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Assessment of the possible adverse effects of oils enriched with n-3 fatty acids in rats; peroxisomal proliferation, mitochondrial dysfunctions and apoplexy

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Possible adverse effects of excess intake of dietary n-3 fatty acids have been reevaluated in rats by measuring peroxisomal proliferation, mitochondrial enzyme activity and incidence of apoplexy. When Sprague-Dawley rats were fed diets containing either fish oil rich in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) or perilla seed oil rich in α-linolenic acid for 4 or 12 weeks, DHA accumulated in phospholipids of liver and heart in the fish oil group, but not in the perilla oil group when compared with safflower and soybean oil groups. Feeding a diet containing 15 weight % of fish oil induced a significant proliferation of peroxisomes compared with safflower oil-diet, but the proliferating activity of perilla oil was much less. The peroxisomal β-oxidation activities were negatively correlated with neutral lipid contents in liver. Heart mitochondrial cytochrome c oxidase activity was not affected significantly by feeding fish oil or perilla oil in the presence of an essential amount of linoleic acid for up to 12 weeks, though the proportion of linoleic acid in heart cardiolipin decreased from >86% in the safflower and soybean oil groups to 81% in the perilla oil group and to 33% in the fish oil group. Feeding n-3-enriched fish oil and perilla oil in 10 weight % of the diets did not accelerate the onset of cerebral bleeding in stroke-prone spontaneously hypertensive rats (SHRSP). Although the proliferating activity of peroxisomes by excess intake of fish oils should be noticed, these results provide evidence that n-3-enriched oils are safe under conditions applicable to human nutrition, considering the levels fed the rats were several fold higher than the anticipated maximal intake in human. © Elsevier Science Inc. 1996 (J. Nutr. Biochem. 7: 542–548, 1996.)

Keywords: peroxisomes; cytochrome c oxidase; apoplexy; fish oil; perilla oil; rats

Introduction

Numerous data supporting that fish oils rich in eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) as well as perilla seed oil rich in α-linolenic acid (18:3n-3) have beneficial effects on the prevention of chronic diseases in the elderly have been accumulated.[1–3] On the other hand, there are rather few studies on possible adverse effects of n-3 fatty acids. Among them, we focused on three possible side effects of excess intake of n-3 fatty acids; peroxisomal proliferation,[4,5] mitochondrial dysfunction associated with the replacement of linoleic acid (18:2n-6) with DHA in mitochondrial cardiolipin molecules[6–8] and the increased incidence of apoplexy as suggested from

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the epidemiological studies of Greenlanders.[9] Fish oil diets increase peroxisomal β-oxidation activity in rat liver,[4] and administration of purified EPA[10] or DHA[11] causes an increase in hepatic peroxisomal β-oxidation activity in rat. The enhancement of peroxisomal β-oxidation may be related to the hypotriglyceridemic effect of fish oils.[10] In contrast, drugs that induce proliferation of peroxisomes and are used as hypolipidemic agents are classified as non-mutagenic hepatocarcinogens,[12] although the mechanism by which the peroxisomal proliferation increases the incidence of hepatocarcinoma in rodents is not yet clear.

Mitochondrial dysfunction has been clearly shown in isolated mitochondria from rats fed fish oil.[6] However, the oil content of the diet used (20 weight %) was too high to correlate the observed effects with human nutrition. Less than 7.5 weight % may be more applicable to humans. A slightly higher incidence of apoplexy seen in Greenlanders compared with Danes may not be due to excess intake of n-3 fatty acids, because a more recent epidemiological study revealed that the incidence of thrombotic diseases and that of apoplexy (cerebral bleeding) are not in a reciprocal relationship among the towns in Greenland.[13] It has been also suggested from the Zutphen Study[14] that low doses of n-3 fatty acids are protective against stroke. Therefore, factors other than fish oil n-3 fatty acids, e.g., a vitamin C deficiency-caused disorder of fibrous proteins synthesis, should be considered for the higher incidence of apoplexy seen in native Greenlanders.

Thus, the suggested side effects of the intake of large amounts of n-3 fatty acids must be reassessed under conditions applicable to human nutrition. In the present study, perilla seed oil rich in α-linolenic acid and fish oils rich in EPA and/or DHA were compared with respect to the effects on peroxisomal proliferation, mitochondrial function, and cerebral bleeding in rats, using soybean oil and/or safflower oil as controls. A strain of stroke-prone spontaneously hypertensive rats (SHRSP) was used as a model for apoplexy because this strain of rats dies mostly of cerebral bleeding.

Methods and materials

Animals and diets

Test diets were prepared by extracting the conventional diet (CE-2) with n-hexane and then supplementing the defatted diet with a vitamin mixture (2%) containing fat-soluble vitamins, DL-choline (0.3%) and safflower seed oil (Ohta Oil Mill Co., Okazaki, Japan), perilla seed oil (Ohta Oil Mill), soybean oil (Ohta Oil Mill), fish oil A (rich in both EPA and DHA) (Nippon Oil Co., Tokyo, Japan) or fish oil B (rich in DHA) (Biox Co., Tokyo, Japan). The validity of using these oil-supplemented diets for rats has been assessed previously by comparing growth curve, reproductive physiology, and teratogenicity.[15] The fish oil diets were supplemented with safflower oil to provide a small amount of an essential fatty acid, linoleic acid. In Experiment I, male Sprague-Dawley rats at 6 weeks of age (Shizuoka Laboratory Animals Co., Shizuoka, Japan) were divided randomly into three groups (n = 6) and given a conventional diet (CE-2; Nippon Clea Co., Tokyo, Japan) for a week and then given test diets containing 7.5 or 15 weight % of either safflower oil, perilla oil, or fish oil A for 4 weeks. In Experiment II, four groups of male rats (n = 4 or 5) were fed test diets containing 10 weight % safflower oil, soybean oil, perilla oil, or fish oil B for 4 weeks from 12 weeks of age or for 12 weeks from 4 weeks of age. The fatty acid compositions of the diets are given in *Table 1*. The diets with peroxide values below 10 mEq/kg were served, and the diets were replaced every day. SHRSP were kindly provided by Prof. Kozo Okamoto, Kinki University School of Medicine (Minamikawachi, Osaka, Japan) and maintained on CE-2. After mating at 12 to 17 weeks of age, the progeny at 4 weeks of age were weaned to test diets and were fed the same diets through life (n = 10 or 12). Animals were maintained in a temperature-controlled room (23 ± 3°C).

Enzyme and protein assay

Livers from individual rats were homogenized in 5 volumes of ice-cold sucrose medium (0.25 M sucrose in 10 mM Tris/HCl, pH 7.4, 1 mM EDTA), centrifuged at 600 g for 10 min to prepare post-nuclear supernatants, which was used as a source for enzyme assays or lipid analyses. The peroxisomal β-oxidation activity was measured using palmitoyl-CoA as a substrate as described.[16] For determination of mitochondrial cytochrome c oxidase activity, heart mitochondria were isolated essentially as described by Yamaoka et al.,[6] and cytochrome c oxidase activity was assayed as described previously.[17] Protein was assayed by the method of Lowry et al.[18] or by the method of Bradford[19] using the Bio-Rad protein dye reagent with bovine serum albumin as a standard.

Lipid analysis

Total lipids were extracted from tissue samples according to the method of Bligh and Dyer.[20] Hepatic phospholipids and neutral lipids were separated by Silica Gel (Merck 60) thin-layer chromatography using a solvent system of diethyl ether/acetic acid (99:1, by vol). Mitochondrial phospholipids were separated by two-dimensional Silica Gel thin-layer chromatography using chloroform/methanol/28% ammonium hydroxide (60:40:6, by vol) and chloroform/acetone/methanol/acetic acid/water (50:20:10:15:5, by vol) as the first and second solvent, respectively. Lipids were visualized by spraying with an ethanolic Rhodamin 6G solution (0.03 w/w %) and the corresponding spots were extracted with chloroform/methanol/3% ammonium hydroxide (6:5:1, by vol). Fatty acids in each fraction were analyzed as methyl esters by a

Table 1 Fatty acid compositions of diets

Fatty acid	Dietary groups				
	Safflower oil	Soybean oil	Perilla oil	Fish oil A	Fish oil B
	Weight % of total fatty acids				
14:0[1]	—[3]	—	—	6.1	3.1
16:0	6.9	11.7	6.9	15.4	18.2
16:1	0.2	—	0.2	7.3	5.0
18:0	2.1	3.4	1.9	2.1	4.0
18:1	20.1	23.6	19.8	12.9	20.3
18:2n-6	68.4	51.1	18.5	8.3	8.5
18:3n-3	0.7	8.8	51.0	—	—
18:4n-3	—	—	—	4.6	1.4
20:1	—	—	—	5.6	2.4
20:4n-6	—	—	—	1.1	1.8
20:5n-3	0.3	0.6	0.3	18.3	6.9
22:6n-3	0.4	0.7	0.4	12.1	26.2
Others	—	—	—	6.2	2.2
n-3/n-6[2]	0.020	0.20	2.3	3.7	3.3

[1]Fatty acids are designated by the number of carbon chain: the number of double bonds, and the first double bond numbered from methyl terminus is indicated as n-3 and n-6.
[2]n-3/n-6 indicates the total n-3 fatty acid/total n-6 fatty acid ratio.
[3]—; not detected.

gas-liquid chromatography on a capillary column coated with DB-225 (0.2 mm diameter, 30 m length, J & W Scientific, Folsom, CA USA), using heptadecanoic acid as an internal standard.

Statistics

Data are reported as means ± SD unless otherwise noted. Significant differences of mean values for fatty acid content and enzyme activities between dietary treatment groups were determined by a one-way analysis of variance followed by Scheffe's F-test. Analysis of survival was conducted using Wilcoxon test, as implemented in JMP 3.0, Statistic Made Visual (SAS Institute, Cary, NC USA). Differences were considered significant at $P < 0.05$.

Results

Effect of oils on hepatic peroxisomal proliferation

Diets containing 7.5 or 15 weight % of safflower oil, perilla oil, or fish oil A were compared for the proliferation of peroxisomes in liver by measuring the oxidation of palmitoyl-CoA in the presence of KCN, an inhibitor of mitochondrial β-oxidation (*Figure 1*). Fish oil A containing both EPA and DHA increased the activity significantly at 15 % and slightly at 7.5 % oil. Perilla oil also tended to increase the peroxisomal β-oxidation activity as compared with safflower oil, although the increase by perilla oil was not significant. Under these conditions, no significant difference was observed in the body weights, but the liver weights (shown in *Figure 1*) roughly paralleled the β-oxidation activities.

Fatty acid compositions of hepatic phospholipids were greatly affected by the type of oils in the diets (containing 15 % oil). The chain length of saturated fatty acids as well as the profile of n-3 and n-6 polyunsaturated fatty acids of liver phospholipids in each dietary group roughly reflected the fatty acid compositions of the diets (*Table 2*). A significant proportion of DHA accumulated in the fish oil A group but not in the perilla oil group. The hepatic content of phospholipids (total fatty acids) in the fish oil group was higher by 15% than that in the safflower oil group.

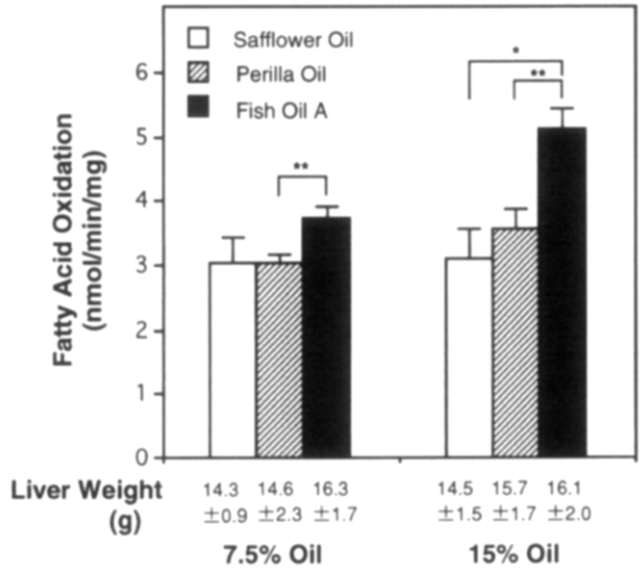

Figure 1 Effects of dietary oils on peroxisomal β-oxidation activity in Sprague-Dawley rats. Livers of rats fed test diets containing 7.5% or 15% oil for 4 weeks were homogenized and the palmitoyl-CoA oxidation activities were measured in the presence of 1 mM KCN. Values are means ± SD (n = 6). *, $P < 0.001$; **, $P < 0.01$.

In the neutral lipid fractions, the levels of saturated and monoenoic fatty acids as well as n-3 and n-6 fatty acids with 18, 20, and 22 carbon chain lengths were all affected by the diets (*Table 3*). Again, DHA accumulated only in the fish oil group. Noteworthy was a great differences in hepatic contents of neutral lipids; the contents of the perilla oil group and fish oil group were 62% and 35% of the value for the safflower oil group, respectively.

Effect of oils on heart mitochondrial enzymes

Cytochrome c oxidase activities were not affected by these diets containing either 7.5 or 15% oil (Experiment I in *Table 4*). Because the feeding period was relatively short (4 weeks) in this experiment, the feeding period was extended to 12 weeks in Experiment II in *Table 4,* and fish oil enriched with DHA (Fish oil B) that was shown to replace linoleate in mitochondrial cardiolipin selectively[6,7] was compared, adding soybean oil as another control all at 10 weight % in the diets. Again, the cytochrome c oxidase activity was not affected by these dietary oils.

Fatty acid compositions of heart mitochondrial phospholipids

Cardiolipin is a cofactor for the cytochrome c oxidase and the decreased cytochrome c oxidase activity in rats fed a

Table 2 Fatty acid composition of liver phospholipids from Sprague Dawley rats fed test diets containing 15% oil for 4 weeks[1]

Fatty acid	Safflower oil	Perilla oil	Fish oil A
	% of total fatty acid		
14:0	0.4 ± 0.3^{ab}	0.2 ± 0.1^{a}	0.4 ± 0.1^{b}
16:0	17.8 ± 2.1^{a}	17.1 ± 1.4^{a}	21.8 ± 0.2^{b}
16:1	tr.^{a}	0.1 ± 0.1^{a}	1.4 ± 0.0^{b}
18:0	23.4 ± 3.7^{ab}	24.2 ± 1.8^{b}	17.9 ± 1.2^{a}
18:1	5.2 ± 0.2^{a}	6.3 ± 0.4^{a}	8.4 ± 1.0^{b}
18:2n-6	14.9 ± 1.4^{b}	15.6 ± 0.9^{b}	8.4 ± 1.0^{a}
18:3n-3	—^{a}	1.9 ± 0.8^{c}	0.2 ± 0.0^{b}
20:3n-6	0.7 ± 0.0^{a}	2.0 ± 0.5^{c}	1.1 ± 0.0^{b}
20:4n-6	28.9 ± 3.2^{c}	13.2 ± 0.5^{b}	9.3 ± 0.3^{a}
20:5n-3	—^{a}	10.2 ± 1.2^{b}	11.0 ± 0.8^{b}
22:4n-6	0.7 ± 0.2^{b}	0.2 ± 0.0^{a}	0.1 ± 0.1^{a}
22:5n-6	0.5 ± 0.1^{b}	tr.^{a}	0.1 ± 0.1^{a}
22:5n-3	0.4 ± 0.1^{a}	2.8 ± 0.2^{b}	3.4 ± 0.3^{c}
22:6n-3	6.2 ± 0.9^{a}	5.1 ± 0.4^{a}	15.8 ± 0.7^{b}
24:0	0.6 ± 0.2	0.6 ± 0.3	0.3 ± 0.3
24:1	0.3 ± 0.2	0.6 ± 0.3	0.6 ± 0.5
n-3	6.7 ± 0.9^{a}	20.0 ± 1.7^{b}	30.4 ± 1.1^{c}
n-6	45.6 ± 4.7^{c}	30.9 ± 1.4^{b}	18.9 ± 1.3^{a}
n-3/n-6	0.1 ± 0.0^{a}	0.6 ± 0.0^{b}	1.6 ± 0.2^{c}
Total fatty acids (mg/g liver)	14.2 ± 0.8^{a}	15.6 ± 0.6^{ab}	16.4 ± 0.4^{b}

[1]Values are means ± SD (n = 3).
Values with different superscripts are significantly different from each other at $P < 0.05$.
—; not detected. tr; trace.

Table 3 Fatty acid composition of liver neutral lipids from Sprague Dawley rats fed test diets containing 15% oil for 4 weeks[1]

Fatty acid	Safflower oil	Perilla oil	Fish oil A
	% of total fatty acid		
14:0	0.3 ± 0.1	0.3 ± 0.0	1.4 ± 0.9
16:0	14.8 ± 2.4[a]	18.9 ± 1.0[b]	21.4 ± 1.9[b]
16:1	tr.[a]	tr.[a]	4.4 ± 0.2[b]
18:0	2.5 ± 0.2	3.3 ± 1.0	5.0 ± 2.3
18:1	12.8 ± 0.2[a]	22.6 ± 0.6[b]	15.5 ± 1.0[a]
18:2n-6	51.5 ± 2.2[c]	16.9 ± 0.6[b]	12.5 ± 0.8[a]
18:3n-3	0.3 ± 0.1[a]	21.8 ± 0.9[c]	1.5 ± 0.1[b]
18:4n-3	0.2 ± 0.1[a]	0.7 ± 0.4[b]	0.9 ± 0.1[b]
20:3n-6	1.2 ± 1.1	0.3 ± 0.1	0.3 ± 0.2
20:4n-6	10.2 ± 1.8[b]	1.1 ± 0.1[a]	1.2 ± 0.2[a]
20:5n-3	0.3 ± 0.2[a]	6.5 ± 0.3[b]	11.9 ± 0.6[c]
22:4n-6	3.6 ± 1.2[b]	—[a]	—[a]
22:5n-6	0.3 ± 0.6	—	—
22:5n-3	0.4 ± 0.2[a]	4.6 ± 0.2[b]	6.7 ± 1.1[c]
22:6n-3	1.7 ± 0.6[a]	2.9 ± 0.3[b]	17.2 ± 3.2[c]
n-3	2.7 ± 0.7[a]	35.8 ± 1.4[b]	37.3 ± 3.4[b]
n-6	66.8 ± 1.9[c]	18.3 ± 0.6[b]	14.0 ± 1.0[a]
n-3/n-6	0.0 ± 0.0[a]	2.0 ± 0.1[b]	2.7 ± 0.3[c]
Total fatty acids (mg/g liver)	46.5 ± 13.2[b]	28.6 ± 8.5[ab]	16.4 ± 6.1[a]

[1]Values are means ± SD (n = 3).
Values with different superscripts are significantly different from each other at $P < 0.05$.
—; not detected. tr; trace.

diet containing 20 weight % fish oil has been shown to be attributed to the replacement of linoleic acid in the cardiolipin molecule with DHA.[6,7] Therefore, in the next experiments, the fatty acid compositions of mitochondrial cardiolipin as well as those of other major phospholipids, phosphatidylcholine, were analyzed. The major fatty acid of cardiolipin was linoleic acid in the safflower oil and soybean oil groups (>87% of the total) after 12-week feeding of the test diet containing 10% oil (*Figure 2*). In the perilla oil group, a small decrease in linoleic acid was compensated for mainly by a small increase in α-linolenic acid. In contrast, the proportion of linoleic acid decreased significantly to 33% of the total, which was compensated for by the increase in DHA, 18:1 and 20:4n-6 in the fish oil-fed group (*Figure 2*).

In phosphatidylcholine from the perilla oil group, the decrease in 20:4n-6 was compensated for by the increase in 22:5n-3, whereas in the fish oil group, the decrease in linoleic acid was compensated for by the increase in DHA as compared with the safflower oil and soybean oil groups (*Figure 3*). These differences in the fatty acid profiles after 12 weeks of the test diet feeding were very similar to those isolated after 4 weeks of feeding (data not shown). The phospholipid contents and heart weights were not different among the dietary groups.

Table 4 Specific activity of cytochrome c oxidase in heart mitochondria from Sprague Dawley rats fed test diets[1]

Exp. I

Dietary groups	Cytochrome c oxidase (s^{-1}/mg protein) 7.5% Oil (n = 6)	15% Oil (n = 6)
Safflower oil for 4 weeks	0.50 ± 0.05	0.48 ± 0.08
Perilla oil for 4 weeks	0.52 ± 0.05	0.47 ± 0.07
Fish oil A (high EPA & DHA) for 4 weeks	0.50 ± 0.05	0.46 ± 0.07

Exp. II

Dietary groups	Cytochrome c oxidase (s^{-1}/mg protein) Feeding for 4 weeks (n = 4)	12 weeks (n = 5)
Safflower oil (10%)	0.44 ± 0.08	0.50 ± 0.06
Soybean oil (10%)	0.46 ± 0.09	0.47 ± 0.08
Perilla oil (10%)	0.43 ± 0.08	0.48 ± 0.08
Fish oil B (10%, high DHA)	0.47 ± 0.10	0.57 ± 0.08

[1]Values are means ± SEM.

Effect of oils on longevity of SHR-SP

Diets containing 10% of either soybean oil, perilla oil, or fish oil B and tap water were ad libitum fed to SHRSP. All the groups grew apparently normally, but the growth of fish oil group was slightly better than the perilla oil and soybean oil groups as shown in *Figure 4* (the difference was not statistically significant).

Survival curves of the dietary groups are shown in *Figure 5*. The mean survival times relative to that of soybean oil group (n = 10) (416 ± 49 days = 100%) were 107 ± 12% and 109 ± 13% for the perilla oil group (n = 12) and fish oil group (n = 12), respectively, although the differences were not significant. Earlier, we reported that the relative mean survival time of safflower oil group was 85.5% of that of the perilla oil group.[21] Cerebral bleeding was observed at 10/10 in the soybean oil group, 10/12 in the perilla oil group, and 12/12 in the fish oil B group, respectively, at autopsy.

Discussion

The tendency to see increased peroxisomal proliferation by feeding fish oil or perilla oil at 15 weight % of diet suggested that the inducibility may not be confined to the 22 carbon fatty acids. Consistently, Kawashima and Kozuka[5] reported a significant induction of peroxisomes in rats by a diet containing 40 energy % of perilla oil. Although the inducible peroxisomal β-oxidation system has been shown to have a relatively broad substrate specificity for various acyl-CoAs[22] the β-oxidation activity increased as the degree of unsaturation increased.[23] We hypothesize that triacylglycerols with liquid nature at body temperature are not suitable as depot fat. Then, the fatty acids with lower melting points such as DHA, EPA, and α-linolenic acid must be β-oxidized preferentially to saturated, monoenoic, and dienoic fatty acids. Peroxisomal proliferation by n-3-enriched oils is therefore interpreted to form a part of adaptive responses to the supply of n-3 fatty acids at amounts beyond the capacities of the mitochondrial β-oxidation and complex lipid synthesizing systems, although the safety of peroxi-

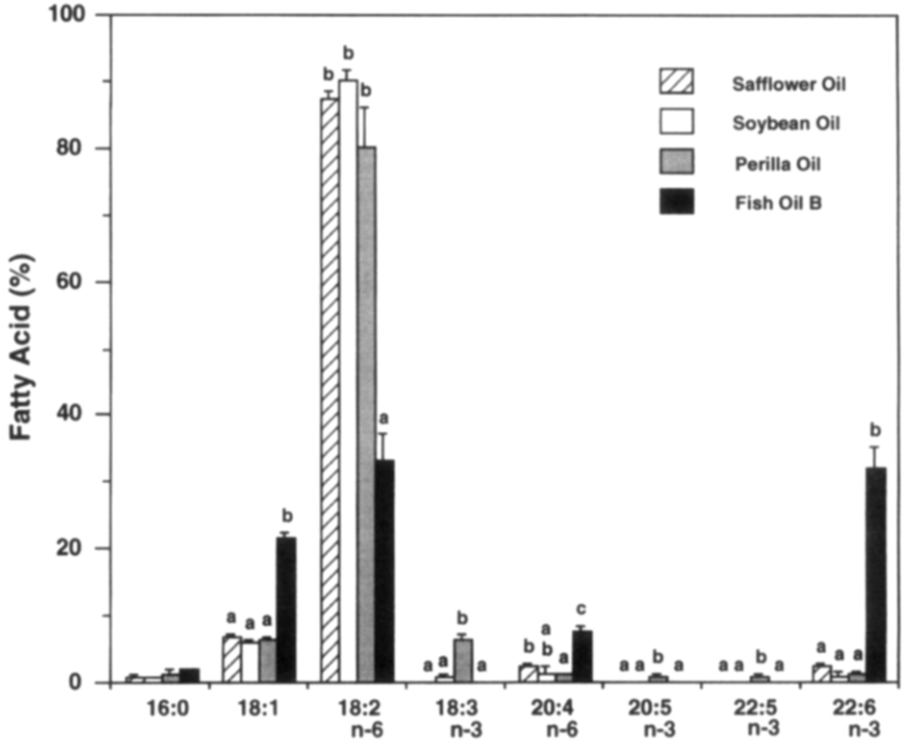

Figure 2 Fatty acid compositions of mitochondrial cardiolipin from Sprague-Dawley rats fed test diets containing either 10% safflower oil, soybean oil, perilla oil, or fish oil B for 12 weeks from 4 weeks of age. Values represent means ± SD for 3 to 4 rats. Values with different superscripts in the same fatty acid columns are significantly different from each other in one-way ANOVA followed by Scheffe's F-test for comparison between groups ($P < 0.05$).

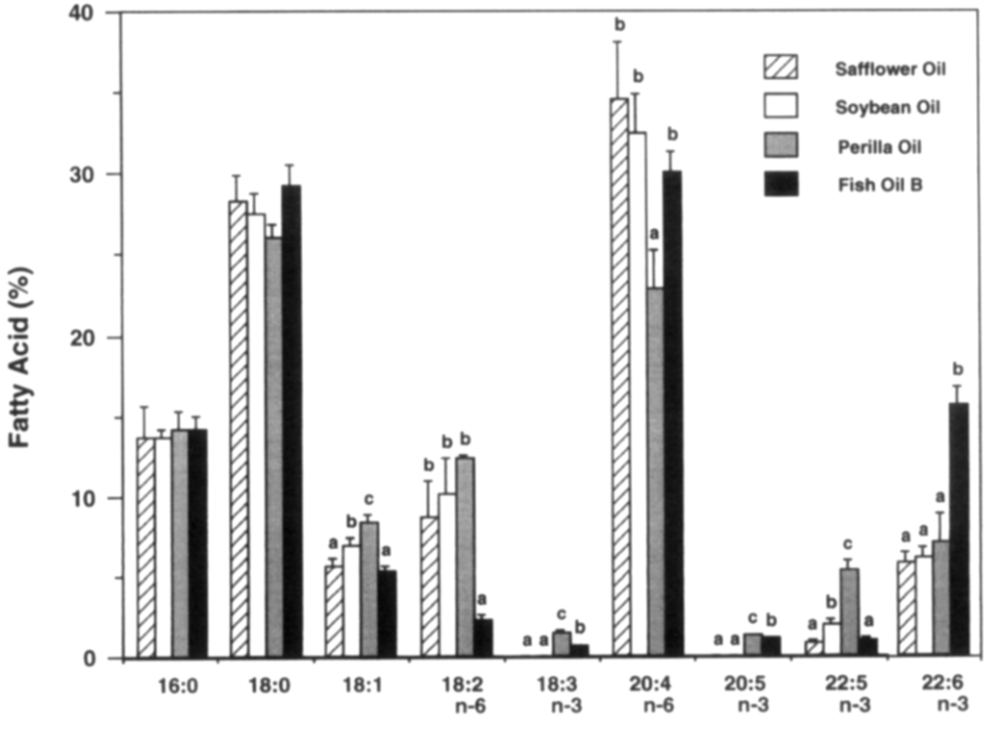

Figure 3 Fatty acid compositions of mitochondrial phosphatidylcholine from Sprague-Dawley rats fed test diets as described in *Figure 2*. Values represent means ± SD for 3 to 4 rats. Values with different superscripts in the same row are significantly different from each other in one-way ANOVA followed by Scheffe's F-test for comparison between groups ($P < 0.05$).

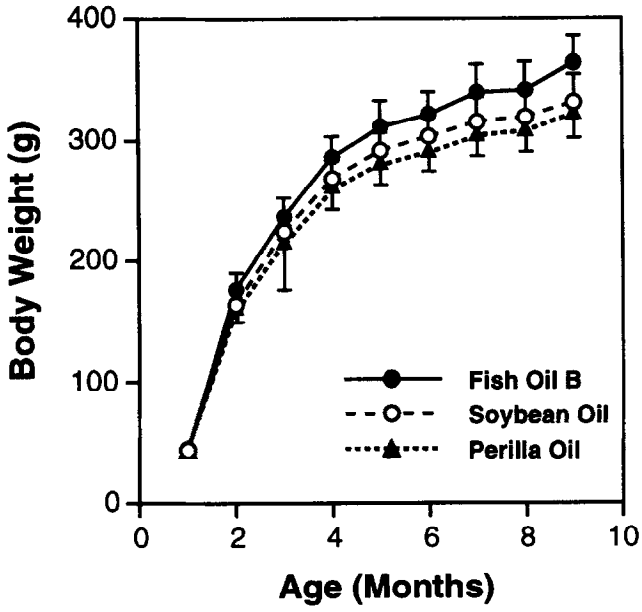

Figure 4 Body weight gains of rats (SHRSP) fed diets containing either 10% soybean oil (n = 10), perilla oil (*n* = 12) or fish oil B (*n* = 12). Values are means ± SD.

some proliferating activity by fish oil must be evaluated further. In fact, the fatty acid contents in hepatic neutral lipids were lower in the n-3 fatty acid-fed group than the safflower oil-fed group. Other endpoints must be measured to verify this hypothesis.

Peroxisome proliferators are known to be non-mutagenic carcinogens. When rat hepatocytes were exposed to artificial proliferators (e.g. clofibric acid), oncogene products, *jun-fos, myc,* and H-*ras,* which are known to be involved in carcinogenesis, were induced.[24,25] By contrast, the c-*myc* oncogene mRNA concentrations were demonstrated to be reduced in the tumor-tissue cells of mice fed n-3 fatty acids,[36] although the excess intake of n-3 fatty acids showed

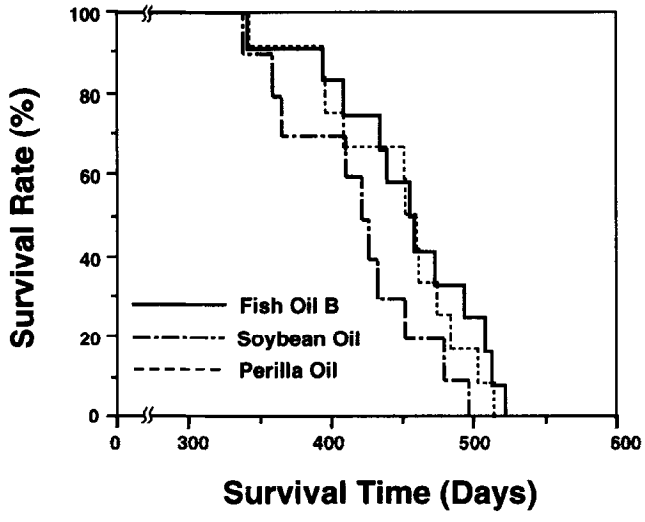

Figure 5 Survival curves of rats (SHRSP) fed diets containing various oils.

a slight peroxisomal proliferating activity. There are several other papers reporting the different effects of clofibrate and n-3 fatty acids. Kawashima and Kozuka[5] reported that perilla oil and fish oil did not depress the detoxification activities of glutathione-dependent enzymes, unlike artificial proliferators. It was also reported that the increase in the activity of hepatic peroxisomal β-oxidation in rats fed partially hydrogenated marine oil was due to the decrease in degradation rate of acyl-CoA oxidase enzyme rather than the increase in the synthetic rate as seen in clofibrate-treated rats.[27] In addition, the experiments using animal models with carcinogen-induced or transplantable tumors have consistently shown that n-3 fish oils delayed tumor appearance and decreased both the rate of growth and the size and number of tumors,[2] as has been shown using perilla oil.[28–30] These observations indicate that the peroxisomal proliferating activity of n-3 fatty acids should be distinguished from that of clofibrate, and that n-3 fatty acids have beneficial effects on tumorigenesis.

When a diet containing 20 weight % of fish oil was fed to rats, cytochrome c oxidase activity of heart mitochondria decreased, F1Fo-ATPase activity increased and mitochondrial function measured as an O_2 consumption rate of heart was impaired significantly.[6] This alteration of enzyme activities was accompanied with a significant decrease of linoleic acid level in mitochondrial cardiolipin; from >80% of the total fatty acids in rats fed corn oil to 14%. However, the diets containing 20 weight % of fish oil and a limited amount of linoleic acid are not comparable to human diets because most of food materials for humans contain significant amounts of linoleic acid and it is difficult to choose human diets containing less than 3 energy % of linoleic acid in industrialized countries. As shown in *Table 4,* no significant decrease in cytochrome c oxidase activity was found when fish oil diets contained 8.3–8.5 weight % of linoleic acid, although the proportion of linoleic acid decreased to 33% of the total fatty acids after 12-week feeding. It is noteworthy that no significant decrease in linoleic acid level of mitochondrial cardiolipin was induced by feeding the perilla oil diet.

A strain of rats, SHRSP, responds to these risk factors and has been used by many researchers as a model for human apoplexy. In the present study using this model, diets containing 10 weight % of fish oil B and perilla oil were shown not to have an adverse effect on the onset of apoplexy. The highest doses (15 weight % oil diets) of fish oil A/B and perilla oil were used in the present study correspond to 10 and 15 energy % of n-3 fatty acids, respectively. According to the new recommended dietary allowance of Japan,[31] the daily allowance of n-3 fatty acids can be figured at about 1.5 energy %, which is much lower compared with the doses used in the present animal study. Although the effects on other types of animal models must also be evaluated, the available evidence indicates that n-3 enriched oils are safe under the conditions applicable to human nutrition.

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References

1 Lands, W.E.M. (1986). in *Fish and Human Health.* Academic Press, New York, NY USA

2 Simopoulos, A.P. (1991). Omega-3 fatty acids in health and disease and in growth and development. *Am. J. Clin. Nutr.* **54,** 438–463

3 Okuyama, H. (1992). Effects of dietary fatty acid balance on behavior and chronic diseases. In *Polyunsaturated Fatty Acids in Human Nutrition* (Bracco, U. and Deckelbaum, R.J., eds.), Nestlé Nutrition Workshop Series, vol. 28, pp. 169–178. Raven Press, New York, NY USA

4 Yamasaki, R.K., Shen, T., and Schade, B. (1987). A diet rich in (n-3) fatty acids increases peroxisomal β-oxidation activity and lowers plasma triacylglycerols without inhibiting glutathione-dependent detoxication activities in the rat liver. *Biochim. Biophys. Acta* **920,** 62–67

5 Kawashima, Y. and Kozuka H. (1993). Dietary manipulation by perilla oil and fish oil of hepatic lipids and its influence on peroxisomal β-oxidation and serum lipids in rat and mouse. *Biol. Pharm. Bull.* **16,** 1194–1199

6 Yamaoka, S., Urade, R., and Kito, M. (1988). Mitochondrial function in rats is affected by modification of membrane phospholipids with dietary sardine oil. *J. Nutr.* **118,** 290–296

7 Yamaoka, S., Urade, R., and Kito, M. (1990). Cardiolipin molecular species in rat heart mitochondria are sensitive to essential fatty acid-deficient dietary lipids. *J. Nutr.* **120,** 415–421

8 Yamaoka-Kosaki, S., Urade, R., and Kito, M. (1991). Cardiolipins from rats fed different dietary lipids affect bovine heart cytochrome c oxidase activity. *J. Nutr.* **121,** 956–958

9 Kromann, N. and Green, A. (1980). Epidemiological studies in the Upernavik district of Greenland. *Acta Med. Scand.* **208,** 401–406

10 Aarsland, A., Lundquist, M., Børretsen, B., and Berge, R.K. (1990). On the effect of peroxisomal β-oxidation and carnitine palmitoyltransferase activity by eicosapentaenoic acid in liver and heart from rats. *Lipids* **25,** 546–548

11 Willumsen, N., Hexeberg, S., Skorve, J., Lundquist, M., and Berge, R.K. (1993). Docosahexaenoic acid shows no triacylglyceride-lowering effects but increases the peroxisomal fatty acid oxidation in liver of rats. *J. Lipid Res.* **34,** 13–22.

12 Reddy, J.K. and Rao, M.S. (1977). Malignant tumors in rats fed nafenopin, a hepatic peroxisome proliferator. *J. Natl. Cancer Inst.* **59,** 1645–1650

13 Dyerberg, J. (1992). Modulation of athero-thrombogenesis by n-3 PUFA's. Refocusing on mechanisms based on epidemiological, observational and experimental data. First IUBMB conference, Biochemistry of Diseases (Abstract) pp. 158

14 Keli, S.O., Feskens, E.J., and Kromhout, D. (1994). Fish consumption and risk of stroke. The Zutphen Study. *Stroke* **25,** 328–332

15 Naito, Y., Ishizaki, K., Yamamoto, H., and Okuyama, H. (1990). Studies on the safety of perilla oil—Effects on reproduction and skeletal teratogenicity. *J. Food Hyg. Soc. Japan* **31,** 251–254

16 Osumi, T. and Hashimoto, T. (1978). Enhancement of fatty acyl-CoA oxidizing activity in rat liver peroxisomes by di-(2-ethylhexyl)phthalate. *J. Biochem.* **83,** 1361–1365

17 Orii, Y. and Okunuki, K. (1965). Studies of cytochrome a. XV. Cytochrome oxidase activity of the Okunuki preparation and its activation by heat, alkali and detergent treatments. *J. Biochem.* **58,** 561–568

18 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275

19 Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye bindings. *Anal. Biochem.* **72,** 248–254

20 Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37,** 911–917

21 Shimokawa, T., Moriuchi, A., Hori, T., Saito, M., Naito, Y., Kabasawa, H., Nague, Y., Matsubara, M., and Okuyama, H. (1988). Effect of dietary alpha-linolenate/linoleate balance on mean survival time, incidence of stroke and blood pressure of spontaneously hypertensive rats. *Life Sci.* **43,** 2067–2075

22 Van Veldhoven, P.P., Vanhove, G., Assselberghs, S., Eyssen, H.J., and Mannaerts, G.P. (1992). Substrate specificities of rat liver peroxisomal acyl-CoA oxidases: palmitoyl-CoA oxidase (inducible acyl-CoA oxidase), pristanoyl-CoA oxidase (non-inducible acyl-CoA oxidase), and trihydroxycoprostanoyl-coA oxidase. *J. Biol. Chem.* **267,** 20065–20074

23 Sørensen, H.N., Gautvik, K.M., Bremer, J., and Spydevold, Ø. (1992). Induction of the three peroxisomal β-oxidation enzymes is synergistically regulated by dexamethasone and fatty acid, and counteracted by insulin in Morris 7800 hepatoma cells in culture. *Eur. J. Biochem.* **208,** 705–711

24 Cherkaoui-Malki, M., Lone, Y.C., Corral-Debrinski, M., and Lattruffe, N. (1990). Differential proto-oncogene mRNA induction from rats treated with peroxisome proliferators. *Biochem. Biophys. Res. Commun.* **173,** 855–861

25 Hsieh, L.L., Shinozuka, H. and Weinstein, I.B. (1991). Changes in expression of cellular oncogenes and endogenous retrovirus-like sequences during hepatocarcinogenesis induced by a peroxisome proliferator. *Br. J. Cancer* **64,** 815–820

26 Fernandes, G. and Venkatraman, J.T. (1991). Modulation of breast cancer growth in nude mice by n-3 fatty acids. In *Health Effects of n-3 Polyunsaturated Fatty Acids in Seafoods.* (Simopoulos, A.P., Kifer, R.R., Martin, R.E., Barlow, S.M. ed.) World Rev. Nutr. Diet Vol. 66, pp 488–503, S. Karger AG., New York, NY USA

27 Horie, S. and Suga T. (1990). Different regulation of hepatic peroxisomal β-oxidation activity in rats treated with clofibrate and partially hydrogenated marine oil. *Biochem. Biophys. Res. Commun.* **166,** 780–786

28 Hori, T., Moriuchi, A., Okuyama, H., Sobajima, T., Tamia-Koizumi, K., and Kojima, K. (1987). Effect of dietary fatty acids on pulmonary metastasis of ascites tumor cells in rats. *Chem. Pharm. Bull.* **35,** 3925–3927

29 Kamano, K., Okuyama, H., Konishi, R., and Nagasawa, H. (1989). Effects of a high-linoleate and a high α-linolenate diet on spontaneous mammary tumorigenesis in mice. *Anticancer Res.* **9,** 1903–1908

30. Hirose, M., Matsuda, A., Ito, N., Kamano, K., and Okuyama, H. (1990). Effects of dietary perilla oil, soybean oil and safflower oil on 7, 12-dimethylbenz[α]anthracene (DMBA) and 1, 2-dimethylhydrazine (DMH)-induced mammary gland and colon carcinogenesis in female SD rats. *Carcinogenesis* **11,** 731–735

31 In *Recommended dietary allowance for the Japanese, 5th revision* (1994). (Health Service Bureau, Ministry of Health and Welfare, Japan, ed.) pp. 56–58, Diichi Shuppan, Tokyo, Japan