Vegetable Oils High in Phytosterols Make Erythrocytes Less Deformable and Shorten the Life Span of Stroke-Prone Spontaneously Hypertensive Rats[1,2]

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ABSTRACT Previous studies have shown that canola oil (CA), compared with soybean oil (SO), shortens the life span of stroke-prone spontaneously hypertensive (SHRSP) rats, a widely used model for hemorrhagic stroke. SHRSP rats are highly sensitive to dietary cholesterol manipulations because a deficiency of membrane cholesterol makes their cell membranes weak and fragile. Phytosterols, abundant in CA but not in SO, can inhibit the absorption of cholesterol and also replace a part of cholesterol in cell membranes. This study was performed to determine whether the high concentration of phytosterols in CA might account for its life-shortening effect on SHRSP rats. Male, 35-d-old SHRSP rats ($n = 28$/group) were fed semipurified diets containing CA, SO, CA fortified with phytosterols (canola oil + phytosterols, CA + P), SO fortified with phytosterols (soybean oil + phytosterols, SO + P), corn oil (CO), olive oil (OO) or a fat blend that mimicked the fat composition of a representative Canadian diet (Canadian fat mimic, CFM; 10 g/100 g diet). These fats provided 97, 36, 207, 201, 114, 27 and 27 mg phytosterols/100 g diet, respectively. Ten rats from each group were killed after 30–32 d for blood and tissue analyses. The remaining rats (18/group) were used for determination of life span. The life span of SHRSP rats fed the high phytosterol oils (CA, CA + P, SO + P and CO) was significantly ($P < 0.05$) shorter than that of CFM- and SO-fed rats. At 30–32 d, the groups fed the high phytosterol oils had greater levels of phytosterols and significantly ($P < 0.05$) higher ratios of phytosterols/cholesterol in plasma, RBC, liver and kidney, and a significantly ($P < 0.05$) lower RBC membrane deformabilty index than the groups fed oils low in phytosterols (SO, OO and CFM). The mean survival times were correlated with RBC deformability index ($r^2 = 0.91$, $P = 0.0033$) and cholesterol concentration ($r^2 = 0.94$, $P = 0.0016$), and inversely correlated with RBC phytosterol concentration ($r^2 = 0.58$, $P = 0.0798$) and phytosterols/cholesterol ($r^2 = 0.65$, $P = 0.0579$), except in the OO group. This study suggests that the high concentration of phytosterols in CA and the addition of phytosterols to other fats make the cell membrane more rigid, which might be a factor contributing to the shortened life span of SHRSP rats. J. Nutr. 130: 1166–1178, 2000.

KEY WORDS: • *canola oil* • *cell membrane deformability* • *life span* • *phytosterols* • *stroke-prone spontaneously hypertensive rats*

In stroke-prone spontaneously hypertensive (SHRSP)[4] rats, a model for human essential hypertension and hemorrhagic-stroke (Yamori et al. 1984a, 1984b, 1987 and 1989, Yamori 1989), various dietary factors influence the development of stroke. Salt loading accelerates the development of stroke, whereas increases in the intakes of potassium, calcium, fiber, protein, fish oil and cholesterol attenuate the deleterious effects of salt (Hamano et al. 1995, Hobbs et al. 1996, Howe et al. 1989, Yamori et al. 1976, 1984a, 1987 and 1989). Recently, a series of studies performed in Japan reported that the type of dietary fat can also influence the life span of these rats (Huang et al. 1996 and 1997, Miyazaki et al. 1998a and 1998b). In these studies, canola oil in particular shortened the survival time in rats given 10 g/L NaCl by ~15–50% compared with soybean oil. The mechanisms and the factors underlying the different effects of dietary fats have not yet been clarified. Nevertheless, a previous study in our laboratory showed that the fatty acid profile of canola oil had no overt influence on the life span of SHRSP rats (Ratnayake et al. 2000). The mean survival time correlated inversely with the level of phytosterols

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[4] Abbreviations used: CA, canola oil; CA + P, canola oil fortified with phytosterols; CFM, Canadian fat mimic; CHD, coronary heart disease; CO, corn oil; DI, deformability index; EI max, maximum elongation; EI min, minimum elongation; GLC, gas-liquid chromatography; OO, olive oil; SHRSP, stroke-prone spontaneously hypertensive rats; SO, soybean oil; SO + P, soybean oil fortified with phytosterols.

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in the diets and tissues. These results suggested a possible antinutritional effect of phytosterols on SHRSP rats.

Research conducted over the last four decades has demonstrated that dietary phytosterols can inhibit the intestinal absorption of dietary and biliary cholesterol, thus reducing serum cholesterol levels (Ling and Jones 1995, Pollak and Kritchevsky 1981). Phytosterols can also replace cholesterol in the cell membranes and thereby alter cell membrane physical properties. In an in vitro experiment, treatment of isolated human RBC with various types of sterols led to partial replacement of membrane cholesterol by sterols, resulting in a significant increase in the osmotic fragility of RBC (Bruckerdorfer et al. 1969). In an in vivo study, the rigidity of liver microsomes was significantly increased in Wistar rats fed a diet enriched with β-sitosterol and campesterol (Leiken and Brenner 1989).

SHRSP rats have defective, abnormal and more fragile cell membranes than other rat strains; this is thought to be a contributing factor in their development of cerebral hemorrhage (Yamori et al. 1980 and 1984a). The cell membrane abnormalities in SHRSP rats are due primarily to a deficiency of cholesterol in blood and in cell membranes. Hamano et al. (1995) demonstrated that incorporation of cholesterol into the diet delays the development of stroke and prolongs the life span of SHRSP rats, which suggests that cholesterol is an essential dietary component for this strain of rats. Most likely, the integrity of the cell membrane was improved by dietary cholesterol.

Considering the above, we suggest that increased intakes of phytosterols might result in partial replacement of cholesterol in cell membranes, making the cell membrane more fragile, thereby exacerbating the development of hemorrhagic stroke and shortening the life span of SHRSP rats. Vegetable oils are the primary sources of phytosterols. Canola oil, compared with soybean oil and other common vegetable oils such as olive oil, contains more phytosterols because of its higher proportions of β-sitosterol, campesterol and brassicasterol. The objective of this study was to determine whether the life-shortening effect of canola oil on SHRSP rats was due to its high concentration of phytosterols. To assess the underlying mechanism of action of phyotsterols, we also investigated the effects of phytosterols on hematologic variables and the deformability of RBC membranes of SHRSP rats.

The study compared the effects of canola oil on the above-mentioned variables in SHRSP rats, with soybean, canola and soybean oils fortified with a mixture of phytosterols isolated from a canola oil deodorizer distillate, corn oil and olive oil. A mixture of fats that mimicked the fat composition of a representative Canadian diet was also included in the study. The Canadian fat mimic contained both cholesterol and phytosterols.

MATERIALS AND METHODS

Preparation of phytosterols from canola oil deodorizer distillate. A mixture of phytosterols was prepared from canola oil deodorizer (CanAmera Foods, Altona, Canada). In a typical preparation, 1 kg of canola deodorizer distillate containing 19.9 g total sterols/100 g material was dissolved in methanol (1 L) and saponified for 1 h with 500 g/L NaOH (196 g). The resulting soaps were acidified with 370 g/L HCl (35 mL) and esterified by adding to the saponification reaction mass 370 g/L HCl (255 mL) and refluxing for 1 h. On cooling to room temperature, the reaction mixture was separated into a top oily layer and a bottom aqueous layer of salt/glycerine/methanol. The aqueous layer was discarded. Methanol (500 mL) and 370 g/L HCl (20 mL) were added to the oily layer and refluxed for 1 h to drive the esterification to completion. The mixture was stirred with cold water (1 L) for 15 min and centrifuged at 1000 × *g* at 15°C. The centrifuged cake was rinsed with 500 mL of a mixture of acetone/methanol/water (75:20:10, v/v/v) and then recrystallized from heptane (1 L). This produced 124 g of 100% pure sterols containing (per 100 g material) 1.0 g cholesterol, 20.5 g brassicasterol, 3.7 g 24-methylene cholesterol, 0.8 g stigmasterol, 29.4 g campesterol, 0.2 g Δ5,23-stigmastadienol, 0.1 g clerosterol, 0.1 g sitastanol, 43.2 g β-sitosterol, 0.4 g Δ5-avenasterol and 0.5 g Δ5,24-stigmastadienol [determined by gas-liquid chromatography (GLC) and TLC].

TABLE 1

Composition of test diets

Ingredient	Test diets CA, SO, CA + P, SO + P, CO and OO	Diet CFM
	g/100 g diet	
Fat[1]	10.0	11.23[2]
Casein[3]	22.0	21.66
Cornstarch[3]	47.95	47.19
Granulated sugar[3]	10.0	10.0
Fiber (Alfa-floc)[3]	5.0	5.0
Mineral mixture AIN-93G[3]	3.5	3.5
Vitamin mixture AIN-93-VX[3]	1.0	1.0
L-Cystine[3]	0.3	0.3
Choline bitartrate[3]	0.25	0.25

[1] Fat sources: CA, canola oil; SO, soybean oil; CA + P, canola oil (100 parts) + 0.974 parts phytosterols (isolated from canola oil deodorizer distillate); SO + P, soybean oil 100 parts + 1.585 parts phytosterols (isolated from canola oil deodorizer distillate); CO, corn oil; OO, olive oil; CFM, a blend of fats that mimicked the fat composition of a representative Canadian diet.

[2] Actual fat content is 10 g. The fat blend of Diet CFM contained only 89.9% fat because of the use of whole-egg powder, butter and margarine in the fat blend (see Materials and Methods section for details). Therefore, the weight of the fat blend used in the preparation of Diet CFM was adjusted to reflect its actual fat content.

[3] Casein containing 96% protein was purchased from the Animal Nutrition Research Council Reference Protein, ICN (Cleveland, OH). Cornstarch, granulated sugar, Alfa-floc, mineral mixture AIN-93G (Reeves et al. 1993) and vitamin Mixture AIN-93-VX (Reeves et al. 1993) were purchased from Harland Teklad Test Diets (Madison, WI); L-cysteine and choline bitartrate were from Sigma Chemical (St. Louis, MO).

Test diets. Seven casein-based semipurified diets containing 10 g fat/100 g were tested (**Table 1**). The fat sources included canola oil (Diet CA), soybean oil (Diet SO), canola oil fortified with the above mentioned phytosterol mixture isolated from canola oil deodorizer distillate (Diet CA + P) and soybean oil fortified with the same phytosterol mixture (Diet SO + P) [the phytosterol fortification of canola and soybean oils was such that the levels of total sterols in dietary oils CA + P and SO + P were two times that in regular canola oil], corn oil (Diet CO), olive oil (extra virgin, Diet OO) and a blend of fat that mimicked the fat composition of a representative Canadian diet (Canadian fat mimic; Diet CFM). All of the dietary oils were purchased from a local supermarket.

The composition of the fat in Diet CFM was based on food intake data from a 24-h recall study reported in the 1990 Nova Scotia (Nova Scotia Department of Health, and Health and Welfare Canada 1993) and Quebec (Bertrand 1995) nutrition surveys. In these surveys, information regarding food intake was recorded, by age group (≥18 y) and sex, 7 d/wk over two seasons (spring and fall 1990). The composition of the representative Canadian diet was calculated by adjusting the food intake data by the population, sex and age distributions reported for Nova Scotia and Quebec in the 1991 Canadian Census (The Canadian Global Almanac 1996). The fat intakes by Canadians were then calculated by coupling the food intake data with the data on fat composition in various foods compiled in the Canadian Nutrient File (Health Canada 1997). The composition of

the diet and fat content of each food item were arranged into 13 food fat groups according to sources of fat. This information was then used to identify the most appropriate fat representative of each fat group for the preparation of the fat blend of Diet CFM. The 13 representative fat sources and their proportions were as follows: (g/112.3 g fat blend); beef tallow, 16.90; chicken fat, 2.80; lard, 8.50; dried whole-egg powder, 6.94; salmon oil, 0.60; butter, 30.25; cocoa butter, 1.50; a mixture of salad oils, 4.80 (canola oil 86%, soybean oil 10.3%, corn oil 1.7%, palm oil 1%); peanut oil, 2.10; a mixture of two types of tub margarines, 10.75 [52% canola oil–based margarine (16% *trans* fatty acid content) and 48% soybean oil margarine (18.5% *trans* fatty acid content)]; print margarine, 0.75 (canola oil–based margarine containing 37% *trans* fatty acids); household shortening, 22.00 (canola oil–based shortening containing 20.9% *trans* fatty acids); and flaxseed oil, 4.50.

The above fat mixture of Diet CFM, in addition to the 100% pure fats and oils, contained whole-egg powder, butter and margarine as sources of fat. These three fat sources contained appreciable amounts of protein and carbohydrates (whole-egg powder: 42% fat, 46% protein, 4.8% carbohydrates and 3.5% ash; butter: 81% fat, 1% protein, 2% ash, 16% water; margarine: 80% fat, 0.5% protein, 16% water). Therefore, to maintain similar levels of protein and carbohydrates in all of the diets, the amounts of casein and cornstarch used in the preparation of Diet CFM were reduced in proportion to the amounts of protein and carbohydrates contributed by whole-egg powder, butter and margarine. Each diet was prepared in 10-kg lots and stored at −4°C.

Animals. SHRSP rats (male, 28 d old) obtained from Seac Yoshitomi (Yoshitomi-Cho, Chikujyo-gun, Fukuoka, Japan) were used. The rats were acclimated to the environment of the animal care facility of Health Canada, Ottawa for 9 d. During this period, they were fed a nonpurified diet (Laboratory Rodent Diet 5001, PMI Feeds, St. Louis, MO). After the acclimation period, rats were placed in the seven dietary groups of 28 rats/group in a randomized block according to body weight (mean body wt 64.3 g). Each group was divided into two subgroups. Ten rats from each group were randomly identified for killing after 30–32 d of dietary treatment (at 65–67 d of age) for collection of blood and tissue samples for biochemical analyses and pathologic examination. The remaining rats (18/group) were used for determination of the life span.

The rats were housed individually in metal cages in a climate-controlled room maintained at 22 ± 1°C and 60% relative humidity with 12-h day:night cycle. Drinking water contained 10 g NaCl/L. The rats had free access to one of the seven diets and drinking water. The diets were replaced every 4 d to minimize deterioration. When a rat in the life span study group was found in pain or judged to be unable to survive overnight, it was killed.

The rats selected for killing at d 30–32 were killed by exsanguination while under 3% isoflurane anesthesia. Blood was withdrawn immediately from the aorta. To ensure that blood samples were processed immediately after being obtained, these were collected from the seven dietary groups over experimental d 30–32; each group was sampled equally on each day. About 7 mL of blood was collected from each rat; 2 mL was stored in EDTA tubes and 5 mL in heparanized polystyrene tubes. The liver, kidneys, heart, a sample of adipose tissue and brain were extracted. Tissue samples intended for lipid analyses were frozen immediately in liquid nitrogen and stored in a freezer maintained at −70°C until analysis. Tissue samples collected for histologic analyses were stored in formalin at room temperature. Health Canada's guide for the care and use of laboratory animals was followed and the study protocol was approved by the Animal Care Committee of Health Canada.

Hematologic measurements. Complete blood cell count was measured using a Coulter Counter S-PLUS IV system (Coulter Electronic, Hialeah, FL) on whole-blood samples collected in EDTA tubes.

Erythrocyte deformability. Erythrocyte deformability was measured on whole-blood samples (collected in heparanized polystyrene tubes) on the day of blood sampling using the ektacytometer (Model 152, Technicon Instruments Corporation, Tarrytown, NY). The principle of the instrument has been described in detail by Clark et al. (1983). Briefly, the ektacytometer provides measurements of cell deformation at constant shear stress as a continuous function of increasing osmolality of the suspending medium. Such ektacytometric measurements, conducted over a wide osmotic range, from low hypotonic to hypertonic concentrations, provide deformability profiles that vary in qualitatively different ways for several RBC disorders. This single measurement can detect relatively minor alterations in RBC membrane rigidity, internal viscosity and the ratio of surface area to volume.

The procedure used for deformability measurements (deformabilty index, DI) was essentially as described by Paterson and Card (1993). Whole blood (100 μL) was suspended in 3.0 mL of a solution containing 0.086 mmol/L polyvinylpyrrolidone (PVP360), 6.3 mmol/L Na_2HPO_4, 2 mmol/L NaH_2PO_4, 6.15 mmol/L NaN_3 (pH 7.35 ± 0.05) and 0.136 mol/L NaCl (290 mOsmol/kg). The viscosity of the suspending solution was adjusted with PVP360 to achieve a final viscosity of 20 ± 1 cP (falling ball viscometer, Gilmont Instruments, Barrington, IL). The erythrocytes were subjected to constant shear stress (160 dyn/cm^2) in the instrument as the osmolality of the suspending medium was increased progressively from 50 to 500 mOsm/kg by varying the amount of NaCl. The osmolality of the gradient solutions was measured using a micro-osmometer (Model 3M0, Advanced Instruments, Needham Heights, MA). The elongation index (also termed deformability index) of cells, which is defined as the ratio of length to width of the diffraction pattern of deforming cells, was obtained with a helium-neon laser beam and then recorded continuously on a linear X-Y recorder. From this elongation vs. osmolality plot, the cell DI was calculated as the difference between the maximum (EI max) and minimum elongation (EI min). The EI max is influenced by membrane deformability and surface area of the cell and is reached at ~290 mOsmol/kg, the physiologic osmolality at which normal erythrocytes have maximum flexibility. The EI min is the elongation cells would have reached just before hemolysis. The EI is measured in hypotonicity.

The other indices measured using the elongation vs. osmolality plot included the following: *1)* O_{hyper}, which gives an estimate of internal viscosity and hence indirectly, the mean corpuscular hemoglobin concentration; *2)* O_{min}, which is the osmolality at EI min. It is the osmolality at which 50% of cells hemolyze in an osmotic fragility assay. It is indicative of the surface area/volume ratio of the cells and reflects the maximum value possible before hemolysis at O_{min}; and *3)* O_{max}, which is the osmolality associated with EI max (Paterson and Card 1993).

Fatty acid and sterol analyses. Whole blood (3 mL) was immediately collected in tubes lined with silicone. Red blood cells were isolated by centrifuging at 2500 × *g* for 20 min. Total lipids in RBC, serum and other tissues were extracted according to Bligh and Dyer (1959). Fat from the diets was extracted using 25 vol of $CHCl_3$/MeOH (1:2, v/v).

For analysis of fatty acid composition, extracted lipid samples were methylated with BF_3/MeOH (140 g/L) and then analyzed by GLC using an SP-2560 flexible fused silica capillary column (100 m × 0.25 mm i.d., Supelco, Bellefonte, PA).

For analysis of sterol composition, ~5–20 mg of the original oil samples or extracted lipid samples were mixed with betulin (GLC internal standard; Lup-20-[29]-ene-3β,28-diol, Sigma Chemical, St. Louis, MO) and the mixture saponified with ethanolic-KOH (0.5 g KOH, 0.4 mL water, 5 mL EtOH). The nonsaponifiable matter was extracted with hexane/methylene chloride (85:15, v/v) and purified by successive washes with water and water/ethanol (80:20, v/v). The extract was dried with anhydrous sodium sulfate, solvents were evaporated in a hot water bath under a stream of nitrogen and then treated with 100 μL of 1-methyl imidazole/*N*-methyl-*N*-(trimethylsilyl)-heptafluorobutyramide (1:20, v/v) for converting the free sterols to trimethyl silyl ether derivative. The content and composition of the silylated sterols were determined in relation to the internal standard by GLC using a DB-1 flexible fused silica capillary column (30 m × 0.25 mm i.d.; J & W Scientific, Folsom, CA). Sterols were identified by comparing their GLC retention times with those of authentic standards (Sigma Chemical) and a mixture of phytosterols derived from canola oil.

Blood lipid and clinical measurements. Blood (~3 mL) was collected from food-deprived rats, centrifuged at 1300 × *g* for 20 min,

TABLE 2

Sterol concentration of the various dietary fats fed to stroke-prone spontaneously hypertensive (SHRSP) rats

	Test diet[1]						
Sterol	CA	SO	CA + P	SO + P	CO	OO	CFM
	mg/100 g diet						
Cholesterol	0.5	0.2	0.7	1.1	0.2	0.0	28.3
Brassicasterol	9.2	0.2	32.6	30.0	0.1	0.0	1.9
24-Methylene cholesterol	2.2	0.2	1.5	0.6	1.9	0.0	0.4
Campesterol	30.6	8.2	65.9	66.9	22.7	1.0	7.7
Stigmasterol	0.5	5.7	1.2	6.6	8.3	0.3	1.1
Δ7-Campesterol	0.8	0.9	0.6	0.7	0.9	0.5	0.5
Clerosterol	1.5	0.0	0.9	1.6	1.3	0.6	0.4
β-Sitosterol	48.1	19.2	98.7	89.7	66.6	21.4	13.9
Sitostanol	0.7	1.5	0.9	0.9	3.5	0.0	0.2
Δ5-Avenasterol	2.5	0.0	2.7	1.2	6.9	3.1	0.7
Δ5,24-Stigmastadienol	1.4	0.5	1.8	1.1	1.7	0.5	0.3
Total phytosterols	97.4	36.3	206.7	200.5	114.0	27.4	27.0
Total sterols	97.9	36.5	207.4	201.6	114.1	27.4	55.2

[1] Test diets: CA, canola oil; SO, soybean oil; CA + P, canola oil + phytosterols; SO + P, soybean oil + phytosterols; CO, corn oil; OO, olive oil; CFM, Canadian dietary fat mimic.

and serum separated. Serum total triglycerides and total sterols (cholesterol + phytosterols) were determined using an Abbott-VP Bichromatic Analyzer, with the A-Gent Triglyceride test and A-Gent Cholesterol test kits (Abbott Laboratories, Mississauga, Canada). HDL total sterols were isolated by selectively precipitating LDL and VLDL total sterols with A-Gent HDL reagent (Abbott Laboratories).

Statistics. Survival data were analyzed using Log-rank and Wilcoxon nonparametric tests for comparing survival curves to provide tests for the effects of diets (Lawless 1982). Note that the Wilcoxon test for differences in the survival function is more sensitive to differences in survival rates near the beginning of the study than is the Log-rank test. Other data were analyzed by ANOVA followed by Tukey's Least Significant Difference test when indicated by the *F*-value using Statistica for Windows (1998; Statsoft, Tulsa, OK). Differences were considered significant when $P < 0.05$. All data in the tables are reported as means and SD. Simple correlations between mean survival times and various blood indices were calculated by linear regression analysis.

RESULTS

Diets. The diets were designed to provide a wide range of phytosterol levels (**Table 2**). Total phytosterol concentrations ranged from 27 to 207 mg/100 g diet with OO and CFM diets providing the lowest levels, and CA + P and SO + P diets providing the highest levels. The unfortified canola oil provided 97 mg sterol/100 g diet, which was ~1.5 times greater than that in unfortified soybean oil. The CO diet had a slightly higher amount of sterols than the CA diet, due primarily to its high β-sitosterol content. In addition to phytosterols, the CFM had a relatively high cholesterol concentration derived from the dairy products and animal fats used in the preparation of this diet. The CFM, compared with other dietary fats, was characterized by high levels of total saturated fatty acids and *trans* fatty acids, and a low level of linoleic acid [18:2(n-6)] (**Table 3**).

TABLE 3

Fatty acid composition of canola oil, soybean oil, corn oil, olive oil and the Canadian fat mimic

Fatty acid	Canola oil (diets CA and CA + P)[1]	Soybean oil (diets SO and SO + P)[1]	Corn oil (diet CO)[1]	Olive oil (diet OO)[1]	Canadian fat mimic (diet CFM)[1]
	g/100 g total fatty acids				
14:0	0.1	0.1	0.0	0.0	3.5
16:0	3.8	9.8	9.5	13.3	19.4
18:0	1.9	4.0	1.8	2.9	9.5
18:1c	60.6	21.1	29.2	67.3	35.0
18:1t	0.1	0.2	0.1	0.1	6.9
18:2(n-6)	20.3	53.8	56.5	13.1	10.4
18:3(n-3)	8.4	8.7	0.9	0.6	4.0
Total saturated	6.9	14.9	12.2	16.9	36.4
Total *cis*-monounsaturated	62.3	21.4	29.7	69.1	36.1
Total polyunsaturated	28.7	62.6	57.4	13.6	15.1
Total *trans*	1.9	1.0	0.6	0.2	9.6

[1] Test diet abbreviations are as indicated in Table 2.

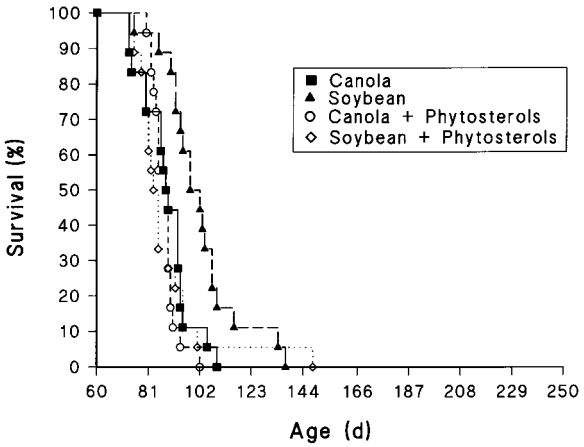

FIGURE 1 Survival curves of stroke-prone spontaneously hypertensive rats (SHRSP) rats fed diets containing canola oil, soybean oil, canola oil + phytosterols and soybean oil + phytosterols. The mean survival time (mean ± SD, n = 18) of each dietary group was 88.4 ± 10.4, 102.1 ± 15.4, 87.6 ± 5.2 and 88.3 ± 16.5 d, respectively. See text for statistical analyses of the survival curves.

Growth. Rats in all groups grew normally, and there were no significant differences in weekly body weights (data not shown). The body, kidney and brain weights of the SHRSP rats killed after feeding for 30–32 d were also not different among the dietary groups (data not shown).

Length of survival. There was a significant effect of diet on survival rates (Log rank and Wilcoxon tests, $P \leq 0.0001$) (**Figs. 1**, **2**). The OO group exhibited the lowest survival rate, whereas the CFM and SO groups exhibited the greatest survival rates. Both CA and CO groups showed significantly lower (Log rank and Wilcoxon tests, $P \leq 0.0001$) survival rates than those of the SO and CFM groups, but higher than that of the OO group, particularly at the later stages of the study (Log rank test, $P = 0.039$; Wilcoxon test, $P = 0.075$). When compared directly with the SO group, the CA group also had a significantly lower survival rate (Log-rank test, $P = 0.0034$; Wilcoxon test $P = 0.0031$). No difference was found between the CA and CO groups (Log rank test, $P = 0.59$; Wilcoxon test, $P = 0.56$). At the beginning of the study, there was no significant difference between the SO and the CFM groups (Wilcoxon test, $P = 0.43$), but the CFM group had a slightly greater survival rate later in the study (Log-rank test, $P = 0.088$).

The phytosterol mixture derived from canola oil deodorizer distillate did not shorten life when added to canola oil. In contrast, a large, significant reduction in survival rate was observed when phytosterols were added to soybean oil (Log rank test, $P = 0.011$; Wilcoxon test, $P = 0.0006$). No difference in the survival rates were found between CA + P and SO + P groups (Log rank test, $P = 0.69$; Wilcoxon test, $P = 0.47$).

General necropsy findings. There were no significant pathologic abnormalities in the rats killed at 30–32 d. In rats that died or were killed with severe clinical symptoms, acute intracranial and/or intracerebral hemorrhage was evident in the majority of cases. The lesions were typical for the SHRSP strain (Yamori 1989) and consistent with those observed in our previous study (Ratnayake et al. 2000). There were no qualitative differences between groups. In some cases, there was evidence of earlier vascular damage accompanied by focal discoloration of brain tissue. Such foci were characterized microscopically by nests of macrophages filled with blood pigments, mixed with small numbers of inflammatory cells. The brain lesions were accompanied by pathologic changes in adjacent small arteries, i.e., atrophy of smooth muscle layers, collagenization of media with infiltration of macrophages leading to a narrowing of the vascular lumen, or obstruction. In the kidneys, there was evidence of a high incidence of interstitial nephritis. Various degrees of ventricular hypertrophy in the heart did not appear to be linked to dietary factors.

Hematologic indices. Dietary phytosterols had a significant effect on platelet characteristics (**Table 4**). SHRSP rats fed CA, CA + P, SO + P and CO (diets high in phytosterols) had lower platelet counts and higher platelet volumes than SHRSP rats fed the diets low in phytosterols (SO, OO and CFM) ($P < 0.0045$). Although CA is high in phytosterols, differences were observed only for platelet volume ($P < 0.0045$). Further evidence for the effect of phytosterols on platelets comes from a comparison between fortified and unfortified canola and soybean oils. Fortification of canola and soybean oils with phytosterols significantly lowered the platelet count and increased the platelet volume. CA + P and SO + P groups had platelet counts and volumes similar to those of the CA and CO groups. There were no differences in the platelet count or volume among the three low phytosterol groups (SO, OO and CFM).

The other hematologic indices examined (mean corpuscular hemoglobin concentration, RBC distribution width, white blood cell count, RBC count, hemoglobin concentration, mean corpuscular volume and mean corpuscular volume hemoglobin) were not significantly affected by the type of fat and quantity of phytosterols in the diet (Table 4). A minor exception was the slightly lower red blood distribution cell width in SHRSP rats fed CFM, compared with those fed CA, CA + P and CO. Red blood distribution cell width measures variability of the RBC width and is elevated during anemia (coefficient >15%).

Erythrocyte deformability profile. In general, the deformability indices (DI) at 30–32d for the high phytosterol dietary groups (CA, CA + P, SO + P and CO) were lower than those of the low phytosterol groups (SO, OO and CFM) (**Table 5**). The differences were significant except between the CA and SO groups ($P = 0.1099$). Furthermore, fortification of soybean oil with phytosterols caused a significant reduction in the DI ($P = 0.0027$).

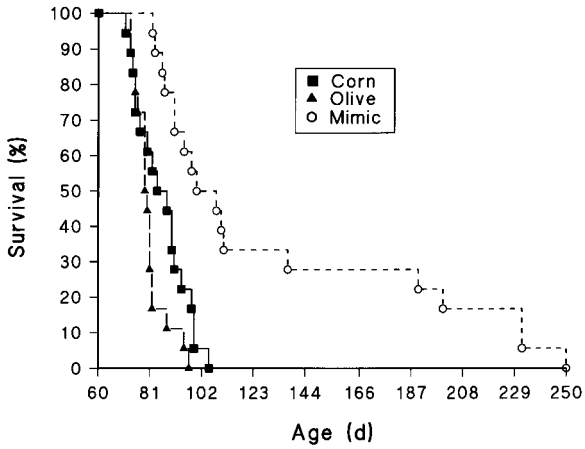

FIGURE 2 Survival curves of stroke-prone spontaneously hypertensive rats (SHRSP) rats fed diets containing canola oil, corn oil, olive oil and Canadian fat mimic. The mean survival time (mean ± SD, n = 18) of each dietary group was 88.4 ± 10.4, 85.8 ± 10.5, 80.8 ± 6.6 and 104.9 ± 28.5 d, respectively. See text for statistical analyses of the survival curves.

TABLE 4

Platelet count (PLT), mean platelet volume (MPV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cell count (WBC), RBC count, hemoglobin (Hb), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) of stroke-prone spontaneously hypertensive (SHRSP) rats fed various dietary fats with differing amounts of phytosterols for 30–32 d[1]

Test diet[2]	PLT	MPV	MCHC	RDW	WBC	RBC	HB	MCV	MCH
	10^9/L	*fL*	*g/L*	*%*	*10^9/L*	*10^{12}/L*	*g/L*	*fL*	*pg*
CA	566.0 ± 35.7^{bc}	7.48 ± 0.25^{b}	344.2 ± 2.0	12.4 ± 0.3^{b}	4.99 ± 1.06	8.77 ± 0.23	158.9 ± 3.57	52.6 ± 0.51	18.1 ± 0.2
SO	624.2 ± 92.8^{c}	6.75 ± 0.22^{a}	345.5 ± 5.4	12.2 ± 0.3^{ab}	5.02 ± 1.22	8.62 ± 0.16	157.7 ± 2.86	53.0 ± 0.40	18.3 ± 0.2
CA + P	429.2 ± 61.7^{a}	8.75 ± 0.40^{c}	343.2 ± 2.4	12.4 ± 0.3^{b}	4.96 ± 0.96	8.93 ± 0.27	161.2 ± 4.89	52.6 ± 0.38	18.0 ± 0.2
SO + P	392.4 ± 83.5^{a}	8.76 ± 0.46^{c}	342.8 ± 4.0	12.3 ± 0.2^{ab}	6.18 ± 1.63	8.83 ± 0.24	160.4 ± 4.03	53.0 ± 0.53	18.2 ± 0.2
CO	453.7 ± 63.2^{ab}	7.89 ± 0.46^{b}	345.2 ± 4.2	12.5 ± 0.2^{b}	5.93 ± 1.43	8.88 ± 0.27	161.9 ± 4.93	52.8 ± 0.40	18.2 ± 0.2
OO	667.1 ± 156.5^{c}	6.67 ± 0.32^{a}	345.1 ± 3.1	12.2 ± 0.4^{ab}	5.60 ± 1.80	8.79 ± 0.47	160.8 ± 6.17	53.0 ± 1.14	18.3 ± 0.4
CFM	669.0 ± 110.6^{c}	6.47 ± 0.15^{a}	343.7 ± 3.5	11.9 ± 0.4^{a}	4.59 ± 1.78	8.55 ± 0.35	156.3 ± 5.92	53.2 ± 1.28	18.3 ± 0.5

[1] Values are means ± SD, $n = 10$. Values in a column not sharing a superscript are significantly different ($P < 0.05$).
[2] Test diet abbreviations are as indicated in Table 2.

Fortification of canola and soybean oils with phytosterols did not affect O_{max} and O_{hyper}, indicating that dietary phytosterols have no important influence on O_{max} and O_{hyper} of SHRSP erythrocytes (Table 5). Nevertheless, the type of dietary fat had a minor effect on these variables. The CO group, compared with all the other groups except the OO group, displayed a significantly higher O_{max} value, whereas the CFM group showed the lowest value, one closest to the physiologic osmolality of 290 mOsmol/kg. O_{hyper} was significantly higher in OO-fed rats, compared with those fed CO and CFM.

O_{min} was slightly affected by the type of dietary fat (Table 5). SO + P–fed rats had significantly higher values compared with those fed the unfortified soybean oil, indicating that they could be most susceptible to lysis. On the other hand, there was no significant difference between the CA and CA + P groups, indicating a minor effect of dietary phytosterols on O_{min}. The CFM group, compared with all the other dietary groups, except the OO group, had significantly lower O_{min} and would be least susceptible to lysis.

Sterol composition of erythrocytes. The cholesterol concentration was significantly ($P < 0.05$) lower and the total phytosterol concentration and the ratio of total phytosterol to cholesterol were higher in erythrocytes of SHRSP rats fed the four high phytosterol diets (CA, CA + P, SO + P and CO) than those fed the low phytosterol diets (SO, OO and CFM) (**Table 6**). A notable exception to this trend was the OO group; the erythrocyte cholesterol concentration of this group was not different from that of the high phytosterol dietary groups and significantly lower compared with the SO and CFM groups. In general, the SHRSP rats fed high phytosterol diets displayed a higher proportion of phytosterols in erythrocytes (20, 32.5, 34.4 and 24% of total sterols in CA, CA + P, SO + P and CO groups, respectively) than in the rats fed low phytosterol diets (12, 8.6 and 8.8 in SO, OO and CFM groups, respectively). There were no significant differences in total phytosterols, cholesterol and the phytosterol/cholesterol ratio between CA and CO groups, between SO and CFM groups and also between CA + P and SO + P groups.

Fortification of canola and soybean oils with phytosterols affected the erythrocyte sterol profile (Table 6). Total phytosterol concentration was significantly higher in CA + P and SO + P groups and cholesterol concentration was lower. The changes in the sterol profile were greatest for the SO group; there was a 280% increase in the ratio when soybean oil was fortified with phytosterols, whereas for canola oil fortification, the increase was only 90%. In both fortified oil groups, the

TABLE 5

The effect of dietary fats on the osmotic deformability profile of RBC of stroke-prone spontaneously hypertensive (SHRSP) rats fed various dietary fats with differing amounts of phytosterols for 30–32 d[1]

Test diet[2]	DI[3]	O_{max}[3]	O_{hyper}[3]	O_{min}[3]
			mOsmol/kg	
CA	0.4049 ± 0.0056^{ab}	304.1 ± 6.5^{b}	412.7 ± 5.8^{ab}	161.5 ± 2.4^{bc}
SO	0.4117 ± 0.0138^{bc}	306.3 ± 5.8^{b}	412.5 ± 3.1^{ab}	161.5 ± 2.3^{bc}
CA + P	0.3980 ± 0.0134^{a}	306.2 ± 6.9^{b}	412.3 ± 5.6^{ab}	161.9 ± 4.4^{cd}
SO + P	0.3987 ± 0.0082^{a}	306.4 ± 4.9^{b}	411.9 ± 5.9^{ab}	164.5 ± 2.9^{d}
CO	0.3968 ± 0.0083^{a}	309.3 ± 5.4^{c}	409.8 ± 7.0^{a}	162.4 ± 3.8^{cd}
OO	0.4145 ± 0.0081^{c}	308.3 ± 8.4^{bc}	415.9 ± 4.9^{b}	158.8 ± 2.0^{ab}
CFM	0.4155 ± 0.0083^{c}	298.8 ± 5.9^{a}	410.3 ± 6.2^{a}	156.3 ± 2.0^{a}

[1] Values are means ± SD, $n = 10$. Values in a column not sharing a superscript are significantly different ($P < 0.05$).
[2] Test diet abbreviations are as indicated in Table 2.
[3] DI, deformability index; O_{max}, the osmolality associated with the maximum deformability of cells; O_{hyper}, an estimate of internal viscosity and mean corpuscular hemoglobin concentration; O_{min}, the osmolality at minimum elongation, which is indicative of the surface area/volume ratio of cells.

TABLE 6

Sterol concentration and ratio of phytosterol to cholesterol of RBC of stroke-prone spontaneously hypertensive (SHRSP) rats fed various dietary fats with differing amounts of phytosterols for 30–32 d[1]

Test diet[2]	Cholesterol	Campesterol	β-Sitosterol	Total phytosterols[3]	Total sterols[3]	Total phytosterols[3]/ cholesterol
			μmol/100 g RBC			
CA	193.9 ± 39.6[a]	25.2 ± 5.5[cd]	15.0 ± 3.1[bc]	46.3 ± 9.1[b]	240.2 ± 52.0[ab]	0.24 ± 0.03[b]
SO	235.6 ± 37.0[b]	13.7 ± 2.7[a]	11.6 ± 2.4[ab]	30.2 ± 6.0[a]	265.8 ± 51.1[ab]	0.13 ± 0.03[a]
CA + P	180.5 ± 38.3[a]	51.2 ± 10.2[e]	23.6 ± 2.5[d]	83.4 ± 7.6[c]	263.9 ± 62.3[ab]	0.46 ± 0.07[c]
SO + P	172.5 ± 45.5[a]	57.7 ± 13.2[e]	22.1 ± 3.4[d]	86.2 ± 16.7[c]	258.7 ± 66.9[ab]	0.50 ± 0.06[c]
CO	172.8 ± 46.0[a]	22.2 ± 6.5[bc]	20.5 ± 2.4[cd]	51.5 ± 7.8[b]	224.3 ± 61.8[a]	0.30 ± 0.05[b]
OO	171.0 ± 39.6[a]	2.7 ± 0.7[a]	7.0 ± 1.4[a]	14.8 ± 4.1[a]	185.8 ± 2.6[a]	0.09 ± 0.01[a]
CFM	274.7 ± 44.0[b]	11.4 ± 3.0[a]	7.7 ± 2.6[a]	24.0 ± 7.8[a]	298.7 ± 63.6[b]	0.09 ± 0.01[a]

[1] Values are means ± SD, $n = 10$. Values in a column not sharing a superscript are significantly different ($P < 0.05$).
[2] Test diet abbreviations are as indicated in Table 2.
[3] The totals for phytosterols and sterols include minor phytosterols (brassicasterol, 24-methylene cholesterol, stigmasterol, Δ7-campesterol, sitostanol, Δ5-avenasterol and Δ5,24-stigmastadienol) which were detected at <2 μmol/100 g RBC.

total sterol concentration was not significantly different from the corresponding unfortified oil groups, indicating that the increased phytosterol/cholesterol ratio was due primarily to replacement of erythrocyte cholesterol by dietary phytosterols. In the two phytosterol-fortified diet groups, phytosterols accounted for 33–34% of total sterols in erythrocytes, whereas for the unfortified canola and soybean oil groups, phytosterols accounted for only 20 and 12% total sterols, respectively.

In rats fed CA, CA + P and SO + P, campesterol was the major phytosterol in erythrocytes, followed by β-sitosterol (Table 6). This is to be expected because campesterol is absorbed more efficiently than β-sitosterol and other common dietary phytosterols (Ling and Jones 1995). In the other dietary groups, both of these sterols were present in almost equal amounts. This is probably because the diets of these groups contained a very large proportion of β-sitosterol and a lower proportion of campesterol compared with the CA, CA + P and SO + P diets (Table 2). The other dietary phytosterols, including brassicasterol, although present in moderate quantities in CA, CA + P and SO + P diets (Table 2), were barely detectable in erythrocytes of all of the dietary groups.

Sterol composition of liver and kidneys. Compared with erythrocytes, liver and kidneys had larger amounts of cholesterol and phytosterols (**Tables 7** and **8**). Nevertheless, the differences in sterol composition among the groups were similar to those in erythrocytes. However, in contrast to erythrocytes, cholesterol concentration in liver and kidneys was significantly higher for the olive group compared with the high phytosterol dietary groups, except the CO group. Additionally, cholesterol concentration in tissues of the CO group were also not significantly different from those of the SO and CFM groups. For the erythrocytes, fortification of canola and soybean oil with phytosterols significantly increased liver and kidney total phyotosterols. Associated with this increase, there was a decrease in the cholesterol concentration of liver with fortification of soybean oil but not with canola oil. In kidneys, however, phytosterol fortification did not affect the cholesterol concentration. These changes, however, increased the phytosterol/cholesterol ratio in both liver and kidney, and the increase was considerable for the soybean + phytosterol group. Furthermore, similar to the erythrocytes, liver total sterols were unaffected by the fortification of canola and soybean oils

TABLE 7

Liver sterol concentration and total phytosterol/cholesterol ratio of stroke-prone spontaneously hypertensive (SHRSP) rats fed various dietary fats with differing amounts of phytosterols for 30–32 d[1]

Test diet[2]	Cholesterol	Campesterol	β-Sitosterol	Total phytosterols[3]	Total sterols[3]	Total phytosterols[3]/ cholesterol
			μmol/100 g liver			
CA	473.3 ± 93.6[a]	68.6 ± 12.5[c]	42.4 ± 6.5[b]	122.3 ± 20.8[c]	595.6 ± 113.0[a]	0.26 ± 0.02[c]
SO	719.8 ± 115.4[b]	38.1 ± 6.5[b]	34.0 ± 7.0[b]	84.0 ± 15.2[b]	803.8 ± 129.4[bcd]	0.12 ± 0.01[b]
CA + P	487.0 ± 38.0[a]	136.0 ± 12.2[d]	76.2 ± 7.2[d]	226.2 ± 20.3[e]	713.2 ± 57.1[abc]	0.46 ± 0.02[d]
SO + P	452.6 ± 37.8[a]	139.3 ± 16.7[d]	65.6 ± 7.2[c]	215.6 ± 25.4[e]	665.2 ± 59.8[ab]	0.48 ± 0.04[d]
CO	644.0 ± 80.4[ab]	71.6 ± 8.5[c]	81.3 ± 9.6[d]	179.8 ± 20.4[d]	823.8 ± 98.4[cd]	0.28 ± 0.02[c]
OO	787.0 ± 103.2[bc]	15.2 ± 6.2[a]	23.4 ± 7.0[a]	48.6 ± 14.8[a]	835.6 ± 100.8[cd]	0.06 ± 0.02[a]
CFM	793.8 ± 74.7[bc]	29.4 ± 5.2[b]	19.0 ± 3.9[a]	58.7 ± 10.6[ab]	852.5 ± 80.6[d]	0.07 ± 0.01[a]

[1] Values are means ± SD, $n = 10$. Values in a column not sharing a superscript are significantly different ($P < 0.05$).
[2] Test diet abbreviations are as indicated in Table 2.
[3] The totals for phytosterols and sterols include minor phytosterols (brassicasterol, 24-methylene cholesterol, stigmasterol, Δ7-campesterol, sitostanol, Δ5-avenasterol and Δ5,24-stigmastadienol) which were detected at <2 μmol/100 g liver.

TABLE 8

Kidney sterol concentration and total phytosterol/cholesterol ratio of stroke-prone spontaneously hypertensive (SHRSP) rats fed various dietary fats with differing amounts of phytosterols for 30–32 d[1]

Test diet[2]	Cholesterol	Campesterol	β-Sitosterol	Total phytosterols[3]	Total sterols[3]	Total phytosterol[3]/ cholesterol
			μmol/100 g kidney			
CA	658.2 ± 124.1[a]	53.2 ± 12.2[c]	32.3 ± 5.8[b]	101.0 ± 23.5[b]	759.2 ± 141.1[a]	0.15 ± 0.03[c]
SO	817.8 ± 57.7[bc]	26.7 ± 1.5[a]	26.0 ± 2.2[a]	64.2 ± 4.7[a]	882.0 ± 56.1[b]	0.08 ± 0.01[b]
CA + P	748.0 ± 41.6[ab]	96.3 ± 7.2[d]	49.9 ± 3.6[c]	163.2 ± 12.1[d]	911.2 ± 50.0[bc]	0.22 ± 0.01[d]
SO + P	806.4 ± 52.8[bc]	116.3 ± 5.7[a]	53.0 ± 6.8[c]	183.8 ± 27.2[d]	990.2 ± 75.2[c]	0.23 ± 0.02[d]
CO	883.5 ± 48.2[cd]	51.2 ± 5.2[c]	55.2 ± 3.6[c]	133.6 ± 10.0[c]	1017.1 ± 54.9[c]	0.15 ± 0.01[c]
OO	964.2 ± 82.2[d]	13.5 ± 4.5[a]	23.1 ± 5.8[a]	52.2 ± 14.2[a]	1016.4 ± 77.7[c]	0.05 ± 0.02[a]
CFM	1179.6 ± 41.3[e]	27.0 ± 4.2[b]	18.1 ± 3.4[a]	55.9 ± 8.8[a]	1235.5 ± 38.4[d]	0.05 ± 0.01[a]

[1] Values are means ± SD, $n = 10$. Values in a column not sharing a superscript are significantly different ($P < 0.05$).
[2] Test diet abbreviations are as indicated in Table 2.
[3] The totals for phytosterols and sterols include minor phytosterols (brassicasterol, 24-methylene cholesterol, stigmasterol, stigmasterol, Δ7-campesterol, sitostanol, Δ5-avenasterol and Δ5,24-stigmastadienol) which were detected at <2 μmol/100 g kidney.

with phytosterols, suggesting that phytosterols are replacing a part of the cholesterol in the liver. In kidneys, however, the total sterol concentration was increased, suggesting that phytosterols are incorporated into the kidney without affecting its cholesterol concentration.

The variations in the phytosterol profile of the liver and kidneys were also similar to the changes described previously for erythrocytes. In the CA, CA + P and SO + P diet groups, campesterol was the major phytosterol, followed by β-sitosterol. In the other dietary groups, both of these phytosterols were present in almost equal proportions. Other dietary phytosterols were also present in the liver lipids but at lower levels.

Sterol composition of plasma. **Table 9** shows plasma lipid data measured according to enzymatic methods. It should be noted here that the enzymatic method for cholesterol is not specific for this sterol or any other individual sterol. The method measures the total sterols; therefore, the values shown in Table 9 for whole-plasma and HDL sterols represent the sum of cholesterol and phytosterols. The composition of individual sterols in whole plasma, however, was measured by GLC and the values, expressed as percentage of total sterols, are shown in **Table 10**.

The plasma total and HDL sterol concentrations were significantly ($P < 0.05$) higher for the four high phytosterol diet groups compared with the three low phytosterol groups (Table 9). A minor exception was the OO group. The plasma total sterol concentration of rats fed OO was not significantly different compared with the CA ($P = 0.780$) and CO groups ($P = 0.190$). The high phytosterol diet groups, compared with the low phyosterol diet groups, had a higher proportion of the various phytosterols and a lower proportion of cholesterol (Table 10), which indicates that the high total sterol in plasma of the high phytosterol dietary groups was due primarily to incorporation of phytosterols into the plasma.

In general, plasma triglyceride concentration was lower for the low phytosterol diet groups compared with the high phytosterol diet groups, but the values were significantly ($P < 0.05$) lower only for the SO group.

TABLE 9

Plasma total and HDL sterol (cholesterol + phytosterols), and triglyceride concentrations of stroke-prone spontaneously hypertensive (SHRSP) rats fed various dietary fats with differing amounts of phytosterols for 30–32 d (values determined by enzymatic methods)[1]

Test diet[2]	Total sterols	HDL sterols	Triglyceride
		mmol/L	
CA	1.89 ± 0.14[b]	1.51 ± 0.10[b]	1.22 ± 0.20[b]
SO	1.39 ± 0.19[a]	1.14 ± 0.15[a]	0.94 ± 0.17[a]
CA + P	2.55 ± 0.15[c]	1.87 ± 0.29[c]	1.40 ± 0.10[b]
SO + P	2.34 ± 0.21[c]	1.86 ± 0.19[c]	1.20 ± 0.12[b]
CO	1.99 ± 0.18[b]	1.58 ± 0.14[b]	1.23 ± 0.27[b]
OO	1.73 ± 0.16[b]	1.16 ± 0.16[a]	1.14 ± 0.18[ab]
CFM	1.41 ± 0.12[a]	1.06 ± 0.10[a]	0.97 ± 0.16[ab]

[1] Values are means ± SD, $n = 10$. Values in a column not sharing a superscript are significantly different ($P < 0.05$).
[2] Test diet abbreviations are indicated in Table 2.

TABLE 10

Plasma sterol composition of stroke-prone spontaneously hypertensive (SHRSP) rats fed various dietary fats with differing amounts of phytosterols for 30–32 d[1,2]

Test diet[3]	Cholesterol	Campesterol	β-Sitosterol
		mol/100 mol sterols	
CA	73.7 ± 1.0[c]	12.7 ± 0.5[e]	11.5 ± 0.5[d]
SO	85.0 ± 0.6[d]	5.5 ± 0.3[c]	8.0 ± 0.3[c]
CA + P	63.9 ± 1.4[a]	19.2 ± 0.8[f]	14.6 ± 0.6[e]
SO + P	61.4 ± 3.4[a]	21.5 ± 1.1[g]	14.6 ± 1.9[e]
CO	69.3 ± 3.8[b]	9.4 ± 0.8[d]	18.2 ± 2.5[f]
OO	91.4 ± 2.9[e]	1.6 ± 0.8[a]	5.3 ± 1.7[b]
CFM	90.9 ± 0.7[e]	4.3 ± 0.4[b]	4.2 ± 0.4[b]

[1] Brassicasterol, 24-methylene cholesterol, stigmasterol, Δ7-campesterol, sitostanol, Δ5-avenasterol and Δ5,24-stigmastadienol were detected in plasma at very low proportions (<1 mol/100 mol total sterols). Their individual values are not shown in this table but are included in the plasma total sterol content shown in Table 9.
[2] Values are means ± SD, $n = 10$. Values in a column not sharing a superscript are significantly different ($P < 0.05$).
[3] Test diet abbreviations are as stated in Table 2.

TABLE 11

Linear regression coefficients (r^2) for relationships among erythrocyte (RBC) sterol contents, deformability index (DI) of RBC, platelet number (PLT) of stroke-prone spontaneously hypertensive (SHRSP) rats fed various dietary fats with differing amounts of phytosterols for 30–32 d and the mean survival times (MST) of SHRSP rats in the life span study groups

Relationship	Including olive oil group		Excluding olive oil group		Positive (+) or negative (−) correlation
	r^2	P	r^2	P	
μmol Cholesterol/100 g RBC vs. MST	0.90	0.0010	0.94	0.0016	+
μmol Total phytosterols/100 g RBC vs. MST	0.08	0.5317	0.58	0.0798	−
μmol Campesterol/100 g RBC vs. MST	0.05	0.6222	0.43	0.1567	−
μmol β-Sitosterol/100 g RBC vs. MST	0.14	0.4131	0.80	0.0160	−
Total phytosterols/cholesterol ratio in RBC vs. MST	0.15	0.3851	0.65	0.0579	−
Campesterol/cholesterol ratio in RBC vs. MST	0.10	0.5003	0.46	0.1235	−
β-Sitosterol/cholesterol ratio in RBC vs. MST	0.25	0.2552	0.81	0.0126	−
μmol Cholesterol/100 g RBC vs. DI of RBC	0.43	0.1102	0.94	0.0014	+
μmol Total phytosterols/100 g RBC vs. DI of RBC	0.77	0.0092	0.71	0.0363	−
μmol Campesterol/100 g RBC vs. DI of RBC	0.63	0.0322	0.54	0.0978	−
μmol β-Sitosterol/100 g RBC vs. DI of RBC	0.95	0.0002	0.95	0.0011	−
Total phytosterols/cholesterol ratio in RBC vs. DI of RBC	0.81	0.0056	0.77	0.0220	−
Campesterol/cholesterol ratio in RBC vs. DI of RBC	0.67	0.0240	0.60	0.0719	−
β-Sitosterol/cholesterol ratio in RBC vs. DI of RBC	0.97	<0.0001	0.97	0.0004	−
DI of RBC vs. MST	0.23	0.2801	0.91	0.0033	+
PLT vs. MST	0.21	0.3006	0.77	0.0218	+
Dietary total phytosterol (mg/100 g diet) vs. PLT	0.90	0.0013	0.87	0.0063	−
DI vs. PLT	0.94	0.0003	0.93	0.0019	+

Phytosterol fortification significantly ($P < 0.05$) increased the plasma total and HDL sterols (Table 9). These increases were greater in particular for the fortified soybean oil group ($P = 0.002$ for both total and HDL sterols). Phytosterol fortification also affected the plasma sterol profile. Cholesterol proportion was decreased significantly with a concomitant increase in the proportion of phytosterols; specifically, there was a large increase in the proportions of campesterol and β-sitosterol (Table 10). Thus, the increase in plasma total sterol with phytosterol fortification could be attributed to increased phytosterol incorporation into plasma.

Plasma triglyceride levels also increased with phytosterol fortification, but this was significant only for the fortified soybean oil group ($P = 0.033$).

Fatty acid composition of erythrocytes. There was a very little variation in the fatty acid composition of erythrocytes among the dietary groups (data not shown). The most noticeable difference was the significantly ($P < 0.05$) lower percentage of (n-3) docosahexaenoic acid in the erythrocytes of the OO and CO groups compared with all other dietary groups. Fortification of canola oil and soybean oil with phytosterols also had no major effect on erythrocyte fatty acid profile. In general, there was no apparent relationship between the erythrocyte fatty acid profile and the dietary level of phytosterols.

Correlations between mean survival times and, erythrocyte sterol content, deformability index of erythrocytes and platelet number. The erythrocyte cholesterol concentration was strongly correlated with the mean survival time as well as with the erythrocyte DI (**Table 11**). There was also a correlation between mean survival time and the erythrocyte DI. These correlations became very strong ($r^2 > 0.90$) and significant ($P < 0.005$) when the data for the OO group were not included in the regression analysis. In contrast to cholesterol, erythrocyte total phytosterols, campesterol and β-sitosterol displayed weak, negative correlations ($r^2 < 0.40$, $P > 0.05$) with mean survival time and the erythrocyte DI. The correlations between erythrocyte phytosterol concentrations and mean survival time, however, were stronger ($r^2 > 0.40$, $P < 0.05$, except for campesterol and campesterol/cholesterol) when the data for the OO group were excluded from the regression analysis. The inverse relationships of erythrocyte campesterol, β-sitosterol and total phytosterol concentrations with the DI, however, were strong ($r^2 > 0.40$) and significant ($P < 0.05$) in both scenarios, i.e., with and without the inclusion of data for the OO group in the regression analysis. Furthermore, there was a very strong negative correlation between the total phytosterol concentration in the diet and platelet number ($r^2 = 0.90$, $P = 0.0013$ with OO data and $r^2 = 0.87$, $P = 0.0063$ without OO data) and a strong positive correlation between DI and platelet number ($r^2 > 0.90$, $P < 0.005$ both with and without OO data).

DISCUSSION

This study demonstrates that dietary fats high in phytosterols as well as the addition of phytosterols to fats naturally low in phytosterols have a significant life-shortening effect on salt-loaded SHRSP rats. Canola oil and corn oil, both of which contain higher amounts of natural phytosterols than many other common vegetable oils, shortened the life span compared with soybean oil or the Canadian fat mimic diet. Furthermore, phytosterol-fortified soybean oil, compared with unfortified soybean oil, reduced life span considerably. These results suggest that the high concentration of phytosterols in canola oil might be responsible for the shorter life span of canola oil–fed SHRSP rats. The lower survivability of SHRSP rats fed phytosterol-enriched diets was not accompanied by any significant changes in body weight, weight gain or the fatty acid profile of RBC. Although blood pressure was not measured in this study, from the results of our previous study on SHRSP rats, it could also be suggested that phytosterols might be shortening the life span of SHRSP rats without altering blood pressure and renal function (Ratnayake et al. 2000).

Feeding phytosterol-enriched diets to SHRSP rats resulted in significant alterations in the sterol profile of various tissues. There was a large incorporation of phytosterols, particularly campesterol and β-sitosterol, in RBC, plasma, liver and kidneys; this was accompanied by a reduction in the cholesterol concentration, which shows that dietary phytosterols replace some of the cholesterol in various tissues in SHRSP rats. Consequently, these changes markedly increased the phytosterol/cholesterol ratio in plasma, RBC membranes, liver and kidney. These changes in the tissue sterol profiles were associated with shorter survival rates and poorer cell membrane integrity, as shown by the lower DI of erythrocytes of SHRSP rats fed the phytosterol-enriched diets.

The DI gives a quantitative measurement of the mechanical stability and flexibility of a population RBC or RBC membranes in response to a constant shear stress as a function of increasing osmolality (Clark et al. 1983). Unstable, less flexible and less deformable cell membranes give low DI values in the ektacytometer. Thus, the low DI values seen in this study for SHRSP rats fed phytosterol-enriched diets suggest that the phytosterols make the cell membrane more rigid and less flexible. Increased membrane rigidity was also observed in liver microsomes of healthy Wistar rats fed a diet supplemented with 3% β-sitosterol + 2% campesterol (Leiken and Brenner 1989). The increase in the membrane rigidity was attributed to partial replacement of liver microsome cholesterol by β-sitosterol and campesterol. In another study, in vitro replacement of a part of the cholesterol complement in human erythrocyte cell membranes by phytosterols caused a significant increase in the osmotic fragility of cell membranes (Bruckerdorfer et al. 1969). Increased RBC membrane phytosterol incorporation, resulting in increased osmotic fragility, has also been observed in patients with phytosterolemia (Wang et al. 1981), a very rare inborn disease due to a defect in cholesterol metabolism (Bhattacharyya and Connor 1974, Miettinen 1980, Wang et al. 1981).

Impaired cell deformability can cause a reduction in cell survival because the inextensible membranes are easily ruptured during physical stress (Weed 1970). This effect may be particularly noticeable in SHRSP rats because they have defective and more fragile cell membranes than other rat strains (Yamori et al. 1980 and 1984b). The cell abnormalities include higher membrane rigidity, increased permeability for Na^+ and K^+, abnormally low Ca^{2+} binding factor and high osmotic fragility. These abnormalities were noted not only in the erythrocytes but also in vascular muscle cells and are believed to be of pathogenic importance in hypertensive vascular lesions because the cerebral hemorrhage and infarction noted in SHRSP rats were commonly caused by arterionecrosis (Yamori 1989). The cell abnormalities are due primarily to the low amount of cholesterol in cell membranes, which is presumably a genetic defect of SHRSP rats. Blood cholesterol concentration is also abnormally low in these rats (Yamori et al. 1980). Consistent with these observations, laboratory feeding studies have shown that dietary cholesterol is beneficial to these rats. Low-to-moderate diet-induced increases in serum cholesterol were associated with a significant reduction in the degree of arterionecrosis and the occurrence of stroke (Ooneda et al. 1978, Yamori et al. 1976). In a more recent study, a diet containing cholesterol (1 g/100 g diet) significantly ($P < 0.05$) delayed the onset of stroke and prolonged the life span of SHRSP rats by ~40% in both NaCl-loaded and unloaded conditions (Hamano et al. 1995). Diets with no added cholesterol greatly shortened poststroke survival. These beneficial effects are most likely due to incorporation of exogenous cholesterol into cell membranes, which leads to an improvement of the cell membrane physical characteristics. Considering the above, we suggest that dietary phytosterols and oils enriched with phytosterols may exacerbate the development of hemorrhagic stroke in SHRSP rats by further weakening the already fragile cell membrane with the replacement of a part of the cholesterol complement in the cell membrane by phytosterols. This could be a possible mechanism by which phytosterols shorten the life span of SHRSP rats. Measuring the deformability index with the ektacytometer provides a unique opportunity to measure the functional properties of the RBC much earlier than that indicated by measures such as a reduction in cell count or an increase in RBC distribution width.

The levels of phyotosterols seen in the RBC, plasma and various tissues of SHRSP rats in this study were very large and at least 5–10 times greater than those reported for healthy rats fed diets containing comparable amounts of phytosterols (Strandberg et al. 1989, Sugano et al. 1977). Furthermore, phytosterols accounted for ~25% of the total sterols in SHRSP tissues, whereas in normal rats and healthy humans, the proportion of phytosterols is ~2–5% of the total sterol (Jones et al. 1997). High levels of phytosterols in serum and RBC are also associated with phytosterolemia (Bhattacharyya and Connor 1974, Miettinen 1980, Wang et al. 1981). These patients have hemolytic anemia and defective RBC membrane morphology, similar to that of SHRSP rats. They develop premature atherosclerotic arterial disease due to excessive accumulation of campesterol, sitosterol and other phytosterols in blood, RBC and body tissues. The high concentration of phytosterols in SHRSP rats may be caused by enhanced absorption of phytosterols, by their impaired removal or by a combination of the two. Whatever the mechanisms by which phytosterols are incorporated, the large accumulation of phytosterols appears to compensate for the naturally lower cholesterol concentration in the tissues. It may be that SHRSP rats cannot synthesize enough cholesterol due to a genetic defect, leading to increased phytosterol absorption and incorporation into tissues as a substitute for cholesterol. This implies that SHRSP rats may not discriminate between phytosterols and cholesterol in intestinal absorption as effectively as normal rats and humans. A similar hypothesis was suggested by Salen et al. (1989) to explain the increased absorption of phytosterols by phytosterolemic subjects.

Like SHRSP rats, humans with a history of hemorrhagic stroke also have cell membrane abnormalities due to lower cholesterol concentration in the cell membranes (Canessa et al. 1980, De Mendonca et al. 1980, Konishi et al. 1982, Tsuda et al. 1992, Yamori et al. 1980 and 1984b). Furthermore, in prospective studies, hemorrhagic stroke has been found to occur at higher rates in persons with low levels of blood total cholesterol than in persons with higher levels. This peculiar lipid-stroke relationship has been reported in Japanese populations (Kimura et al. 1972, Komachi et al. 1977, Kroes and Ostwald 1971, Lin et al. 1984, Okada et al. 1976, Shimamoto et al. 1989, Tanaka et al. 1982, Ueda et al. 1988, Ueshima et al. 1980), in Japanese men living in Hawaii (Iribarren et al. 1995, Kagan et al. 1980, Yano et al. 1989) and Caucasian men and women in the U.S. (Gordon et al. 1981, Iribarren et al. 1996, Iso et al. 1989, Neaton et al. 1992). Although the reasons behind these associations are unknown, it is quite plausible that, as in SHRSP rats, the weakening of cell membranes due to low circulating cholesterol may influence arterionecrosis. Thus, it will be important for future studies to consider whether large intakes of phytosterols would exacerbate the development of hemorrhagic stroke in humans, as seen in SHRSP rats of this study. A parallel situation exists in

patients with phytosterolemia (Bhattacharyya and Connor 1974, Miettinen 1980, Wang et al. 1981).

In addition to the detrimental effects on RBC membranes, phytosterols markedly influenced platelet characteristics. The SHRSP rats fed canola oil and other dietary fats enriched with phytosterols had lower platelet counts than those fed soybean oil and the other two diets low in phytosterols. The more pronounced reduction in platelet count compared with RBC counts in the phytosterol-fed rats is not unexpected because platelets have a half-life of 3–5 d compared with 25–40 d for RBC. A similar lower platelet count was reported in phytosterolemia (Salen et al. 1989) and apolipoprotein E–deficient mice fed phytosterol-enriched diets (Moghadasian et al. 1999). In a clinical trial involving healthy humans, feeding phytosterol-enriched margarine for 12 wk led to a small, nonsignificant reduction in platelet number (-15.4×10^9/L, $P > 0.05$) (Plat and Mensink 1998). Canola oil, compared with soybean oil, also significantly ($P < 0.05$) reduced the platelet number in studies involving very young piglets (Kramer et al. 1994, Innis and Dyer 1999). The correlation between platelet number and the survival time observed in this study suggests that the lowering of platelet number by dietary phytosterols may also be associated with the early death of SHRSP rats. Unfortunately, we did not analyze the sterol composition of platelets; therefore it is not known whether phytosterols incorporated into the platelet membrane could have affected the flexibility of platelet membrane, similar to the effect observed for RBC.

Although the four phytosterol-enriched diets contained different levels of phytosterols, the survival rates, RBC DI and platelet count of SHRSP rats fed these diets were not significantly different. Particularly, there was no significant difference between rats fed canola oil and those fed canola oil fortified with phytosterols. On the other hand, fortified soybean oil produced more adverse effects than unfortified soybean oil. This might imply a threshold limit to the influence of phytosterols on biological variables of SHRSP rats. According to this logic, the threshold might already have been reached in unfortified canola oil, which provided 974 mg phytosterols/100 g oil. It appears that an increase in the dietary phytosterol concentration beyond what is found in canola oil does not produce any further changes. Soybean oil, on the other hand, has a low concentration of phytosterols (363 mg phytosterols/100 g oil). This appears to lie below the threshold limit because fortified soybean oil had significant adverse nutritional effects beyond those observed for unfortified soybean oil.

In this study, the rats fed the Canadian fat mimic diet exhibited the greatest survival rate; this is clearly attributable to its high cholesterol concentration and low phytosterol to cholesterol ratio, relative to the other dietary fats tested. Most likely, dietary cholesterol counteracted the unfavorable biological effects of phytosterols. The longer life span of SHRSP rats fed fish oil and lard found in the study of Huang et al. (1997) may also be explained by the presence of cholesterol in these dietary fats.

Of all the dietary fats tested in this study, olive oil–fed rats had the lowest survival rates despite a very low phytosterol concentration . A similar observation was made by Huang et al. (1997) who found no significant difference in survival rates between animals fed canola and olive oils. These results suggest that another factor (other than phytosterols) is shortening the life span of olive oil–fed SHRSP rats. This factor must operate by another mechanism because the RBC membrane and platelet characteristics were similar to those of the other rats fed low phytosterols. In addition to olive oil, Huang et al. (1997) observed that oils such as high oleate sunflower, high oleate safflower and evening primrose, which contain low-to-moderate levels of phytosterols, exhibit life-shortening activities comparable to those of canola oil. Previous studies in our laboratory showed that there is no significant association between the fatty acid composition of dietary fats and the life span of SHRSP rats (Ratnayake et al. 2000). Some of these results indicate that in addition to phytosterols, another minor component in the nonsaponifiable matter in some vegetable oils also affects the life span of SHRSP rats.

Unlike in SHRSP rats and phytosterolemic subjects, dietary phytosterols are usually considered safe and beneficial for both normal humans and common laboratory animals (Ling and Jones 1995). This difference between SHRSP rats and phytosterolemics, and other species might be attributed primarily to the poor intestinal absorption and very low incorporation of phytosterols into plasma and tissues by normal humans and laboratory animals. The beneficial effect of phytosterols is due mainly to their ability to interfere with the intestinal absorption of both dietary and biliary cholesterol. Consumption of β-sitosterol for 4 y by healthy humans resulted in no adverse health effects as determined by kidney, liver functions tests, hematology, urinalysis, electrocardiogram records and gallbladder visualization (Shipley et al. 1968). Long-term (22 mo) oral administration of β-sitosterol produced no detrimental effects in rats, rabbits and dogs (Shipley et al. 1968). Chronic administration of β-sitosterol subcutaneously to either male or female rats was well tolerated without any evidence of tissue abnormalities (Malini and Vanithakumari 1990). In apolipoprotein E–deficient mice, phytosterols (2 g/100 g diet) were antiatherogenic; this beneficial effect of phytosterols was accompanied by less fragile RBC, unlike the situation in SHRSP rats and sitosterolemia (Moghadasian et al. 1997 and 1999). The observed decrease in the RBC osmotic fragility of apolipoprotein E–deficient mice was attributed to the decrease in plasma cholesterol level because of the inhibitory effect of phytosterols on cholesterol absorption in the intestine and no significant accumulation of phytosterols in plasma and tissues (Moghadasian et al. 1999). Furthermore, in contrast to SHRSP rats, which are sustained by dietary cholesterol, the cholesterol-fed rabbits (0.5 g/10 g diet for 1 y) suffered from a RBC deformability, compared with controls fed a cholesterol-free diet (Kanakaraj and Singh 1989). These observations might indicate that more attention should be given to the cholesterol balance in stabilizing membrane function and the fact that different species of laboratory animals may respond differently to the cell membrane cholesterol balance. The various studies also point out that phytosterols that exert beneficial antiatherogenic effects in humans and some animal species, may be toxic to some other species, viz., SHRSP rats and phytosterolemic subjects, when a gene defect induces a cholesterol-related membrane abnormality.

Although canola oil, corn oil and olive oil reduced the life span of SHRSP rats, their safety in human nutrition for the general population is unquestionable because all of these oils have been used in many countries over several centuries or decades without any adverse health consequences. Moreover, SHRSP rats are a model for intracerebral hemorrhagic stroke (Yamori et al. 1987), which is not a major health issue in many countries. In Canada, intracerebral hemorrhagic stroke incidence is very low and declining (Mayo et al. 1996) despite a high increase in the consumption of canola oil over the last two decades. Currently, in Canada, intracerebral hemorrhagic stroke accounts for 6.77 deaths per 100,000 population. Coronary heart disease (CHD), on the other hand, is the leading cause of death in most industrialized countries, and its importance as a major health problem is increasing in developing

countries (Tunstall-Pedoe et al. 1999). High serum cholesterol is one of the main risk factors for CHD, and a high intake of saturated fatty acids is the main determinant of serum cholesterol concentration (Hegsted et al. 1993). Lowering the intake of saturated fat is one of the approaches used for maintaining a desirable serum cholesterol concentration. Research conducted over the last four decades has firmly established the hypocholesterolemic effects of phytosterols (Ling and Jones 1995). Thus, canola oil, because of its low concentration of saturated fatty acids and high phytosterol concentration, could be considered more beneficial than other common dietary fats in lowering the blood lipid risk factors for the development of CHD.

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