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Effects of Hydrophilic and Lipophilic β-Sitosterol Derivatives on Cholesterol Absorption and Plasma Cholesterol Levels in Rats

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The effects of two phytosterol derivatives of β-sitosterol, a lipophilic derivative (LPSS) and a hydrophilic derivative (HPSS), on cholesterol uptake and blood lipoprotein levels in rats were compared with those of β-sitosterol. LPSS and HPSS have solubilities of up to 0.05 g/mL in edible oil and 0.15 g/mL in water at 25 °C, respectively. The intragastric administration of either 30 or 50 mg of phytosterols with 10 mg of [4-^{14}C]-cholesterol per kg of body weight once a day for 3 consecutive days reduced cholesterol uptake by approximately 30% compared to controls that received cholesterol alone. Feeding a cholesterol-enriched diet containing 1% or 3% β-sitosterol, LPSS, or HPSS for 2 and 4 weeks resulted in lowered levels of total blood cholesterol and reduced the atherogenic index in all groups. These results indicate that LPSS and HPSS have comparable effects to β-sitosterol in lowering blood cholesterol levels but they differ from β-sitosterol in having a solubility advantage.

KEYWORDS: β-Sitosterol; hydrophilic derivative; lipophilic derivative; cholesterol absorption; plasma cholesterol level

INTRODUCTION

Phytosterols (plant sterols) are present in all foods of plant origin and are particularly abundant in seeds, nuts, and vegetable oils at concentrations of up to 5%. They resemble cholesterol in function (essential constituents of cell membranes) and structure. Whereas the side chain of cholesterol is composed of 8 carbon atoms, most phytosterol side chains contain 9 or 10 carbons. Phytosterols can be classified as stigmasterol, spinasterol, campesterol, and sitosterol, and also, for example, sitosterol has α-, β-, γ-type.

Among the phytosterols, β-sitosterol (24-ethyl-5-cholestene-3-ol) has demonstrated the greatest potential for the production of steroidal drugs and food ingredients that function as cholesterol-lowering agents (*1*). Since β-sitosterol was found to lower serum cholesterol levels by inhibiting the absorption of cholesterol in the intestines through competition with LDL-cholesterol (LDL-C) in both animal and human studies (*2–4*), numerous studies have investigated the potential applications of β-sitosterols as blood cholesterol-lowering agents not only for pharmaceutical formulations but also for use as a food ingredient. Their effectiveness and safety have also been demonstrated, as summarized in a recently published review (*5*). However, free forms of phytosterols are soluble neither in water nor in various plant oils. Due to their insolubility in water and poor solubility in oil, phytosterols are restricted in their applications.

To overcome this problem, many studies have focused on the chemical modification of β-sitosterol, mainly through the 3-hydroxy group. The first approaches to increase the solubility of β-sitosterol in fats were carried out by esterification with fatty acids (*6*), and many synthetic methods using fatty acid anhydride (*7*), acyl chloride (*8*), or a basic catalyst (*9*) were investigated. Lipase-catalyzed synthesis of β-sitosterol esters has also been reported (*10, 11*). β-Sitosterol ester compounds were reported to have the same cholesterol-lowering effects as the parent molecule, and margarine containing a β-sitosterol ester (*12, 13*) produced by transesterification of β-sitosterol with a fatty acid methyl ester derived from canola oil finally entered the United States market in 1999. On the other hand, approaches to increase the water solubility of β-sitosterol for potential applications in nonlipid-based foods were carried out by formulations with emulsifiers. Lecithin-emulsified micelles of β-sitosterol were reported to have a similar cholesterol-lowering effect (*14, 15*). Recently, we have synthesized two derivatives of β-sitosterol by chemical modification: lipophilic derivative (LPSS) and hydrophilic derivative (HPSS). LPSS is an oil-soluble β-sitosterol derivative which is esterified with oleic acid (*9*). HPSS is a new type of hydrophilic phytosterol derivative

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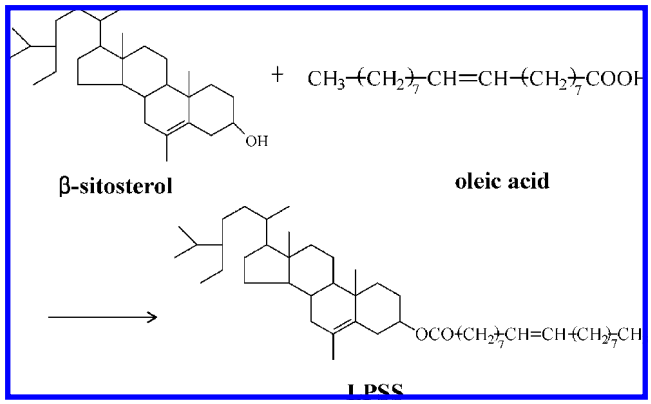

Figure 1. Synthesis of LPSS.

that is synthesized by coupling β-sitosterol with a hydrophilic polymer (*16*).

In this study, the effects of these two phytosterol derivatives, LPSS and HPSS, on the cholesterol uptake and blood lipoproteins levels in rats were compared with those of β-sitosterol.

MATERIALS AND METHODS

Materials. β-Sitosterol, poly(ethylene glycol) (PEG, MW = 1500), succinic anhydride, 1,3-dicyclohexylcarbodiimide (DCC), and 4-dimethylaminopyridine (DMAP) were purchased from Aldrich and used without further purification. Cholesterol and cholic acid were purchased from Sigma (St. Louis, MO). [4-^{14}C]-Cholesterol (specific activity, 51 mCi/mol; concentration, 0.04 mCi/ml in ethanol) was obtained from NEN Life Science Products (Boston, MA). Ultima Gold scintillation cocktail was purchased from Packard Instruments (Meriden, CT). Tubes used for collecting blood were from the Vaccutainer system (Becton Dickinson, U.S.A.). Diethylether was purchased from Junsei Co. (Japan).

Preparation of LPSS. LPSS was prepared as previously described (*9*). Briefly, β-sitosterol was esterified with oleic acid in the presence of a basic catalyst (DMAP) and dehydrating agents (DCC) at 25 °C, and the product was purified by recrystallization in ethyl alcohol (**Figure 1**). The structure of LPSS was confirmed by ^{1}H NMR in $CDCl_3$.

Preparation of HPSS. HPSS was synthesized by a two-step reaction as previously described (*16*) (**Figure 2**). The first step was the synthesis of an intermediate (carboxyethyl-β-sitosterol, CES), which affords carboxylic functionality to β-sitosterol, and the second step was the coupling of the hydrophilic polymer (PEG), having a molecular weight of 1500, to CES in the presence of DMAP and DCC. The characterizations of CES and HPSS were carried out by ^{1}H NMR and matrix-assisted laser desorption ionization mass spectrometry.

Preparation of Diet. To prepare the cholesterol-enriched diet, the pellet diet for rodents (Purina Korea, Korea) was crushed to a powder using a mill, and given amounts of HPSS, LPSS, or sitosterol along with 1% cholesterol and 0.5% cholic acid were added and mixed very well by passing the powder through a sieve.

Animal Treatment. The 4 or 6 week old SPF male Sprague−Dawley rats were obtained from Dai-Han animal breeding center (Eumsung, Korea). Rats were caged under a supply of filtered, pathogen-free air at a temperature between 20 and 23 °C with a 12 h light−dark cycle and a relative humidity of 50%. Animals were allowed free access to tap water and the pellet diet (Jeiljedang, Korea) during a 1 week acclimatization period. Animal studies were conducted in accordance with the institutional guidelines for care and use of laboratory animals.

For cholesterol absorption experiments, 7 week old rats were randomly divided into eight groups consisting of five animals each. The grouping and treatment of the rats are summarized in **Table 1**. [4-^{14}C]-Cholesterol was administered intragastrically once per day for 3 consecutive days with the indicated dose of phytosterol. After deprivation of the diet from 9 a.m. to 2 p.m., the rats were dosed with the cholesterol and phytosterol via oral gavage and were allowed free access to the diet and water 3 h later.

To determine the cholesterol-lowering effects of the phytosterol derivatives, hypercholesterolemia was induced in the rats by feeding a cholesterol-enriched diet containing 1% cholesterol and 0.5% cholic acid. Forty 5 week old male rats were randomly divided into eight groups. After an overnight fast, each group was administrated the following diet for 2 and 4 weeks: (1) background control (BC) = normal powder diet, (2) control (NC) = high-fat powder diet that contains 1% cholesterol + 0.5% cholic acid, (3) 1× HPSS = high-fat powder diet containing 1% HPSS, (4) 3× HPSS = high-fat powder diet containing 3% HPSS, (5) 1× LPSS = high-fat powder diet containing 1% LPSS, (6) 3× LPSS = high-fat powder diet containing 3% LPSS, (7) 1× sitosterol = high-fat powder diet containing 1% β-sitosterol, (8) 3× sitosterol = high-fat powder diet containing 3% β-sitosterol. Rats were starved overnight before being sacrificed, and blood was collected from the heart under ether anesthesia.

Measurement of [4-^{14}C]-Cholesterol in Blood. Collected Blood was centrifuged at 2000*g* for 20 min to prepare plasma. Amounts of 1.5 mL of supernatant (plasma) and 10 mL of scintillation cocktail were mixed and counted using a liquid scintillation counter (Tri-Carb 2100 Tr, Packard) for 2 min per sample.

Analysis of Blood Lipoproteins and Clinical Markers. Blood was centrifuged at 1500*g* for 20 min at 4 °C to prepare plasma. The lipoproteins (total cholesterol, TC; HDL-cholesterol, HDL-C; LDL-cholesterol, LDL-C; triglyceride, TG), alanine aminotransferase (ALT), albumin, and creatine in the plasma were analyzed using an automatic analyzer Hitachi model 747 in the clinical laboratory at the Korea University Medical Center.

Data Analysis. All data are represented as mean ± SD. Statistical differences were calculated using a one-way analysis of variance (ANOVA), followed by the Student's *t* test. Statistical significance was defined as a probability of less than 0.05.

RESULTS AND DISCUSSION

Preparation of LPSS and HPSS. Lipophilic derivatives of β-sitosterol are usually synthesized using fatty acids (*6*) or fatty acid methyl esters (*17, 18*). However, LPSS in this study is composed of β-sitosterol and an unsaturated fatty acid (oleic acid) in order to compare the solubility of LPSS with a derivative containing a saturated fatty acid (stearic acid). In fact, the solubility of LPSS in edible oil was 3 times higher than that of a derivative with stearic acid (*9*).

HPSS was synthesized by coupling a hydrophilic polymer (PEG), having a molecular weight of 1500, to an intermediate having carboxylic groups. Since one chain of PEG has two −OH groups at both ends, two sterol moieties can be introduced into one PEG chain. However, according to our previous results (*16*), an HPSS derivative with two sterol moieties at both ends of the PEG chain was not soluble in water, and the water solubility significantly depended on the degree of substitution (DS, average molar number of β-sitosterol moieties per molecule of HPSS). In this study, we used an HPSS molecule with a DS = 1.08, which is soluble in water at concentrations of up to 0.15 g/mL at 25 °C.

Effect of Phytosterol Derivatives on Cholesterol Uptake in Rats. Although the cholesterol-lowering mechanism of phytosterols is not fully understand, they appear to inhibit the intestinal absorption of cholesterol by competing with cholesterol for incorporation into mixed micelles or hindering cholesterol esterification in the intestinal mucosal cells (*17*). HPSS and LPSS are synthetic β-sitosterol derivatives with water- or lipid-soluble side chains, and the size of each molecule is bigger than that of β-sitosterol.

In order to compare the ability of these synthetic phytosterol derivatives to inhibit cholesterol uptake with that of β-sitosterol, rats were treated with 30 (3×) or 50 mg (5×) of β-sitosterol, LPSS, or HPSS, along with 10 mg of [4-^{14}C]-cholesterol per kg body weight once a day for 3 consecutive days. The 3× and

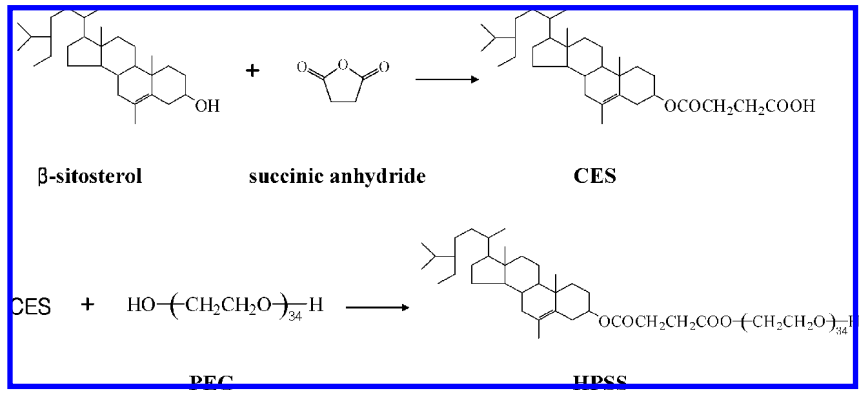

Figure 2. Synthesis of HPSS.

Table 1. Experimental Groups and Treatments of Rats for Cholesterol Uptake Experiments

	control		positive control		LPSS		HPSS	
experimental groups	background control (BC)	negative control (NC)	3× sitosterol	5× sitosterol	3× LPSS	5× LPSS	3× HPSS	5× HPSS
amount of cholesterol	10 mg cold cholesterol in 0.5 mL corn oil	0.025 mg [4-^{14}C]-cholesterol in 0.08 mL of ethanol and 10 mg cold cholesterol in 0.5 mL of corn oil						
samples			β-sitosterol		LPSS		HPSS	
amounts of samples			30 mg	50 mg	30 mg	50 mg	30 mg	50 mg

5× doses of β-sitosterol similarly inhibited cholesterol uptake by 30.6% (±8.85%) and 30.1% (±3.47%), respectively (**Figure 3**). The inhibition of cholesterol absorption by β-sitosterol was relatively low compared with the results of Ikeda et al. (*18*). They reported that the intragastric administration of a single emulsified lipid meal containing 25 mg of [^{3}H] cholesterol and 25 mg of sitosterol inhibited the lymphatic absorption of cholesterol by 57% in 24 h. In this study, we administered a corn oil suspension containing 10 mg each of cholesterol and β-sitosterol to rats for 3 consecutive days. Several mechanisms for the lowering of blood TC were assumed. Phytosterol-mediated inhibition of cholesterol absorption was suggested to function by competing with cholesterol at steps essential for absorption including micellar solubilization, uptake by the brush border membrane, intracellular esterification, and/or incorporation into chylomicrons. Additional phytosterol-specific mechanisms at absorption sites on enterocytes or within enterocytes cannot be ruled out (*19, 20*). Among these hypotheses, the displacement of cholesterol from micelles may represent the major mechanism of plant sterol-mediated inhibition of cholesterol absorption (*18, 19, 21*). Thus, the difference in levels of inhibition between this experiment and other studies might be due to variations in micellar solubilization by the formulations of β-sitosterol and/or cholesterol.

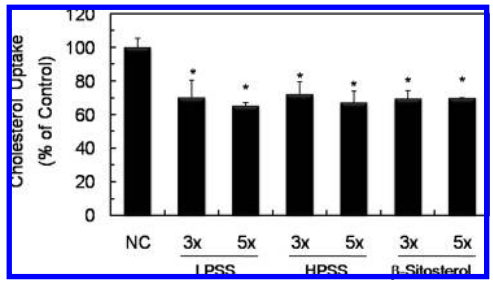

Figure 3. Inhibition of exogenous cholesterol absorption by β-sitosterol and phytosterol derivatives, HPSS and LPSS. Male Sprague−Dawley rats were intragastrically administered a suspension of [4-^{14}C]-cholesterol at a concentration of 10 mg/kg either alone (negative control, NC) or with 30 or 50 mg/kg doses of β-sitosterol suspension, LPSS solution, or HPSS solution once a day for 3 consecutive days. Rats in the background control group (BC) were intragastrically treated the cold cholesterol (10 mg/kg) alone. Blood was collected and analyzed for sterol radioactivity by liquid scintillation counting. Results are the mean ± SD for $n = 5$. * and ** represent significant differences from the control group (NC) with $P < 0.05$ and $P < 0.01$, respectively.

Administration of LPSS or HPSS inhibited cholesterol uptake in a dose-dependent manner. The plasma radioactivity was lowered 29.8% (±14.63%) and 34.8% (±5.51%) by 3× and 5× LPSS treatment, respectively. Administration of 3× and 5× HPSS reduced cholesterol uptake by 27.8% (±11.7%) and 32.9% (±11.0%), respectively (**Figure 3**). LPSS and HPSS are both esterified derivatives and were dissolved in corn oil and water, respectively. The use of fatty acid esters of plant sterols is based on their efficient hydrolysis by pancreatic cholesterol esterases (EC 3.1.1.13), which release free plant sterols, the form that actively reduces cholesterol absorption (*22*). HPSS and LPSS might be hydrolyzed to β-sitosterol in the intestine in order to inhibit the uptake of cholesterol. The inhibition of cholesterol uptake by HPSS, LPSS, and β-sitosterol did not show significant differences, and a 3-fold ratio of phytosterol to cholesterol seemed to result in a maximal inhibition of cholesterol uptake by around 30%. Thus, their hydrolysis rates might not differ significantly.

Effect of Phytosterol Derivatives on the Levels of Blood Lipoproteins in Rats. Hypercholesterolemia was induced in rats by feeding a cholesterol-enriched diet consisting of 1% cholesterol, 0.5% cholic acid, and phytosterol derivatives. The dosages of phytosterols were chosen as 1× and 3× the amount of cholesterol based on the data obtained for cholesterol uptake. Yang and Koo (*23*) reported that cholic acid (0.5%) increases micelle formation and facilitates intestinal absorption of cholesterol. Previous studies have shown that feeding rats with diets supplemented with 1% cholesterol and 0.5% cholic acid for 5 (*23*) or 10 days (*24*) was sufficient to induce hypercholesterolemia. In this experiment, the serum TC level was increased to 206.6 ± 53.1 mg/dL at 2 weeks in the control group and there were no significant changes during the feeding period from 2 weeks to 4 weeks (**Figure 4A**). This result is in accord with a previous report (*23*) that feeding rats with 1% cholesterol and 0.5% cholic acid for 5 days was sufficient to elevate the serum

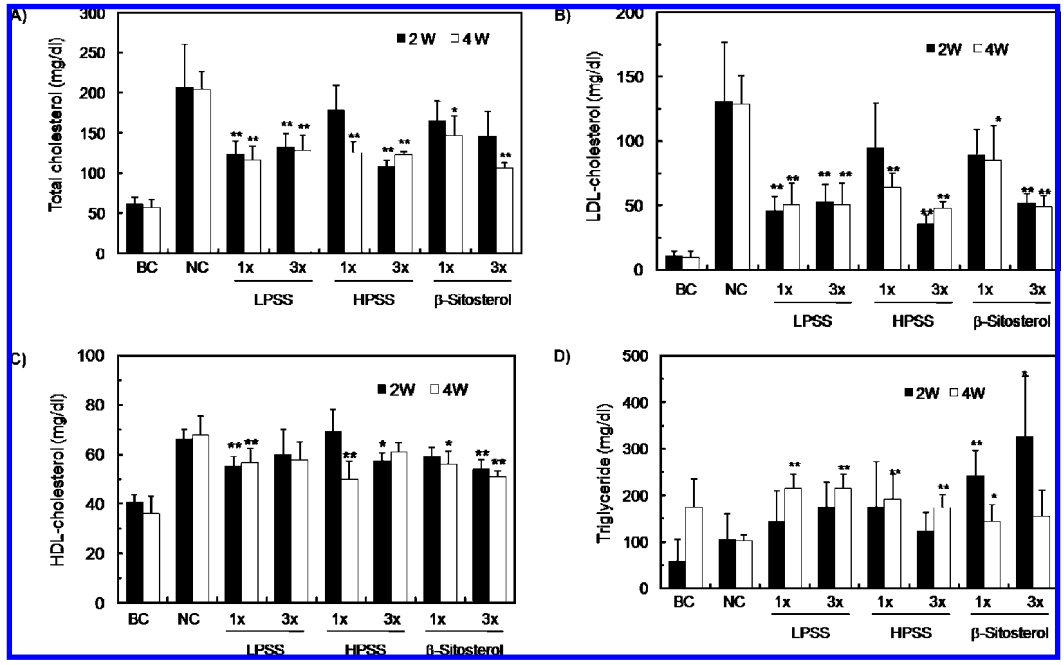

Figure 4. Effect of phytosterol derivatives on the plasma levels of (**A**) total cholesterol, (**B**) LDL-C, (**C**) HDL-C, and (**D**) triglycerides in rats fed the cholesterol-supplemented diet for 2 or 4 weeks. Values are expressed as mean ± SD of five rats in each group. ∗ and ∗∗ represent significant differences from the control group (NC) with $P < 0.05$ and $P < 0.01$, respectively.

TC level to 238 mg/dL and that no further increase was observed when the duration of feeding was extended to 10 days.

Lipoprotein levels, particularly TC and LDL-C, in the NC group were significantly increased compared to the BC group, except for the triglyceride levels, and most of the increase in TC was due to LDL-C (**Figure 4**). Thus, feeding rats the cholesterol-supplemented diet reduced the ratios of HDL-C/LDL-C and HDL-C/TC ($P < 0.01$) and elevated the atherogenic index ($P < 0.01$) from 0.49 ± 0.15 and 0.58 ± 0.10 to 2.09 ± 0.65 and 2.04 ± 0.37 at 2 and 4 weeks, respectively (**Figure 5**). These data concur with the results of many studies showing that phytosterol decreased intestinal cholesterol absorption and subsequently, LDL-C (*18, 20, 25*).

Although the level of serum TC was not significantly lowered in the 1× LPSS and β-sitosterol groups at 2 weeks, it was significantly lowered in all groups at 4 weeks (**Figure 4A**). These results support numerous previous reports that plant sterols significantly reduced not only the total concentration of cholesterol but also LDL-C levels in rats fed a cholesterol-supplemented diet (*18, 25, 26*). HPSS significantly reduced the levels of TC and LDL-C at both doses after 2 weeks ($P < 0.05$). The levels of TC and LDL-C in the 3× HPSS group were 38.9% (±6.84%) and 50.2% (±8.48%) lower than those of the NC group, respectively, at 2 weeks ($P < 0.01$), but they were not lower in the 1× HPSS group at 2 weeks (**Figure 4**, parts **A** and **B**). Although the 1× β-sitosterol group did not have an improved AI or HDL-C/TC ratio at 4 weeks, the 3× β-sitosterol group did show improvements in a time-dependent manner (**Figure 5**, parts **B** and **C**). The LPSS and 3× HPSS groups had improved ratios of HDL-C/LDL-C and HDL-C/TC and an improved AI at 2 weeks. 1× HPSS lowered the AI by 25.7% at 4 weeks ($P < 0.05$) but not at 2 weeks. These results might indicate that the solubility of phytosterols in lipids can affect the time required to lower the levels of blood cholesterol, which is consistent with a report by Ikeda and Sugano (*27*). According to their results, β-sitosterol did not disturb cholesterol absorption until micelles in the intestinal lumen were saturated with sterols.

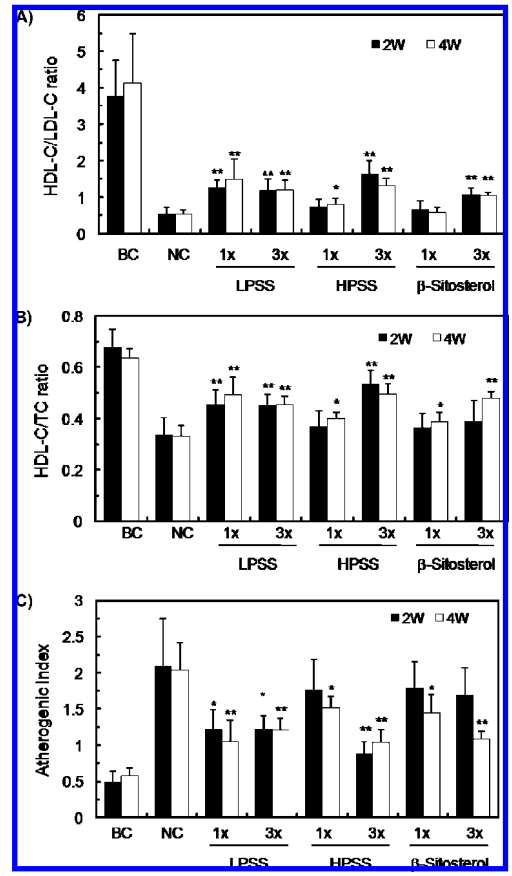

Figure 5. Effect of phytosterol derivatives on the (**A**) HDL-C/LDL-C ratio, (**B**) HDL-C/total cholesterol ratio, and (**C**) atherogenic index in rats fed the cholesterol-supplemented diet for 2 or 4 weeks. Atherogenic index = (TC − HDL-C)/HDL-C. Values are expressed as mean ± SD of five rats in each group. ∗ and ∗∗ represent significant differences from the control group (NC) with $P < 0.05$ and $P < 0.01$, respectively.

They suggested that restriction of the micellar solubility of cholesterol, rather than inhibition of uptake from the brush

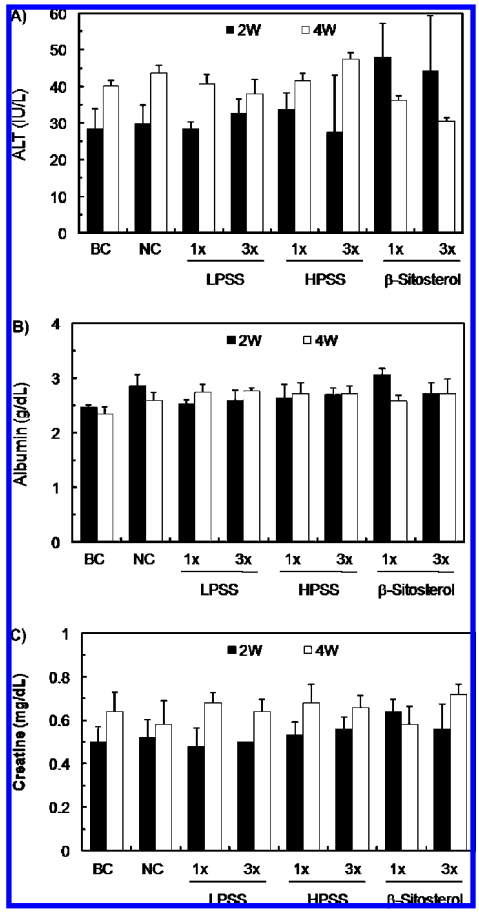

Figure 6. Effect of phytosterol derivatives on liver function as determined by levels of (**A**) alanine−leucine transferase and (**B**) albumin and on kidney function as determined by levels of (**C**) creatine, in rats fed the cholesterol-supplemented diet for 2 or 4 weeks. Values are expressed as mean ± SD of five rats in each group. * and ** represent significant differences from the control group (NC) with $P < 0.05$ and $P < 0.01$, respectively.

border membrane, is the major determinant for the interference of β-sitosterol with cholesterol absorption.

Cholesterol feeding or phytosterol derivative treatments imposed no adverse effects on the apparent growth of the rats, and there were no differences in the percentage increase in body weight and food consumption when compared with the NC group (data not shown). The average food intake for the 4 week period of the eight dietary regimes was 18 g/day/rat. Functional changes in the liver and kidneys of the rats did not appear to be significant based on the blood levels of albumin, ALT, and creatine in all eight groups during the administration of the cholesterol-supplemented diet containing phytosterols (**Figure 6**). Numerous studies in humans and animals have shown that plant sterols or stanol esters are safe and do not have any adverse effects, including gastrointestinal effects (*19*). Our results also showed that HPSS and LPSS are safe in rats fed for 4 weeks.

The solubility of β-sitosterol is extremely low in edible oil and fats and is also low in water. To overcome the solubility problem of β-sitosterol, plant sterols, or plant stanol esters with fatty acids derived from vegetable oils were prepared. Due to their fatlike properties, phytosterol esters can easily be added into a wide variety of food products to provide a means of introducing the daily amount of phytosterol needed for optimal reduction of cholesterol absorption without affecting the taste of the final product (*19*). The cholesterol-lowering effects of plant sterols or plant stanol esters in several products such as margarine, milk, and yoghurt on hyperlipidemic subjects have been widely studied. The wide variety of reported LDL-C responses (reductions of LDL-C by 5−15.5%) to stanols/sterols may be accounted for by different doses (1.6−3.0 g/day), differences in their esterification levels, differences in how they are ingested (with or without a meal), and differences in the genotypes of the subjects (*28–31*). Although there are extensive studies on lipid-soluble phytosterol esters in combination with several types of vegetable oils, there are currently no reports on water-soluble phytosterol esters. In this study, we reported that HPSS, a water-soluble phytosterol ester, showed a cholesterol-lowering efficacy similar to β-sitosterol in rats.

In summary, this study revealed that HPSS and LPSS supplements decreased the cholesterol uptake and improved the AI index in rats fed a cholesterol-supplemented diet via interference with fat absorption similar to β-sitosterol. The findings of this study suggest that HPSS can be added as a supplement to many kinds of foods such as milk, drinks, steak sauces, or coffee and that it might help prevent atherosclerotic diseases.

ABBREVIATIONS USED

HPSS, hydrophilic derivative of β-sitosterol; LPSS, lipophilic derivative of β-sitosterol; B.W., body weight; PEG, poly(ethylene glycol); DCC, 1,3-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; CES, carboxyethyl-β-sitosterol; DS, the degree of substitution; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglyceride; ALT, alanine aminotransferase.

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