols or 1× and 2× PSMP supplementation showed no effect, but 5 × PSMP caused a significant reduction in HDL-C ($p < 0.01$). The ratio of LDL-C/HDL-C is an index of atherogenicity, we noticed a significant increase of this value in high fat high cholesterol diet (control group) when compared with the basal. Comparing with the control LDL-C/HDL-C, there was a significant decrease in the groups with dietary supplementation of 0.72% phytosterols ($p < 0.05$) or 2× ($p < 0.001$) and 5× ($p < 0.001$) PSMP. The reduction of atherogenic index (LDL-C/HDL-C) by dietary intake of phytosterols was also noticed by previous researchers[22,32].

Dietary cholesterols must be incorporated in bile salt micelles in order to be absorbed by intestinal mucosa[67]. The chemical structures of phytosterols are similar to cholesterol, they can displace cholesterol from bile salt micelles and compete with the site for absorption in the brush border[60], thus suppress the absorption of exogenous (dietary) cholesterol. Most human and animal studies demonstrated that dietary phytosterols significantly reduce plasma cholesterols, especially LDL-C concentration without affecting HDL-C or plasma TG concentrations[5,6,20,27,35,68,69]. Our results showed that the higher the PSMP quantities in the hamster's diet,

Table 3. Plasma cholesterols (mg/dL) and triacylglycerol (mg/dL) concentrations in different groups of test animals

	Base[a]	C	PC	1×	2×	5×
Triacylglycerol	157.0 ± 34.4	328.7 ± 178.7$^{\#\#\#}$	376.3 ± 199.3	361.4 ± 80.9$^{\#\#\#}$	348.5 ± 93.7$^{\#\#\#}$	244.3 ± 83.6$^{\#\#\#}$
Total cholesterol	149.1 ± 27.8	468.9 ± 88.8$^{\#\#\#}$	385.6 ± 92.6$^{\#\#\#}$	398.0 ± 54.9$^{\#\#\#}$*	324.0 ± 56.8$^{\#\#\#}$**	231.8 ± 37.6$^{\#\#\#}$***
(VLDL+IDL)-C	21.3 ± 7.6	134.8 ± 61.9$^{\#\#\#}$	118.9 ± 30.8$^{\#\#\#}$	108.9 ± 16.0$^{\#\#\#}$	87.8 ± 25.5$^{\#\#\#}$*	84.6 ± 29.3$^{\#\#\#}$*
LDL-C	27.8 ± 6.2	123.0 ± 35.6$^{\#\#\#}$	78.9 ± 29.3#*	117.9 ± 32.2$^{\#\#\#}$	59.1 ± 15.3$^{\#\#\#}$***	33.3 ± 8.8***
HDL-C	64.4 ± 11.8	111.9 ± 22.3$^{\#\#\#}$	104.7 ± 15.8$^{\#\#}$	116.2 ± 9.9$^{\#\#\#}$	131.0 ± 29.7$^{\#\#\#}$	93.4 ± 8.6$^{\#\#\#}$**
LDL-C/HDL-C	0.46 ± 0.09	1.12 ± 0.49$^{\#\#\#}$	0.73 ± 0.18$^{\#\#\#}$*	1.12 ± 0.41$^{\#\#\#}$	0.45 ± 0.03***	0.37 ± 0.07$^{\#}$***

[a]Base line (initial) vaule.

Each value represents mean ± SD, n = 8. Plasma samples from both hamsters in the same cage were combined and lipoproteins were isolated by continuous density gradient ultracentrifugation.

means significantly different when compared with the Base group ($p < 0.05$) by Student's t test, ## and ### are at $p < 0.01$ and $p < 0.001$ levels.

* means significantly different when compared with the C group ($p < 0.05$) by Student's t test, ** and *** are at $p < 0.01$ and $p < 0.001$ levels.

the higher were the reductions of hamster's plasma TC, non HDL-C concentrations and LDL-C/HDL-C ratio, while plasma TG level was not affected. The hypocholesterolemic effect of the 1×, 2× and 5× PSMP diet was believed to come from PSMP since the fiber contents of all the diets were kept at around 5% as shown in the lower part of Table 1. These results were consistent with the previous research findings[17,35,59]. Although in our study the concentration of HDL-C was reduced in the 5× group, cholesterol fraction in HDL-C (HDL-C/TC) was increased (HDL-C/TC in control was 30.3% and in 5× group was 44.2%). Some studies indicated that dietary phytosterols could raise lecithin:cholesterol acyltransferase (LCAT), a major component in HDL-C, activity[22,70] and thus HDL-C/TC[5].

IV. *Liver Weight and Liver Lipid Composition*

After feeding high fat and high cholesterol diets for 4 weeks, all hamsters had developed fatty liver by visual observation after sacrifice except for 5× PSMP group. We also noticed that liver weights were decreased as PSMP contents increased in diet (Table 2). The relative liver weight in normal hamsters was 3.2 g/100 g body weight[72]. In our experiment, a high fat high cholesterol diet caused an increase in relative liver weight to 5.6 g/100 g (control group) and was reduced by dietary supplementation of 0.72% phytosterols (PC group) or PSMP (the relative liver weight decreased by 18% in PC group and 8, 12.5, 30% in 1×, 2× and 5× PSMP groups, respectively). Ntanios and Jones[73] reported that hamsters fed with high cholesterol diet and 1% sitostanol could reduce 21% relative liver weight, which was similar to our findings. Table 4 displayed the hepatic lipid profiles in different groups of test animals. Hepatic cholesterol and TG concentrations were significantly increased in the control group as compared with base group. Feeding of phytosterols or PSMP significantly decreased hepatic cholesterol (decreased by 29.2% in PC group and 34.7, 37.3, 65.0% in 1×, 2× and 5× PSMP groups, respectively) and TG (decreased by 24.4% in PC group and 19.9, 50.0, 49.4% in 1×, 2× and 5× PSMP groups, respectively) concentrations. Lin[35] indicated that 0.24% phytosterol esters could decrease hepatic cholesterol concentration by 44.3% in hamsters which were fed with high fat, high cholesterol diet for 5 weeks. It was suggested that the increase in hamster's relative liver weight by high fat, high cholesterol diet might be due to increased accumulation of cholesterol and TG in liver[35]. Dietary phytosterols and PSMP were observed to reduce hepatic cholesterol and TG levels in this experiment and thus assumed to be the reason for the decreased relative liver weight.

Phytosterols are poorly absorbed, interfere with biliary cholesterol absorption, and accelerate bile acid and biliary cholesterol elimination[4,5]. Then, the conversion of cholesterol to bile acid is greatly enhanced to maintain the pool of bile acid[74]. This may accelerate the hepatic cholesterol lowering rate. Furthermore, phytosterol was able to reduce liver acetyl-CoA carboxylase and malic enzyme activities[70], alter lipid metabolism in liver, and possibly decrease fatty synthesis. This might explain

Table 4. Hepatic lipid profiles in different groups of test animals

	Base	C	PC	1×	2×	5×
Cholesterol (mg/g liver)	6.4 ± 0.2	34.3 ± 8.0$^{###}$	24.3 ± 2.1$^{###}$**	22.4 ± 3.8$^{###}$***	21.5 ± 3.9$^{###}$***	12.0 ± 3.8$^{###}$***
(mg/liver)		270.4 ± 80.50	173.5 ± 13.3**	167.3 ± 23.6***	148.1 ± 19.6***	74.2 ± 15.5***
Triacylglycerol (mg/g liver)	4.8 ± 1.1	17.6 ± 6.2$^{###}$	13.3 ± 1.8$^{###}$*	14.1 ± 1.8$^{###}$*	8.8 ± 0.8$^{###}$**	8.9 ± 2.4$^{###}$**
(mg/liver)		138.5 ± 62.4	94.7 ± 11.3*	104.6 ± 11.5	61.2 ± 4.2***	54.5 ± 9.6***

Each value represents mean ± SD, n = 16.

means significantly different when compared with the Base group ($p < 0.05$) by Student's t test, ### is at $p < 0.001$ level.

* means significantly different when compared with the C group ($p < 0.05$) by Student's t test, ** and *** are at $p < 0.01$ and $p < 0.001$ levels.

Table 5. Fecal cholesterol and β-sitosterol contents of different groups of test animals

	Base	C	PC	1×	2×	5×
Cholesterol (mg/g feces)	1.0 ± 0.5	6.7 ± 2.0$^{###}$	17.6 ± 2.1$^{###}$***	12.4 ± 1.6$^{###}$***	22.6 ± 1.7$^{###}$***	41.0 ± 3.6$^{###}$***
β-sitosterol (mg/g feces)	ND[a]	3.1 ± 1.1	41.0 ± 7.9***	13.6 ± 1.4***	34.7 ± 3.4***	102.0 ± 8.4***

[a]Not detected.

Each value represents mean ± SD, n = 8. Fecal samples from both hamsters in the same cage were combined.

means significantly different when compared with the Base group ($p < 0.05$) by Student's t test, ### is at $p < 0.001$ level.

* means significantly different when compared with the C group ($p < 0.05$) by Student's t test, *** is at $p < 0.001$ level.

why hepatic TG concentrations were decreased in the PC and PSMP groups.

V. *Fecal Cholesterol and Phytosterols Analysis*

Fecal cholesterol in normal hamsters before experiment (base group) was 1.0 mg/g dry weight (Table 5) and β-sitosterol was not detected in feces. At end of the study, fecal cholesterol in the control group rose to 6.7 ± 2.0 mg/g dry weight ($p < 0.001$) and β-sitosterol was 3.1 ± 1.1 mg/g dry weight. The risen fecal cholesterol might be due to the higher cholesterol contents in control diet, and fecal β-sitosterol might be related to corn oil in the diet. Refined corn oil contains 7.15~9.52 g/kg of phytosterols and 6.90 g/kg of β-sitosterol, which are higher than sunflower, cottonseed, soybean, olive and palm oils[8]. Comparing with control group, diets with phytosterols or PSMP significantly increased fecal cholesterol and β-sitosterol concentrations, in proportion to the dietary PSMP contents.

An elevated plasma cholesterol concentration, especially LDL-C, increases the risk of atherosclerosis and coronary heart disease (CHD)[1,2]. The net effect of dietary cholesterol absorption, endogenous cholesterol synthesis and biliary cholesterol excretion regulates body cholesterol balance[74,75]. Results of this study confirmed that the cholesterol-lowering effect of phytosterols is not only due to the inhibition of intestinal dietary cholesterol absorption but also due to the interference of biliary cholesterol re-absorption.

CONCLUSIONS

Feeding with high fat (15%) and high cholesterol (0.5%) diet for 4 weeks successfully induced hypercholesterolemia in hamsters. There were no adverse effects of plant sterols/stanols-containing milk powder (PSMP) on the growth and health condition of hamsters. Even when high dose PSMP (64.75%, w/w) was fed, hamsters had the highest body weight gain and lowest plasma and liver lipid contents. PSMP showed significant effect on lowering plasma TC, non HDL-C concentrations, atherogenic index, hepatic lipid level, and relative liver weight as well as raising fecal cholesterol and phytosterols contents.

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