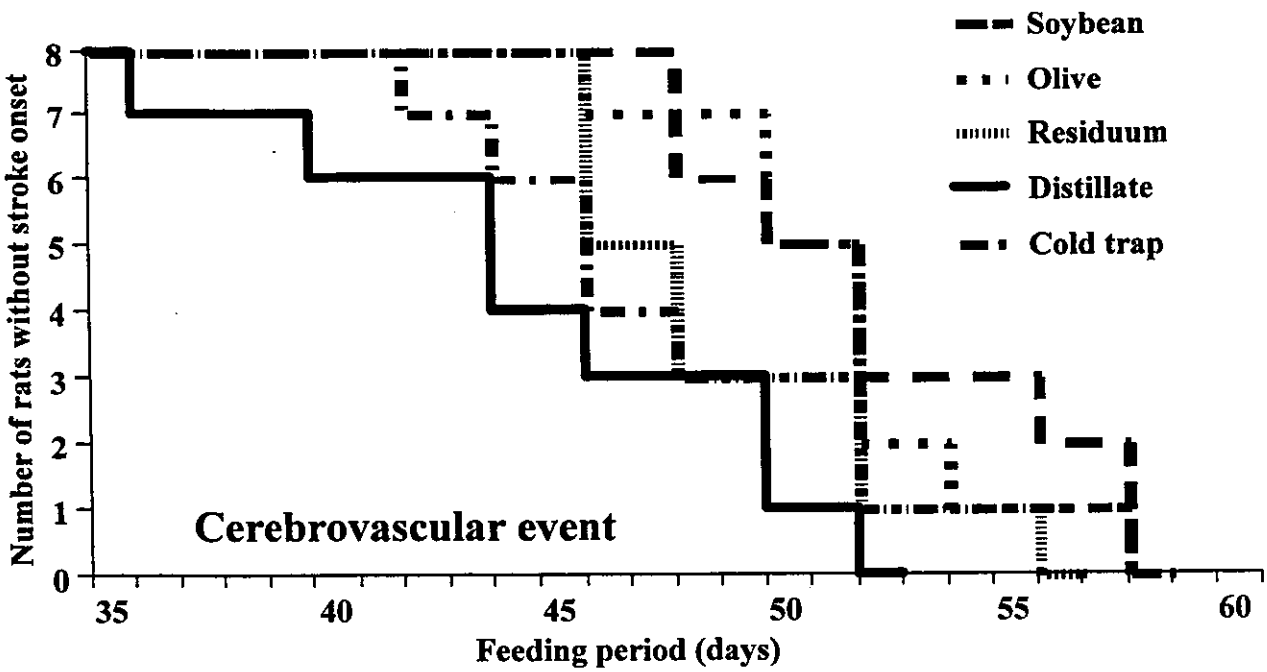

Fig. 4 Effects of test oils on stroke onset in the salt-loaded SHRSP

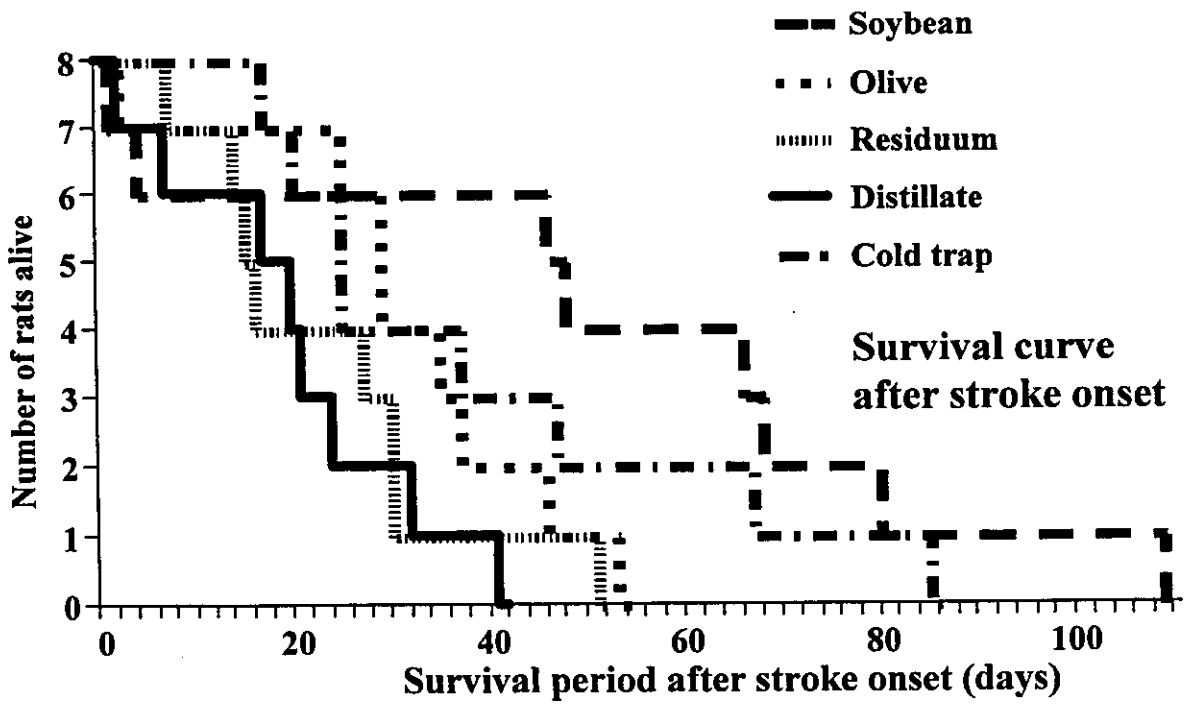

Fig. 5 Effects of test oils on survival period after stroke onset in the salt-loaded SHRSP

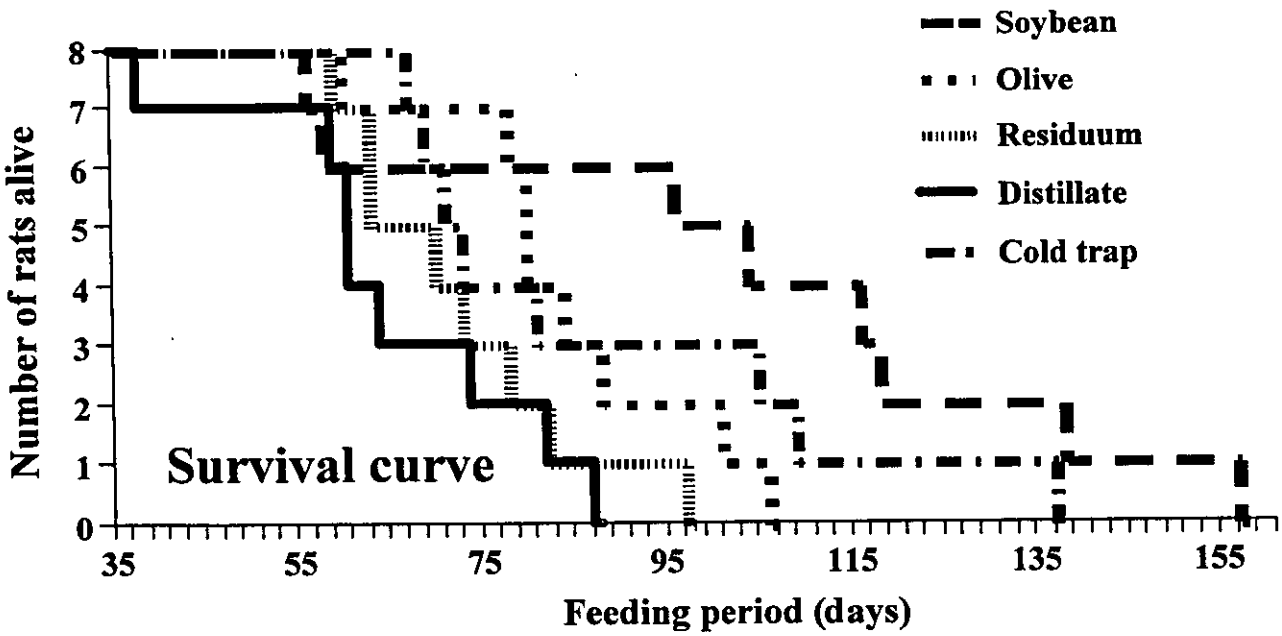

Fig. 6 Effects of test oils on survival period in the salt-loaded SHRSP

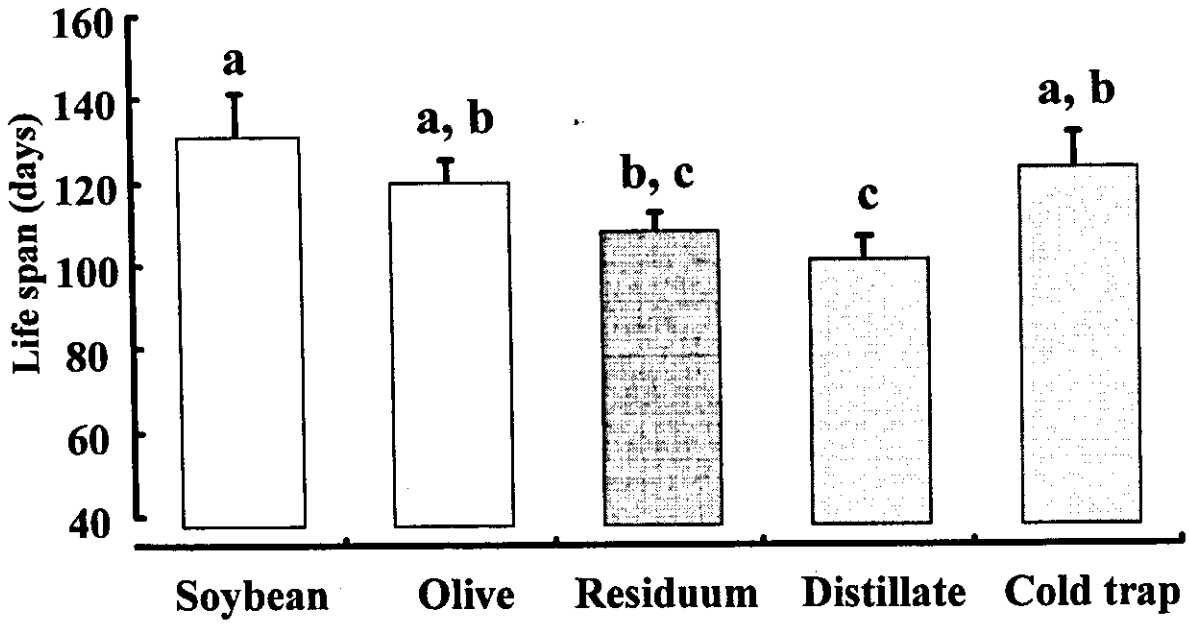

Fig. 7 Effect of test oils on life span of the salt-loaded SHRSP

Data are the mean ± standard error of the mean (n=8 in each group).
Values not sharing common superscript letter are significantly different at $p<0.05$.

Ⅲ. 研究成果の刊行に関する一覧表

雑　誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tatematsu,K., Hirose.N., Ichikawa,Y., Fujii,Y., Takami,A. and Okuyama,H.	Nutritional Evaluation of an Inter-Esterified Perilla Oil and Lard in Comparison with Butter and Margarine Based on the Survival of Stroke-Prone Spontaneously Hypertensive (SHRSP) Rats.	J.Health Sci.	50(1)	108-111	2004
Tatematsu,K., Fuma,S., Satoh,J., Ichikawa, Y., Fujii,Y., and Okuyama,H.	Dietary canola oil and soybean oil fed to SHRSP rat dams differently affect the growth and survival of their male pups.	J.Nutr.	134	1347-1352	2004
Tatematsu,K., Fuma,S., Nagase,T., Ichikawa, Y., Fujii,Y., and Okuyama,H.	Factors other than phytosterols in some vegetable oils affect the survival of SHRSP rats.	J.Chem.Toxicol.	42	1443-1451	2004
内藤　由紀子、大原　直樹	菜種油摂取による脳卒中易発症高血圧自然発症ラットの短命化の要因に関する基礎研究	秦野研究所年報	27	49-60	2005
小林　哲幸	ストレス応答とステリルグルコシド	脂質栄養学	13	36-43	2004

Ⅳ. 研究成果の刊行物・別刷

Rapid Communication

Nutritional Evaluation of an Inter-Esterified Perilla Oil and Lard in Comparison with Butter and Margarine Based on the Survival of SHRSP Rats

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Some kinds of vegetable oil and a partially-hydrogenated oil shorten the survival of the stroke-prone spontaneously hypertensive (SHRSP) rats compared with perilla seed oil, soybean oil and lard. The n-3/n-6 ratio of constituent fatty acids, phytosterol content and other factors in these oils have been proposed to affect the survival of this strain. Here, we examined the safety of a fat produced by the inter-esterification of perilla oil and lard (Perilla-Lard) on the bases of the survival of SHRSP rats. The mean survival time decreased in the order of the butter, the Perilla-Lard, the lard, the margarine and the partially-hydrogenated soybean oil (Hyd.Soy) group. The correlations between survival time and cholesterol content or phytosterol content in the diet were analyzed, and the probable health benefits of the new margarine-type fats made of animal fats and oils with high n-3/n-6 ratios were discussed.

Key Words: Safety, butter, perilla oil, lard, SHRSP rat, survival

INTRODUCTION

The stroke-prone spontaneously hypertensive (SHRSP) rat strain, established from the SHR and Wistar/Kyoto strains, develops hypertension and cerebral hemorrhage, particularly when a salt solution in given as drinking water. These three strains develop phytosterolemia more easily than other rat strains possibly due to a mutation in a ABC transporter.[1,2] The addition of cholesterol to the diet prolongs the survival of this strain.[3] We found that canola oil, olive oil and some other oils shorten the survival of this strain by 15 to 40% depending on salt concentration as compared with soybean oil and perilla oil.[4,5]

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Partially-hydrogenated soybean oil (Hyd.Soy) has a survival-shortening activity comparable to that of canola oil[4] but lard is as safe as soybean oil. Fats and oils have different effects on survival of SHRSP rats that can not be accounted for by the difference in their fatty acid composition. Thus we assumed the presence of minor components in some vegetable oils (survival-shortening factors) to explain such differences.[5] Recently, phytosterols have been proposed to form part of the survival-shortening factors. Along this line, olive oil is exceptional in that its phytosterol content is the lowest but survival-shortening activity is the highest among the oils examined.[6]

Efforts to identify the principle of survival-shortening factors have been continued, but it is possible to obtain a new

type of fat without such survival-shortening activity by the inter-esterification of animal fat and perilla seed oil or flaxseed oil. The expected health benefits of such new types of margarine are as follows; (a) their melting point can be modified by changing the ratio of fats and oils, and (b) high n-3/n-6 ratios can be maintained by choosing low-linoleic acid fats and oils. Cholesterol in animal fats has long been considered as a detrimental factor for atherosclerosis-related diseases, but recent epidemiological studies revealed that serum cholesterol level negatively correlates with all-cause mortality and cancer mortality.[7] Therefore, cholesterol in animal fats is unlikely to be a risk factor for diseases in elderly. Moreover, saturated and monounsaturated fatty acids are not converted to eicosanoids, the over and unbalanced production of which is a major risk factor for these diseases. On the basis of this background, we prepared margarine-type fats by the inter-esterification of animal fats and perilla seed oil, and compared the effect of an inter-esterification product (Perilla-Lard) on the survival of SHRSP rats with those of butter, lard, a commercially available margarine and Hyd.Soy.

MATERIALS AND METHODS

Diets and animals Test diets were prepared by mixing a basal conventional diet (CE-2; Central Laboratory of Experimental Animals, Clea Japan, Tokyo) with one type of fat at a 9:1 ratio. Butter (Y-Brand) and margarine (N-brand) obtained from a local market were treated with hexane. The hexane layer was washed with water and the solvent was evaporated to obtain the fat fraction. Partially-hydrogenated soybean oil (mp., 30℃, a product for human consumption) was purchased from Hamari Chemicals, Ltd. (Osaka). SHRSP rats were purchased from Seack Yoshitomi Co. (Fukuoka, Japan) and bred in our laboratory. Male rats from the same litter were divided randomly into different dietary groups and introduced to a test diet at 4 wks old age. The rats were kept under specific pathogen-free conditions. Each of dietary groups comprised 12 animals and 0.25% NaCl was given as drinking water. This experiment was approved by the Ethical Committee of the Graduate School of Pharmaceutical Sciences, Nagoya City University.

Analysis of sterols and fatty acids in diets Sterols extracted from diets with hexane/chloroform (4:1) after saponification were treated with a trimethyl-silylating reagent (Tokyo Kasei Kogyo Co., Ltd., Tokyo), and the trimethylsilyl ethers were analyzed by gas-liquid chromatography (GLC). The fatty acids of the diets were analyzed as methylesters by GLC using heptadecanoic acid as an internal standard as described previously.[8]

Inter-esterification between lard and perilla oil The inter-esterification reaction between lard and perilla oil (1:1) was performed with sodium methoxide as catalyst according to a conventional method used in cooking oil production. The degree of inter-esterification was monitored on the basis of the appearance of new molecular triacylglycerols using an HPLC system (LC-VP ver.6.12 SP3) equipped with an ELSD detector (Shimadzu, Kyoto).[9]

Table 1 Fatty acid and sterol compositions of test diet

	Butter	Perilla-Lard	Lard	Margarine	Hyd. Soy
Fatty acid (%)					
Saturated fatty acid					
12:0	1.7	0.0	0.0	0.1	0.0
14:0	8.8	0.9	1.3	0.6	0.4
15:0	1.0	0.1	0.1	0.1	0.0
16:0	31.8	16.3	23.3	20.1	15.6
18:0	8.5	6.1	10.6	4.2	5.9
20:0	0.2	0.2	0.2	0.3	0.3
22:0	0.1	0.0	0.0	0.2	0.3
Monounsaturated fatty acid					
14:1	0.7	0.1	0.0	0.0	0.0
16:1	1.5	1.3	1.8	0.4	0.5
18:1cis	23.3	28.3	36.4	32.5	28.2
18:1trans	1.8	0.0	0.0	5.2	19.3
20:1	0.3	0.7	0.8	0.6	0.5
22:1	0.2	0.2	1.7	0.1	0.1
24:1	0.1	0.0	1.8	0.1	0.0
n-6 Polyunsaturated fatty acid					
18:2n-6	16.8	21.1	18.6	30.1	25.9
n-3 Polyunsaturated fatty acid					
18:3n-3	1.4	23.1	1.1	3.2	1.3
20:5n-3	1.0	0.9	1.8	1.0	1.1
22:6n-3	0.6	0.5	0.5	0.8	0.6
Sterol (mg/100g diet)					
Cholesterol	75.5	62.3	65.2	53.2	54.1
Campesterol	23.0	27.2	23.9	32.3	29.6
Stigmasterol	8.4	11.0	9.3	12.6	14.0
β-Sitosterol	47.1	71.0	49.9	66.0	82.0
Total phytosterol	78.5	109.2	83.1	110.8	125.7
Total sterol	153.9	171.5	148.3	164.1	179.8

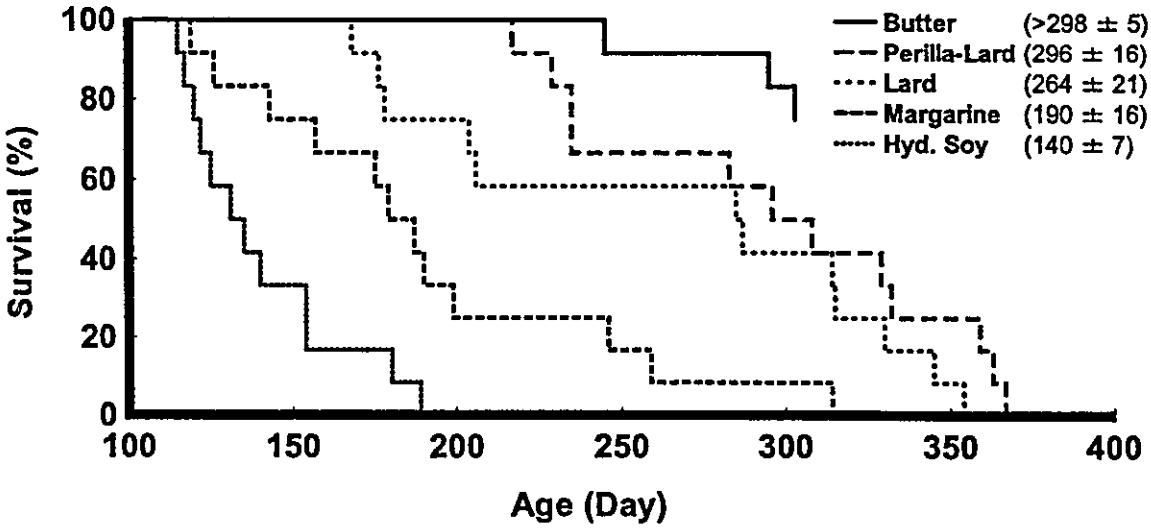

Figure 1 Comparison of the survival curves and mean survival days shown in parentheses

Statistical analyses Data were presented as means ± SEM. Statistical analysis of the survival data was performed by the Log-Rank and nonparametric Wilcoxon's signed rank tests. The computer program KyPlot ver. 2.0 (Kyence Inc., Tokyo) was used.

RESULTS AND DISCUSSION

The fatty acid and sterol compositions of the diets are shown in Table 1. Butter, lard and Perilla-Lard exhibited typical expected fatty acid compositions. From the fatty acid composition of margarine, it appears to be of the hard type composed of partially-hydrogenated vegetable oil(s) and untreated vegetable oil(s). The proportions of *trans* octadecenoic acid, 5.2% in the margarine and 19.2% in Hyd.Soy, can be a rough measure of the ratio of hydrogenated oil to untreated oil.

The mean survival time decreased in the order of butter, Perilla-Lard, lard, margarine and Hyd.Soy groups (Fig.1). The mean survival time of the Hyd.Soy group was comparable to that of the canola oil group, and those of the butter and soybean oil groups were similar (data not shown).

The difference in the mean survival time between butter and Perilla-Lard groups was not significant ($p=0.066$ in Log-Rank test and $p=0.051$ in the Wilcoxon's signed rank test), the mean survival time of Perilla-Lard group tended to be longer than that of lard group ($p=0.053$ in the Log-Rank test and 0.033 in the Wilcoxon's signed rank test), the mean survival times of the butter and Perilla-Lard groups were significantly longer than that of the margarine group ($p<0.002$ in both tests), and the difference between lard and margarine groups was significant ($p<0.02$ in both tests). The mean survival time of the Hyd.Soy group was significantly shorter than those of the butter, Perilla-Lard and lard groups ($p=0.000$ in both tests), and also shorter than that of the margarine group ($p<0.01$ in both tests).

Phytosterols, which are competitive inhibitors of cholesterol absorption in the intestine, have been shown to shorten the survival of SHRSP rats, but dietary cholesterols have been shown to prolong it.[3,6] The contents of cholesterol and major phytosterols in the diets are shown in Table 1. The basal diet (CE-2) contained cholesterol derived from fish meal and skimmed milk. In addition, butter, Perilla-Lard and lard contain cholesterol. The mean survival time correlated positively with cholesterol content in the diet and negatively with phytosterol content, except for the Perilla-Lard group; the phytosterol content in the Perilla-Lard diet was comparable to that in the margarine diet but the survival of the former was much longer than that of the latter ($p<0.02$). This

could be attributed to α-linolenic acid-rich perilla oil, which is known to prolong the survival of SHRSP rats.[8] Similarly, high-linoleic and high-oleic types of safflower oil had similar phytosterol contents but their survival times were very different.[4,10] Thus, phytosterol is a factor affecting the survival of SHRSP rats, but other factors are also involved.

The SHRSP, SHR and WKY rat strains are unique in that they develop phytosterolemia more easily than other rat strains.[1,2] Until now, the impact of the observed survival-shortening activity of some fats and oils on human nutrition remains inconclusive.[11] However, new types of fat (margarine) made of animal fats and perilla oil or flaxseed oil may be accepted particularly when the elevation of n-3/n-6 ratio in foods is recommended and cholesterol intake is shown not to positively correlate with mortality from cardiovascular diseases.[12]

Acknowledgment This work was supported in part by a Health and Labour Science Research Grant from the Ministry of Health, Labour and Welfare, Japan.

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Dietary Canola and Soybean Oil Fed to SHRSP Rat Dams Differently Affect the Growth and Survival of Their Male Pups[1]

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ABSTRACT Canola oil (Can), as well as some other oils, shortens the survival of SHRSP rats compared with soybean oil (Soy). Although detrimental factors other than phytosterols have not been identified, they are likely to be hydrophobic and transmittable to pups. To test this possibility, female SHRSP rats (F0) were fed a diet supplemented with Can or Soy and mated at 11 wk of age. The growth of suckling pups (F1) from the Can-fed dams was significantly retarded compared with that of pups from the Soy-fed dams. Half of the male pups (F1) were weaned to the same diet as their dams (Can→Can and Soy→Soy groups) and the rest were weaned to the other diet (Can→Soy and Soy→Can groups). The survival rate of the male pups (F1) was significantly lower in the Can→Can group than in the Soy→Can group, and in the Can→Soy group than in the Soy→Soy group, indicating that the oils fed to dams differently affected the growth and survival of pups. There were fewer pups per dam in the Can-fed dams (F0) than in the Soy-fed dams, and in the dams (F1) of the Can→Can and Soy→Can groups than in those of the Can→Soy and Soy→Soy groups. Although Can is nutritionally detrimental to SHRSP rats compared with Soy, no direct evidence has been obtained thus far relating these observations to human nutrition. J. Nutr. 134: 1347–1352, 2004.

KEY WORDS: • *SHRSP rat* • *canola oil* • *soybean oil* • *survival* • *reproduction*

Vegetable oils contain various minor components such as fat-soluble vitamins, phytosterols, isoflavonoids, tocopherols, and environmental chemicals (1). Fat-soluble substances are generally secreted into breast milk and are likely to affect the pups' physiology (2). The spontaneously hypertensive rat, stroke prone (SHRSP)[3] strain, derived from the SHR and Wistar-Kyoto (WKY) strains, develops hypertension and dies of stroke frequently, particularly when salt is added to their drinking water. SHRSP rats exhibit various other anomalies such as renal injury (3), peroxidative injury (4), developmental disorders (5), and reproductive physiologic disorders (6,7). Using this strain, we showed that dietary perilla seed oil, flaxseed oil, and fish oil with very low (n-6)/(n-3) ratios prolong survival by ~10% compared with safflower and soybean oils with high (n-6)/(n-3) ratios (8,9); however, canola oil (Can), with a relatively low (n-6)/(n-3) ratio (~2.5), markedly shortens survival (~40% in the absence of NaCl in the drinking water) compared with soybean oil (Soy). In addition, several other vegetable oils (e.g., olive oil, corn oil, high-oleic safflower oil, high-oleic sunflower oil, evening primrose oil, hydrogenated Soy, and hydrogenated Can) were shown in our laboratory and by others to shorten survival similarly to Can (10–12). Decreased platelet number (13), increased red cell fragility (14), severe renal injury involving lesions in blood vessels (15), and elevated blood pressure (16) are associated with dietary Can. Antinutritional activities of Can were observed in other strains of rats (16) and mice (17), and in other species. For example, platelet number was decreased and mortality was increased in iron-injected piglets fed a milk replacer diet that contained Can (18,19).

Extensive effort has been made to identify the antinutritional factor associated with some vegetable oils. Ratnayake et al. (20,21) found that the phytosterol content of oils is involved in shortening survival because Can and corn oil, with higher phytosterol contents than Soy, exhibit such activities. Olive oil is an exception, however, in that it has the lowest phytosterol content but shortens survival the most among the oils examined. A purified phytosterol fraction from Can shortened survival when it was added to a Soy diet at 2 times the concentration of the Can diet, suggesting that the phytosterol content is a factor contributing to the shortening of survival (20). However, olive oil was not a single exception for the proposed association between survival and phytosterol content. Indeed, we found other exceptions (10,12), and Ogawa et al. (22) found that an amount of phytosterol comparable to that of Can is not sufficient to reproduce the activity of Can, indicating that factors other than the major phytosterols are involved in shortening survival.

We postulated that the factors that shorten survival are

[1] Supported in part by a Health and Labour Science Research Grant from the Ministry of Health, Labour and Welfare Japan.

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[3] Abbreviations used: Can, canola oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SHRSP, spontaneously hypertensive rat, stroke prone; Soy, soybean oil; WKY, Wistar-Kyoto.

Manuscript received 11 December 2003. Initial review completed 25 January 2004. Revision accepted 4 April 2004.

TABLE 1

Effect of dietary oils fed to dams on reproductive variables in rat dams (F0) fed Can or Soy diet and pups (F1) weaned to the same diet (Can→Can and Soy→Soy) or to the other diet (Can→Soy and Soy→Can), and on the survival of the 4 dietary groups of male pups (F1)

Generation	F0		F1			
Dietary group	Can	Soy	Can→Can	Soy→Can	Can→Soy	Soy→Soy
Diet for F0	Can	Soy	Can	Soy	Can	Soy
Diet for F1			Can		Soy	
Delivered dams/mated female, *n/n*	30/41	30/39	17/27	22/25	22/27	23/25
Male pups, *n*	106	140	57	85	107	100
Female pups, *n*	130	126	84	94	106	113
Total pups, *n*	236	266	141	179	213	213
Pups/dam,[1] *n*	7.9	8.9*	8.2	8.1	9.7	9.3

[1] 2-way ANOVA: the effect of the F1 diet was significant ($P = 0.016$), whereas those of the F0 diet ($P = 0.409$) and their interaction ($P = 0.801$) were not. * Different from Can, $P = 0.037$ (Student's t test).

likely to be hydrophobic and transmittable to the next generation. Here, we examined this possibility by feeding Can and Soy to SHRSP rats through 2 generations.

MATERIALS AND METHODS

Animal and diets. The conventional basal diet (CE-2, standardized for the proliferation and maintenance of rats and mice) was composed of soybean meal, fishmeal, skimmed milk, corn, wheat, wheat bran, alfalfa meal, a vitamin mixture, and a mineral mixture (The Central Laboratory for Experimental Animals Japan, Clea Japan). The basal diet consisted of 24.8% protein, 4.4% fat, 3.5% dietary fiber, 7% ash, and minerals and vitamins.[4] The basal diet (CE-2) and Can or Soy were mixed at a 9:1 ratio and the mixture was pelleted (Clea Japan). These test diets were stored at −20°C for <3 mo. Vegetable oils available for human consumption were purchased from local markets. The test diets supplied were replaced every 2 d to keep the peroxide values of the served food below 100 mEq/kg. The pelleted conventional diet was more stable to peroxidation than the pelleted semipurified diet (data not shown).

SHRSP rats were obtained from Seack Yoshitomi and maintained in our laboratory. Rats from the same litter were randomly divided into different dietary groups and kept under specific pathogen-free conditions. The temperature and humidity in the room were maintained at 23 ± 3°C and 50 ± 3%, respectively, with a 12-h day:night light cycle. For survival tests, the number of rats was 10–12 in each dietary group, and a 5 g/L NaCl solution was given as drinking water (NaCl-loaded). This study was approved by Ethical Committee of the Graduate School of Pharmaceutical Sciences, Nagoya City University.

Reproductive physiology. Female rats at 4 wk of age were randomly divided into the Can and Soy groups (F0, n = 9–12). At 11 wk of age, they were mated with male SHRSP rats fed the basal diet for 1 wk. The rats were kept for 4 d at a male:female ratio of 1:2 and then the male/female combinations were changed for another 4 d (Fig. 1). Parturition and lactation were observed every 12 h. Dams and pups were fed the same diet for 3 wk after parturition and the weight gain of the pups was estimated. At 4 wk of age, the pups (F1) in each dietary group were randomly divided into 2 groups; half were weaned to the same diet as their dams (Can→Can and Soy→Soy groups) and the other half were weaned to the other diet (Can→Soy and Soy→Can groups), and the survival of the male pups (F1) of the 4 dietary groups was monitored. The female SHRSP rats (F1) of the corresponding 4 dietary groups were mated at 11 wk of age. These experiments were repeated 2 or 4 times to obtain sufficient data for statistical analysis.

Collection of breast milk. Eight hours after separation from their pups, the dams at 3 wk of lactation were pretreated i.p. with 5 U of oxytocin (Wako Chemicals) and anesthetized with pentobarbiturate; their milk was collected with a Pasteur pipette and then frozen at −70°C until analysis.

Lipid analysis. The fatty acid compositions of the test diets, serum lipids, and milk lipids were analyzed after extraction of total lipids according to the method of Bligh and Dyer (23). Fatty acids were converted to their methyl esters by treatment with 1.37 mol/L HCl in methanol and were quantified by GC using a capillary column (DB-225, J&W Scientific) (12). Heptadecanoic acid was used as an

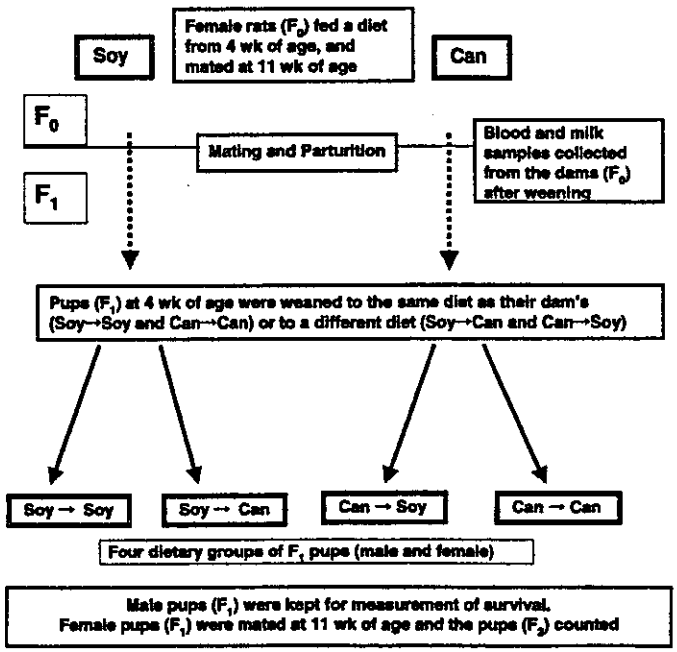

FIGURE 1 Protocol for the assessment of the reproductive physiology of rat dams (F0) fed Can or Soy diet and pups (F1) weaned to the same diet (Can→Can and Soy→Soy) or to the other diet (Can→Soy and Soy→Can), and of the survival of the 4 dietary groups of male pups (F1).

[4] The basal diet contained the following minerals and vitamins (/kg): calcium (12.5 g), phosphorus (10.6 g), magnesium (3.4 g), potassium (11.1 g), manganese (113 mg), iron (316 mg), copper (8.6 mg), zinc (52 mg), sodium (3.8 g), retinol (4.3 mg), α-tocopherol (110 mg), thiamine (15.8 mg), riboflavin (15 mg), pyridoxine (13 mg), vitamin B-12 (0.035 mg), ascorbic acid (280 mg), pantothenic acid (30.5 mg), niacin (17.3 mg), folic acid (1.6 mg), choline (2.65 g), biotin (0.391 mg) and inositol (2.08 g).

internal standard. Sterols were determined as trimethylsilyl ether derivatives by GC as described by Ratnayake et al. (20).

Statistical analyses. Data are presented as means ± SEM. Statistical analyses of the survival rates were performed by Log-rank test, which is interpreted to reflect relatively more of the difference in the late phase than of the early phase of the survival curves, and by Wilcoxon signed rank tests (nonparametric) reflecting more of the difference in the early phase. Student's *t* test was used for the comparison of 2 groups, i.e., the difference in the numbers of pups/dam between the Can and Soy groups (F0), and the difference in the fatty acid and sterol compositions of diets and tissue lipids between the 2 dietary groups. The difference in the number of pups/dam of the 4 dietary groups (F1) was analyzed by 2-way ANOVA with F0 diet and F1 diet as factors. Difference in the weights of male and female pups (F1) was analyzed by two-way ANOVA with sex and diet as factors. The computer program KyPlot ver. 2.0 (Kyence) was used. Probability values < 0.05 were considered to indicate significant difference.

RESULTS

Reproductive physiology. Rats in the 2 dietary groups became pregnant at similar rates (Table 1). The number of pups (F1) per dam was lower in the Can group than in the Soy group ($P < 0.05$).

The pups grew normally. However, the weight gains of the male and female pups (F1) before weaning at 4 wk of age were lower in the Can group than in the Soy group ($P < 0.01$, Table 2), even though the mean number of pups per dam was lower in the Can group (Table 1). There were fewer pups (F2) per dam (F1) in the Can→Can and Soy→Can groups than in the Can→Soy and Soy→Soy groups ($P < 0.05$, Table 1).

Survival of male pups (F1). The mean survival time of the male pups (F1) increased in the order of Can→Can, Soy→Can, Can→Soy and Soy→Soy groups (Fig. 2, Table 3). The effect of dietary oil fed to dams (Can or Soy) on the survival of the pups (F1) was analyzed for the following 2 combinations. The difference in the survival rates of the Can→Can and Soy→Can groups was significant in the Wilcoxon test but not in the Log-rank test (Table 3). The difference in the survival rates of the Can→Soy and Soy→Soy groups was significant in both tests.

Fatty acid and sterol compositions of dams' serum and milk lipids. The serum of the Can and Soy groups contained similar concentrations of fatty acids (Table 4). The serum lipids of the Can group contained significantly more octadecenoic and less linoleic acid than those of the Soy group, reflecting the dietary fatty acid compositions. Interestingly, the proportion of arachidonic acid and the arachidonate/linoleate ratio were significantly higher in the Can group than in the Soy group, even though the Can diet contained less linoleic acid than the Soy diet (Table 4). Similarly, the proportions of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as well as the (EPA plus DHA)/α-linolenate ratio were greater in the Can group than in the Soy group.

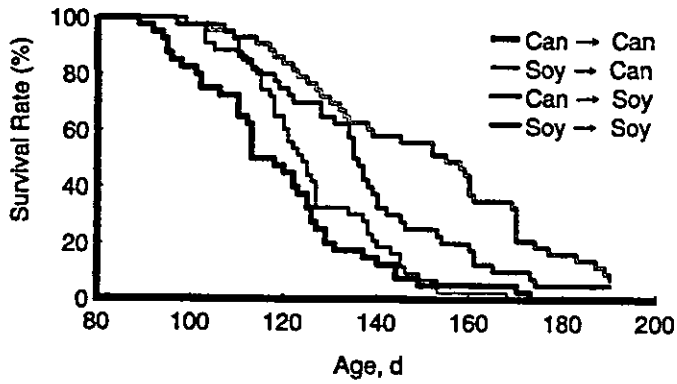

FIGURE 2 Effect of feeding Can or Soy diets to rat dams (F0) on the survival of their male pups (F1) weaned to the same diet as their dams (Can→Can and Soy→Soy) or to the other diet (Can→Soy and Soy→Can) and given a 5 g/L NaCl solution as drinking water. The results of statistical analyses of the survival rates are presented in Table 3.

The dams' milk from the 2 dietary groups (F0) contained similar concentrations of fatty acids. The fatty acid composition of the milk was very similar to that of each diet, except that the medium-chain fatty acids (10:0, 12:0, and 14:0), which were likely synthesized in the dams, were relatively higher. Because the proportions of 20- and 22-carbon highly unsaturated fatty acids were relatively small in the milk, the dietary effects on the elongation and desaturation reactions were not apparent in milk lipids.

The basal diet itself contained cholesterol and phytosterols; hence the difference in sterol concentrations between the 2 diets was smaller than that between the 2 oils (Table 5). Stigmasterol and brassicasterol were present in the diets but were not detectable in the serum and milk. The proportion of phytosterols in the total sterol fraction and the phytosterol/cholesterol ratio were greater in the Can diet than in the Soy diet, but in the milk lipids, the differences in these variables between the 2 dietary groups were less pronounced than in the diets. The phytosterol/cholesterol ratio decreased in the order of diets > serum lipids > milk lipids. The phytosterols were not concentrated into milk lipids compared with diet because the phytosterol/cholesterol ratio of the milk from the Can group (0.16) was even smaller than that of the milk of dams fed the control Soy diet (1.5). Similarly, the phytosterol/energy ratio of the milk from the Can group (~25 μg/kJ, calculated based on the energy contents of cows' and human

TABLE 2

Body weights of male and female F1 pups fed Can or Soy diets from weaning until 4 wk of age[1]

	Male		Female		2-way ANOVA, *P*-value		
Age, *d*	Can (*n* = 53)	Soy (*n* = 67)	Can (*n* = 65)	Soy (*n* = 59)	Sex	Diet	Interaction
7	12.1 ± 0.2	12.4 ± 0.2	11.6 ± 0.2	12.2 ± 0.2	0.032	0.001	0.487
14	22.6 ± 0.2	23.7 ± 0.2	21.6 ± 0.2	23.1 ± 0.2	0.001	<0.001	0.437
21	33.8 ± 0.4	36.6 ± 0.4	32.5 ± 0.3	35.3 ± 0.4	<0.001	<0.001	0.992
28	49.7 ± 0.7	55.0 ± 0.6	46.6 ± 0.5	51.2 ± 0.7	<0.001	<0.001	0.546

[1] Values are means ± SEM.

TABLE 3

Survival time of 4 dietary groups of male F1 rats fed Can or Soy diets from weaning until 4 wk of age[1,2]

Dietary group		Can→Can	Soy→Can	Can→Soy	Soy→Soy
Mean survival time, *d*		119 ± 3	126 ± 2	134 ± 4	150 ± 4
vs. Can-Can	Log-rank[2]		0.251	0.002	<0.001
	Wilcoxon[3]		0.033	0.001	<0.001
vs. Soy-Can	Log-rank			0.023	<0.001
	Wilcoxon			0.067	<0.001
vs. Can-Soy	Log-rank				0.003
	Wilcoxon				0.012

[1] Values are means ± SEM, n = 36–38/group.
[2] The Log-rank test reflects relatively more of the late phase than of the early phase of survival curves.
[3] The Wilcoxon test reflects more of the early phase.

milk) was even less than that of the milk of dams fed the control Soy diet (100 μg/kJ).

DISCUSSION

Phytosterols shorten the survival of SHRSP rats (20,21), but a 5-fold greater amount of phytosterol is required to produce an effect similar to the survival-shortening effect of Can (23). The SHRSP rat strain, as well as the parent WKY strain, develops phytosterolemia more easily than some other rat strains (24,25), and the subcutaneous administration of phytosterols at ~0.5 mg/kg daily for up to 48 d affects the fertility of male rats (26). However, the administration of phytosterol esters for 2 generations did not affect the reproduction of male and female rats (Wistar) at 8% of diet, a

TABLE 4

Fatty acid compositions of diets and dam's milk collected at 3 wk of lactation and of dam's serum at 6 wk of age in rats fed the Can or Soy diet[1–3]

	Diet		Serum		Milk	
	Can	Soy	Can	Soy	Can	Soy
			g/100 g total fatty acids			
Saturated fatty acids						
10:0	—	—	—	—	6.3 ± 1.0	6.2 ± 1.3
12:0	—	—	—	—	4.2 ± 0.6	3.7 ± 0.6
14:0	0.3	0.3	—	—	2.7 ± 0.4	2.2 ± 0.3
16:0	9.3	13.2	14.5 ± 0.4	16.4 ± 0.3**	11.5 ± 1.6	13.8 ± 2.3
18:0 (dimethylacetal)	—	—	0.3 ± 0.1	0.3 ± 0.1	1.8 ± 0.4	3.4 ± 0.7*
18:0	2.1	4.1	12.7 ± 0.3	12.0 ± 0.6	0.2 ± 0.0	0.1 ± 0.0*
20:0	0.5	0.3	0.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:0	0.2	0.3	—	—	—	—
24:0	—	—	0.3 ± 0.1	0.0 ± 0.0*	—	—
Monounsaturated fatty acids						
14:1	0.0	0.1	—	—	—	—
16:1	0.5	0.4	0.0 ± 0.0	0.3 ± 0.1**	0.6 ± 0.1	0.6 ± 0.1
18:1(n-9)	49.3	24.1	22.4 ± 0.6	12.8 ± 0.9**	48.5 ± 6.9	24.4 ± 4.6**
18:1(n-7)	—	—	1.1 ± 0.3	0.5 ± 0.1*	—	—
20:1	1.3	0.5	—	—	1.0 ± 0.1	0.4 ± 0.1**
22:1	—	—	—	—	0.1 ± 0.0	0.1 ± 0.0
24:1	—	—	0.8 ± 0.1	0.7 ± 0.2	—	—
(n-6) PUFA						
18:2(n-6)	27.8	49.4	20.5 ± 0.4	33.7 ± 0.8**	24.1 ± 3.3	45.1 ± 7.9*
18:3(n-6)	0.6	0.0	0.4 ± 0.1	0.6 ± 0.0*	0.3 ± 0.1	0.5 ± 0.1
20:3(n-6)	—	—	—	—	0.2 ± 0.0	0.4 ± 0.1
20:4(n-6)	—	—	20.9 ± 0.5	17.4 ± 1.1*	0.7 ± 0.1	1.0 ± 0.2
22:4(n-6)	—	—	—	—	0.1 ± 0.0	0.1 ± 0.0
(n-3) PUFA						
18:3(n-3)	6.6	5.7	1.4 ± 0.1	2.0 ± 0.2**	5.5 ± 0.7	4.8 ± 0.8
20:5(n-3)	—	—	1.5 ± 0.1	1.0 ± 0.0**	0.4 ± 0.0	0.4 ± 0.1
22:5(n-3)	0.9	0.8	—	—	0.2 ± 0.0	0.2 ± 0.1
22:6(n-3)	0.8	0.7	2.8 ± 0.1	2.3 ± 0.1**	0.4 ± 0.1	0.5 ± 0.1
Total FA, *mg/mL*			5.33 ± 0.35	5.99 ± 0.32	108.9 ± 14.5	108.1 ± 18.8

[1] Values are means ± SEM, n = 18 (serum) or 10 (milk); —, not detectable.
[2] Asterisks indicate different from Can: * $P < 0.05$; ** $P < 0.01$ (Student's t test).
[3] The total fatty acid concentration in the diets was 12.4 g/100 g (n = 2).

TABLE 5

Sterol concentrations of diets and dams' milk collected after 3 wk of lactation and dams' serum taken at 6 wk of age in rats (F0) fed the Can or Soy diet[1,2]

	Diet		Serum		Milk	
Diet group	Can	Soy	Can	Soy	Can	Soy
	μmol/g diet		*mmol/L*			
Cholesterol	1.77	1.69	1.70 ± 0.21	1.52 ± 0.22	1.25 ± 0.14	1.26 ± 0.19
Brassicasterol	0.18	—	—	—	—	—
Campesterol	1.28	0.42	0.22 ± 0.03	0.13 ± 0.03*	0.11 ± 0.02	0.07 ± 0.01
Stigmasterol	0.12	0.17	—	—	—	—
β-Sitosterol	2.40	1.72	0.21 ± 0.03	0.16 ± 0.03	0.08 ± 0.01	0.07 ± 0.01
Total phytosterol	1.63	0.95	0.47 ± 0.03	0.33 ± 0.04*	0.20 ± 0.02	0.15 ± 0.02
Total sterol	2.31	1.61	2.36 ± 0.11	2.04 ± 0.16	1.57 ± 0.10	1.54 ± 0.17
Phytosterol/Cholesterol	2.38	1.46	0.25 ± 0.01	0.19 ± 0.01**	0.15 ± 0.01	0.11 ± 0.01

[1] Values are means ± SEM, n = 18 (serum) or 10 (milk); —, not detectable.
[2] Asterisks indicate different from Can: * $P < 0.05$; ** $P < 0.01$ (Student's t test).

concentration that is much higher than that in the Can diet (0.16%) (27,28). The data presented in Table 5 support the interpretation that factors other than the major phytosterols are involved in the retarded growth and shortened survival of pups from dams fed the Can diet.

Eicosanoids derived from arachidonic acid and linoleic acid play pivotal roles in growth and reproductive physiology (29). Although the linoleic acid/α-linolenic acid ratio of the Can diet was smaller than that of the Soy diet, the proportion of arachidonic acid in serum lipids was greater in the Can group than in the Soy group (Table 4), indicating that the retardation of growth of the F1 pups from the Can-fed dams was not due to a lack of eicosanoid precursors.

In piglets fed a milk replacer containing Can, increased requirements for vitamin E after iron injection were revealed (19). However, the hepatic vitamin E content was greater in the Can group than in the Soy group (unpublished observations), indicating that the tissue vitamin E level is not a critical factor for the shortening of survival in SHRSP rats. Although the Can used was a double-low variety of rapeseed oil with reduced contents of erucic acid and glucosinolates, it still contains hydrolysis products of glucosinolates such as isothiocyanates, oxazolidinethione, indole derivatives, and other minor components (30).

After death, cerebral bleeding was observed in most of male SHRSP rats from both dietary groups (10). The color of the lung was darker in the Can-fed rats, particularly in those with shorter survival times, compared with that of the Soy-fed rats, which was indicative of hemorrhage and/or hemostasis. In kidney, impaired blood vessels and glomerular structures were observed microscopically and the severity of nephropathy symptoms was greater in the Can group than in the Soy group (15 and unpublished observations). All of these observations could be related to survival, and the causes of death appeared to be complex.

A beneficial effect of Can was shown in the Lyon Diet Heart Study (31); Can and olive oil were effective in the secondary prevention of coronary heart diseases, possibly due to the reduced intake of linoleic acid and increased intakes of oleic acid and α-linolenic acid. Although the use of relatively crude, high-erucic rapeseed oil has been associated epidemiologically with increased pulmonary adenocarcinoma in China (32), no other lines of evidence have been presented to date to suggest detrimental effects of Can on human health. However, the unusual effects of Can in rodents (10,13,17,20) and piglets (18,19), as well as those observed in the present experiments, warrant further studies to identify the detrimental factors other than phytosterols and/or to produce Can with reduced shortening of survival in SHRSP rats, because its fatty acid composition seems to be beneficial in human nutrition.

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Factors other than phytosterols in some vegetable oils affect the survival of SHRSP rats

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Abstract

Unusual survival-shortening activities of some vegetable oils were detected in stroke-prone spontaneously hypertensive (SHRSP) rats, and phytosterol (PS) in the oils and the tissue tocopherol status have been suggested to be the factors for the activities. Here, we re-evaluated the contribution of PS to the survival-shortening, and examined the hepatic tocopherol status. A basal diet for rodents and a test oil were mixed at a 9:1 ratio, and the diet was given to male SHRSP rats upon weaning. The total and major PS contents of the diets and tissue lipids did not correlate with relative survival time. The free fatty acid fractions obtained by lipase and alkaline hydrolyses of canola oil (Can) and the original Can contained PS in comparable amounts but the free fatty acid fractions did not exhibit survival-shortening activities compared with the soybean oil (Soy) group. The activity was not detected in the ethyl acetate extracts of the aqueous phase after the hydrolysis. When a commercially available PS preparation was added to the Soy diet at an amount 2.8-fold higher than that in the Can diet, the mean survival time was shortened but was still significantly longer than that of the Can group. The hepatic tocopherol level was significantly higher in the Can group than in the hydrogenated Soy group and Soy group, but the former two groups exhibited a survival-shortening activity. These results indicate that factors other than PS, tocopherol status and fatty acid composition in some vegetable oils are critical for the survival-shortening activity observed in SHRSP rats.

Keywords: Survival; Phytosterol; SHRSP rat; Canola oil; Soybean oil

1. Introduction

Oil from traditional rapeseed variety contains two anti-nutritional factors, erucic acid and thyrotoxic sulfur compounds from glucosinolates. In 1974, a new rapeseed cultivar that is low in both erucic acid and glucosinolate contents was released (Stefansson and Kondra, 1975), which is now available as canola. Even with the use of rapeseed oil of the low-erucic type as dietary supplement, myocardial lesions were observed in rats (McCutcheon et al., 1976). However, a triacylglycerol fraction, highly purified from low-erucic rapeseed oil by repeated molecular distillation, resulted in similar cardiac lesions even though the preparation lacked phytosterol (PS) (Kramer and Sauer, 1983). Based on the results of extensive studies, the observed myocardial lesions in male rats (Sprague-Dawley, Wistar and Sherman) were ascribed to triacylglycerols and their particular fatty acid composition (Kramer and Sauer, 1983).

Perilla seed oil, flaxseed oil and fish oil with very low $n-6/n-3$ ratios extended the survival of the SHRSP rats, an animal model of human-cerebral hemorrhages, by approximately 10%, and exhibited other beneficial effects compared with high-linoleic oils (Shimokawa et al., 1988; Okuyama et al., 1996; Miyazaki et al., 2000; Minami et al., 1997), indicating that dietary $n-3$ fatty acids are beneficial to SHRSP rats. Unexpectedly, canola oil (Can) with a relatively

Abbreviations: SHRSP rat, stroke-prone spontaneously hypertensive rat; Can, canola oil; Soy, soybean oil; PS, phytosterol

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low $n-6/n-3$ ratio (~2.5) shortened the survival time of SHRSP rats compared with the Soy diet (Huang et al., 1996). Some other oils such as high-erucic rapeseed oil, olive oil, evening primrose oil, high-oleic safflower oil, high-oleic sunflower oil, corn oil, hydrogenated Can and hydrogenated Soy (Hyd.Soy) also exhibited comparable survival-shortening activities (Huang et al., 1996, 1997; Miyazaki et al., 1998a,b; Ratnayake et al., 2000a,b). However, the free fatty acid fractions obtained by the lipase hydrolysis of Can, Hyd.Soy and high-oleic safflower oil did not exhibit such an activity, although the fatty acid compositions, tissue fatty acid compositions and growth rates were very similar between the groups fed these oils and derived free fatty acid fractions. These observations suggested that minor components other than fatty acids are responsible for the survival-shortening activity (Miyazaki et al., 1998a,b). In neonatal piglets raised on a Can-based milk replacer diet, tissue tocopherol levels were lower and signs of vitamin E deficiency were severer than those in piglets raised on a Soy-based milk replacer diet (Sauer et al., 1997). However, the factors in Can that increased the demand for vitamin E have not yet been determined.

Recently, the SHRSP rat strain as well as its parent WKY strain has been reported to exhibit severe phytosterolemia in comparison with other rat strains (Wistar, WKA) (Ikeda et al., 2001), the condition is associated with a mutation in an ATP-binding cassette (ABC) transporter with a presumed activity of excreting PS into the intestine (Scoggan et al., 2003). By comparing some vegetable oils, phytosterol (PS) was proposed to be involved, at least in part, in the survival-shortening activity, although olive oil was exceptional in that its PS content was the lowest but resulted in the shortest survival time among the oils examined (Ratnayake et al., 2000a). A mixture of PS, extracted from canola deodorant distillates and recrystalized, was shown to exhibit a survival-shortening activity (Ratnayake et al., 2000a). If PS is the major factor affecting survival, the impact of the observed survival-shortening activity of some vegetable oils on human nutrition may be considered relatively small because the effects may be confined to particular animal strains, and PS is generally recognized as a beneficial nutrient for its ability to suppress cholesterol absorption in the intestine. However, our data (Huang et al., 1996, 1997; Miyazaki et al., 1998b) were not consistent with this interpretation; hence, we re-evaluated the correlation between survival time and PS content in dietary and tissue lipids, attempted to extract the presumed factors from the aqueous phase after the lipase and alkaline hydrolyses of Can by modified methods, and evaluated hepatic tocopherol status in relation to the reported vitamin E deficiency in piglets (Sauer et al., 1997).

2. Materials and methods

2.1. Diet and animals

The basal conventional diet (CE-2, Central Laboratory of Experimental Animals, Clea Japan, Tokyo) consisted of soybean meal, fish meal, skimmed milk, soybean oil, corn, wheat, wheat bran, alfalfa meal, a vitamin mixture and a mineral mixture; the diet contained endogenous fatty acids at 2.7%. The experimental diets were prepared by mixing CE-2 with one type of oil or a free fatty acid fraction obtained by the alkaline or lipase hydrolysis of Can at a 9:1 ratio. These diets were stored at −20 °C for less than 3 months before serving. Vegetable oils, commercially available for human consumption were purchased from local markets. Hyd.Soy (mp 30 °C) was a product for human consumption. A commercially available phytosterol preparation, a mixture of phytosterols from soybean containing β-sitosterol (51.0%), stigmasterol (27.3%), campesterol (13.2%) and a small amount of dihydrobrassicasterol (S5753, Sigma, St Louis, MO, US), was added to the basal diet at 0.4% for the evaluation of its effect on survival. The test diets supplied were replaced every 2 days so as to keep the peroxide value of the ingested diet below 100 meq/kg.

SHRSP rats were obtained from Seack Yoshitomi Co. (Fukuoka, Japan) and bred in our laboratory. Male rats from the same litter were randomly divided into different dietary groups and introduced at 4 week of age to a test diet. The rats were kept under specific pathogen-free conditions. The temperature and humidity in the room were maintained at 23 ± 3 °C and 50 ± 10%, respectively, with a 12-h day:night light cycle. In survival tests, the number of animals was 12 in each group unless otherwise indicated and the drinking water provided contained 0.5% NaCl. One set of experiments for measuring survival time included the Can, Can/Lip (a free fatty acid fraction obtained by lipase hydrolysis of Can), Can/Alk (a free fatty acid fraction obtained by alkaline hydrolysis of Can), ethyl acetate extracts from aqueous phase (Can/EA) and Soy groups, and another set included the Can, Can/PS (Can supplemented with phytosterol from soybean), Soy/PS and Soy groups. Other survival data were taken from previous experiments as noted in the legend to Fig. 2. Although survival time was determined under the SPF conditions described above, the mean survival time of a given dietary group varied significantly due to unknown factors. Therefore, both the Can and Soy groups were included as controls in each set of experiments, and mean survival time was expressed relative to that of the Soy group.

For the determination of tissue PS and tocopherols, test diets were fed for 8 week after weaning at 4 weeks of age. After anesthetization with diethylether, the cervical vertebrae of the rats were dislocated, and serum and

tissue samples were collected and stored in liquid nitrogen until lipid analysis.

This experiment was approved by the Ethical Committee of Nagoya City University Graduate School of Pharmaceutical Sciences.

2.2. Analysis of sterols, fatty acids and tocopherols in diets and tissues

The diets or tissue homogenates (equivalent to about 1 mg of total sterol) were mixed with 1.0 mg of betulin (an internal standard). A saponification mixture (0.5 g of KOH in 5 ml of ethanol) was added and the mixture was placed in a boiling water bath for 2 h. The non-saponifiable matter was recovered by extraction with hexane/chloroform (4:1, v/v) and purified by successive washing with water/ethanol (80:20, v/v). After dehydration with anhydrous sodium sulfate, the solvent was evaporated with nitrogen gas. The non-saponifiable matter was treated with 100 μl of trimethylsilylating reagent (Tokyo Kasei Kogyo Co., Ltd., Tokyo) and the trimethylsilylated sterols were determined by gas–liquid chromatography using a DB-1 flexible fused-silica capillary column (30 m×0.25 mm; J & W Scientific, Folsom, CA). Total lipids were extracted from tissue samples by the method of Bligh and Dyer (Bligh and Dyer, 1959), and a defined amount of heptadecanoic acid, an internal standard, was added to the extracts for the quantification of the amount of fatty acids. Fatty acids were analyzed as methylesters by gas–liquid chromatography. Tissue tocopherol level was determined by high performance liquid chromatography according to the method of Abe et al. (1975).

2.3. Preparation of free fatty acid fractions and ethyl acetate extracts

For the preparation of a free fatty acid fraction from Can by lipase hydrolysis, Lipase AY 30 (2 g, 90,000 I.U., Amano Pharmaceutical Co., Nagoya, Japan) was dissolved in 2 l of 0.1 M acetate buffer (pH = 5.6)–20 mM $CaCl_2$, and mixed with 3 kg of Can. The mixture was stirred at room temperature for 3 days and extracted with 7.5 l of hexane. The hexane layer was collected, and the solvent was evaporated under reduced pressure to obtain a free fatty acid fraction (Can/Lip). Polyvinylalcohols, used as emulsifiers in the previous experiments (Miyazaki et al., 1998b), were omitted to avoid difficulties in treating the aqueous phase after lipase treatment. After the hexane extraction of the free fatty acid fraction, the aqueous phase was treated with ethyl acetate, and the solvent was evaporated. This extraction was repeated five times, and the pooled extract was used as Can/EA fraction.

In order to prepare a free fatty acid fraction in a shorter incubation time, Can was treated under alkaline conditions. The treated oil (3.5 kg) was mixed with 1 N NaOH in EtOH:H_2O (10:1) solution for 1 h at room temperature (20–25 °C). The hydrolysis was followed by silica gel thin-layer chromatographic analysis of the aliquots, using a mixture of petroleum ether, diethyl ether, and acetic acid (80:30:1 v/v) as solvent. After neutralization, the free fatty acid fraction was obtained by extraction with 3.5 l of hexane. The solvents were removed using a rotary evaporator (Can/Alk). The abbreviations of the test diets (oils) are listed in Table 1.

Table 1
Abbreviations of the test oils

Can	Canola oil
Can/Lip	Free fatty acid fraction obtained by lipase hydrolysis of Can
Can/Alk	Free fatty acid fraction obtained by alkaline hydrolysis of Can
Soy	Soybean oil
Can/PS	Can supplemented with phytosterol at 4% of oil
Soy/PS	Soy supplemented with PS at 4% of oil
Hyd.Soy	Partially hydrogenated soybean oil
Olv	Olive oil
Epo	Evening primrose oil
Per	Perilla seed oil
HO-Saf	High-oleic safflower oil
HL-Saf	High-linoleic safflower oil

2.4. Statistical analysis

All data were presented as means ± SEM. Statistical analysis of the survival data was performed by the Log-Rank method and Wilcoxon signed rank method (a non-parametric method). Other data were evaluated by one-way ANOVA using Bonferroni's multiple comparison. Correlations between mean survival time and phytosterol content were determined by linear regression analysis. A computer program KyPlot ver. 2.0 (Kyence Inc., Tokyo, Japan) was used.

3. Results

3.1. Major fatty acids and sterols in diets

The fatty acid composition of the diet is shown in Table 2, and the sterol content in Table 3. The Can diet and the diets containing the free fatty acid fractions obtained by lipase hydrolysis (Can/Lip), extraction by ethylacetate (Can/EA) and alkaline-hydrolysis (Can/Alk) had similar fatty acid compositions. Cholesterol in the diet was derived mostly from the basal diet (CE-2) consisting of fish meal and skimmed milk. Brassicasterol was present in Can but not in the other oils listed in Table 3. Stigmasterol was absent in Can but was detectable in all the diets, which was derived from the

Table 2
Fatty acid composition of the test diet[a,b]

	Can	Can/Lip	Can/EA	Can/Alk	Soy	Can/PS	Soy/PS	Hyd.Soy	Olv	Epo
Saturated fatty acid										
14.0	0.3	0.2	0.2	0.3	0.3	0.3	0.3	0.4	0.2	0.0
16:0	8.0	8.3	9.0	8.3	11.9	8.8	13.1	13.6	11.9	9.7
18:0	1.8	2.0	2.0	1.8	3.2	1.8	4.5	12.5	3.0	1.9
20:0	0.4	0.4	0.4	0.4	0.2	0.4	0.3	0.3	0.3	0.0
Monounsaturated fatty acid										
16:1	0.5	0.5	0.5	0.5	0.4	0.5	0.4	0.4	0.8	0.4
18:1[c]	50.8	57.4	52.1	50.5	23.7	50.8	24.9	55.8	62.9	16.5
20:1	1.2	0.9	0.9	1.2	0.4	1.1	0.4	0.8	0.5	0.5
n − 6 Polyunsaturated fatty acid										
18:2*n* − 6	29.0	22.7	26.8	29.1	52.3	26.9	48.5	14.4	18.0	63.4
18:3*n* − 6	0.6	0.5	0.5	0.6	0.0	0.7	0.0	0.0	0.0	4.6
n − 3 Polyunsaturated fatty acid										
18:3*n* − 3	6.3	5.4	6.0	6.0	6.1	7.1	6.0	1.0	1.3	1.5
20:5*n* − 3	0.7	1.0	0.6	0.8	0.6	0.7	0.7	0.3	0.7	0.9
22:6*n* − 3	0.5	0.4	1.0	0.5	0.5	0.6	0.7	0.6	0.5	0.6

[a] The test diet was prepared by mixing the basal diet (CE-2) with a test oil at a 9:1 ratio. Long-chain fatty acids (20:5*n* − 3 and 22:6*n* − 3) were derived from fish meal in the basal diet. The values are expressed as percent of the total fatty acids.
[b] Abbreviations of the test diets are listed in Table 1.
[c] The 18:1 fatty acids includes the *cis* and *trans* isomers in the case of hydrogenated soybean oil (Hyd.Soy).

Table 3
Sterol content of the test diet

	Can	Can/Lip	Can/EA	Can/Alk	Soy	Can/PS	Soy/PS	Hyd.Soy	Olv	Epo	CE-2
Sterol (mg/100 g)[a]											
Cholesterol	47.3	60.2	50.7	54.9	48.4	53.0	51.4	49.9	46.9	40.6	41.4 ± 2.6
Brassicasterol[b]	6.2	6.3	7.2	5.9	ND	10.1[b]	6.9[b]	ND	ND	ND	ND
Campesterol	33.7	38.4	40.7	37.7	22.5	107.3	103.8	17.3	16.1	15.8	16.8 ± 0.5
Stigmasterol	11.2	15.6	7.0	8.1	14.1	34.5	45.4	10.0	8.9	5.3	7.0 ± 0.2
β-sitosterol	78.6	93.4	71.5	65.8	55.9	206.8	211.6	44.6	49.2	83.1	34.1 ± 1.2
Total PS	129.8	153.7	126.4	117.7	92.6	358.6	360.8	71.8	74.2	104.2	57.9 ± 1.4
Total sterol	177.1	213.9	177.1	172.6	141.0	411.6	412.2	121.7	121.1	144.8	99.3 ± 4.1

[a] Averages of two determinations are presented, except for the basal diet, CE-2 ($n = 3$, mean SD). Abbreviations are the same as those in Table 1. ND, not detectable.
[b] These values included unidentified component.

basal diet containing soybean meal. The difference in the PS content between the test diets was less prominent than that between the oils tested, due to the endogenous cholesterol and PS in the basal diet. The PS content in the Can/Lip diet was slightly higher but that of Can/Alk was slightly lower than that in the Can diet.

3.2. Mean survival time affected by the dietary oils

Although the free fatty acid fraction obtained by the lipase hydrolysis of Can did not exhibit the survival-shortening activity (Miyazaki et al., 1998b), it is possible that the presumed factors were degraded during prolonged incubation (days) in the presence of detergents. To test this possibility, the free fatty acid fraction was obtained by the lipase hydrolysis of Can in the presence of $CaCl_2$ but without using detergents (Can/Lip), and also by alkaline hydrolysis (Can/Alk). The ethyl acetate extract obtained from the aqueous phase after alkaline hydrolysis was dissolved in Soy (Can/EA). The mean survival time of the Can group was significantly shorter than that of the Soy, Can/EA and Can/Alk groups, but the differences in mean survival time between the latter three groups were not significant (Fig. 1A, Table 4). The Can/Lip and the ethyl acetate extract from the lipase hydrolysis did not exhibit survival-shortening activity (data not shown).

Next, we examined the effect of phytosterol of soybean origin (PS) on the survival of SHRSP rats. At a total PS content 2.8-fold greater than that in the Can diet, the content which was comparable to that examined by Ratnayake et al. (2000a), the survival time of the Soy/PS group was significantly shorter than that of the Soy group, but was significantly longer than that of the Can group (Fig. 1B, Table 4). The addition of PS to the Can diet did not further shorten the survival time.

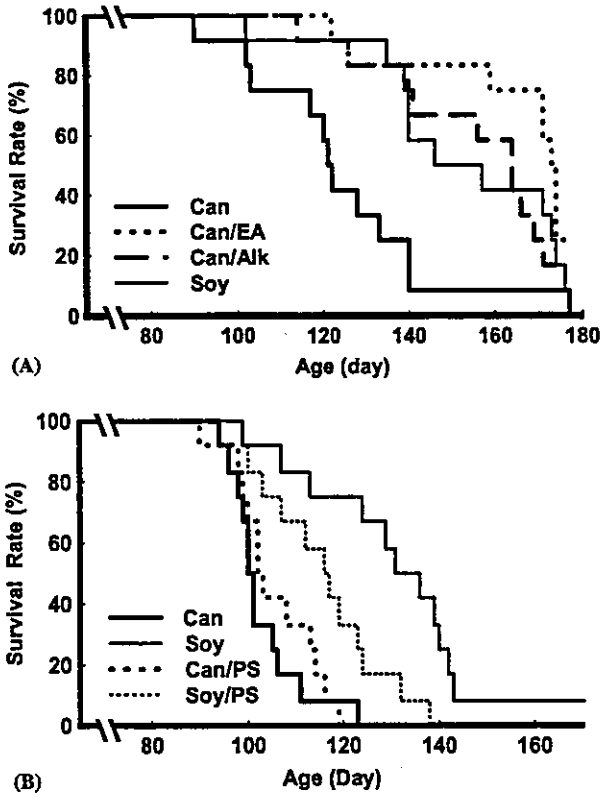

Fig. 1. Survival curves for SHRSP rats fed a diet supplemented with a free fatty acid fraction (Can/Alk) or an ethyl acetate extract (Can/EA) of aqueous phase obtained after Can hydrolysis (A), and a free fatty acid fraction (Can/Alk) or an ethyl acetate extract (Can/EA) of aqueous phase obtained after Can hydrolysis (B). A mixture of phytosterols from soybean (PS) was added to the Can or Soy diet at 0.4% (A). A free fatty acid fraction was obtained by the alkaline hydrolysis of Can (Can/Alk) as described in the text. After hydrolysis and hexane extraction of the free fatty acid fraction, the aqueous phase was extracted with ethyl acetate and the extract was mixed with Soy to obtain the Can/EA fraction. There were 12 rats in each dietary group, and the results of statistical analyses are presented in Table 4.

3.3. Correlation between relative mean survival time and dietary phytosterol content

Some of the survival times were taken from our previous experiments determined using a conventional substrain of the SHRSP rat (Fig. 2A), and from the present experiments using an SPF substrain of the SHRSP rat (Fig. 2B). The survival times are shown relative to that of the Soy group, and their correlations with PS contents in the diets (Table 3) are plotted in Fig. 2. No significant negative correlation was observed between relative survival time and PS content in the diet, and the content of any major PSs (campesterol, stigmasterol and β-sitosterol).

3.4. Correlation between relative survival time, tissue PS content and hepatic tocopherol content

Sterol contents in sera and livers taken from the rats fed the test diets for 8 weeks were determined (Table 5). Campesterol and β-sitosterol constituted more or less 10% of the total sterol, but other PSs in the diets were not detectable in these tissues. Because the basal diet included endogenous cholesterol as well as PS, the differences in hepatic cholesterol and PS content were relatively small, and the hepatic total PS content decreased in the order of the Soy/PS, Can, Can/Alk, Soy, Epo, Hyd.Soy and Olv groups.

The order of hepatic PS content did not correlate with that of the mean survival times of these dietary groups (Fig. 2). Indeed, the groups with similar hepatic total PS contents exhibited marked differences in survival time, e.g., Soy group vs. Hyd.Soy group; Can group vs. Can/Alk group. Hepatic campesterol content and β-sitosterol content did not correlate with the mean survival times (Fig. 2, Table 5). The serum total PS,

Table 4
Statistical analysis of the effect of phytosterol (PS) on survival time

Experiment 1		Can	Can/EA	Can/Alk	Soy
Mean survival time (Days ± SEM)		122 ± 5	>165 ± 4	155 ± 6	>153 ± 7
vs. Can	Log-rank		0.001>	0.001>	0.001>
	Wilcoxon		0.001>	0.001>	0.001>
vs. Can/EA	Log-rank			0.138	0.158
	Wilcoxon			0.105	0.170
vs. Can/Alk	Log-rank				0.866
	Wilcoxon				0.840
Experiment 2		**Can**	**Can/PS**	**Soy/PS**	**Soy**
Mean survival time (Days ± SEM)		103 ± 2	106 ± 2	116 ± 4	>130 ± 4
vs. Can	Log-rank		0.514	0.003	0.001>
	Wilcoxon		0.326	0.005	0.001>
vs. Can/PS	Log-rank			0.017	0.001>
	Wilcoxon			0.037	0.001
vs. Soy/PS	Log-rank				0.008
	Wilcoxon				0.027

There were 12 rats in each dietary group.