[29, 31]. We here evaluated consumption and plasma concentrations of FAs, calculated their correlations, and assessed magnitude of seasonal differences using data from the JADE Study referred to above [9].

Subjects and methods

Subjects and design

In order to validate an evidence-based semi-quantitative food frequency questionnaire (SQFFQ) developed on the basis of multiple regression and contribution analyses [32], we executed 28 day WDRs, as described elsewhere [9]. Briefly, in autumn 1996, we first mail-administered the SQFFQ to 106 (21 male and 85 female) middle-aged Japanese dietitians living in Aichi Prefecture, in Central Japan, and self-administered 7 consecutive day WDRs approximately a week later, and at about 3 month intervals in winter, spring and summer of 1997. Overnight fasting venous blood was collected on the next morning of the last day of the respective 7 day WDRs [9, 33].

Male dietitians were excluded because they were rather small in number. The data presented in this paper were based on the 28 day WDRs provided by 71 female dietitians without overt fat-related diseases who gave informed consent. Characteristics of the study subjects on enrollment were shown in Table 1 as compared with those for 40–49 years of age in the National Nutrition Survey in 1996 [34].

Table 1. Characteristics of 71 Japanese female dietitians on enrollment compared with the National Nutrition Survey in 1996

	This study	NNS[a] (1996)
Height[b] (cm)	156.0 (5.2)[c]	154.7 (5.3)
Weight (kg)	52.4 (5.6)	54.6 (8.1)
BMI[d] (kg/m^2)	21.5 (2.2)	22.8 (3.2)
Age (Years of age)	47.7 (8.2)	40–49
30–39 years (Number)	13	
40–49 years	30	
50–59 years	21	
60 years	7	
Drinking		
Non/former/current		
Number	52/1/18	
%	73.2/1.4/25.3	88.5/1.6/9.9
Smoking		
Non/former/current		
Number	66/3/2	
%	93.0/4.2/2.8	88.1/2.0/9.9
Exercise		
Non/current		
Number	28/43	
%	39.4/60.6	81.4/18.6

[a] National Nutrition Survey.
[b] Height and weight were measured at the enrollment health checkups.
[c] Mean (SD).
[d] Body mass index.

Fatty acids selected

For intake and plasma concentrations for FAs, the following 13 FAs were chosen: 14:0 (myristic acid); 16:0 (palmitic acid); 18:0 (stearic acid); 16:1 (palmitoleic acid); 18:1 (oleic acid); 18:2 n-6 (linoleic acid: LA); 18:3 n-6 (γ-linolenic acid); 20:3 n-6 (dihomo-γ-linolenic acid); 20:4 n-6 (arachidonic acid: AA); 18:3 n-3 (α-linolenic acid: ALA); 20:5 n-3 (eicosapentaenoic acid: EPA); 22:5 n-3 (docosapentaenoic acid: DPA) and 22:6 n-3 (docosahexaenoic acid: DHA). Furthermore, total FA (SFAs + MUFAs + PUFAs), saturated FAs (SFAs = myristic acid + palmitic acid + stearic acid), monounsaturated FAs (MUFAs = palmitoleic acid + oleic acid), n-6 polyunsaturated FAs (PUFAs = LA + γ-linolenic acid + dihomo-γ-linolenic acid + AA), n-3 PUFAs (= α-linolenic acid [ALA] + EPA + DPA + DHA) and n-3 highly unsaturated FAs (HUFAs = EPA + DPA + DHA) were also computed.

Calculation of fatty acid intake

We assessed average daily consumption of the 13 FAs, contributing 79.1 ± 3.7% of the total FA intake, by multiplying the food intake (in grams) or serving/portion size and the nutrient content per 100 g of food as listed in the Standard Tables of Food Composition, Version 4 [35–37] and the Follow-up of the Standard Tables of Food Composition [38].

Fatty acid analysis

As reported in detail elsewhere [33], in brief, plasma prepared in tubes with EDTA-2Na was stored at −80 °C until analysis of FAs by gas chromatography. We measured FAs in the whole plasma, including cholesterol ester, phospholipid and triglyceride fractions. The 13 FAs, mentioned above, were determined using heptadecanoic acid as an internal standard [39]. Each FA value was expressed as an absolute concentration (mg/dl).

The precision of FA measurements in plasma was also covered elsewhere [33]. In brief, coefficients of variation within the series ranged from 1.8 to 4.8% for the 13 FAs and coefficients for day to day variation ranged from 2.5 to 7.2%.

Statistical methods

As is well known, intake of FAs is related to energy, adjustment for energy was made according to regression analysis [7]. In order to detect confounding effects, we computed Pearson's correlation coefficients (CCs) between age, BMI, exercise, alcohol and

smoking habits vs. energy-adjusted consumption and plasma concentrations, respectively. Using age- and energy-adjusted intake and age-adjusted plasma concentrations for each season, we made comparisons with ANOVA for repeated measures [40], followed by Tukey's multiple *t*-test. Spearman's partial rank CCs were calculated between age- and energy-adjusted consumption and age-adjusted plasma concentrations of FAs for each season [7]. Furthermore, inter-seasonal Spearman's partial rank CCs were computed for age- and energy-adjusted intake and age-adjusted plasma concentrations of FAs. All tests were two-sided and p values of 0.05 or less were regarded as statistically significant.

Results

Relations of age with consumption and concentrations

There were inverse Pearson's CCs for energy-adjusted intake of FAs, except for dihomo-γ-linolenic acid and FAs provided by fish/marine foods (but not statistically significant), with age, while consistently positive CCs for plasma concentrations (Table 2). Inconsistent Person's CCs were observed for BMI, exercise, alcohol and smoking habits with consumption and plasma concentrations, respectively (data not shown).

Seasonal differences in consumption and concentrations

Statistically significant seasonal differences were observed in age- and energy-adjusted consumption for most FAs, except for myristic acid, MUFAs, oleic acid, n-6 PUFAs, LA, γ-linolenic acid, ALA, PUFAs/SFAs, and n-6 PUFAs/n-3 PUFAs (Table 3), and in age-adjusted plasma concentrations for most FAs, except for stearic acid, γ-linolenic acid, n-3 PUFAs, ALA, EPA, DHA and n-3 HUFAs (Table 4).

Spearman's partial rank CCs between intake and plasma concentrations

Statistically significant Spearman's partial rank CCs between age- and energy-adjusted consumption and age-adjusted plasma concentrations of FAs were noted for EPA, DHA, n-3 HUFAs, n-6 PUFAs/n-3 PUFAs and n-6 PUFAs/n-3 HUFAs for almost all seasons (Table 5).

Inter-seasonal Spearman's partial rank CCs for consumption and plasma concentrations

There were statistically significant inter-seasonal Spearman's partial rank CCs for most selected FAs, not only in regarding age- and energy-adjusted

Table 2. Pearson's correlation coefficients between age vs. energy-adjusted intake and plasma concentrations of fatty acids

		Intake				Plasma concentrations			
		Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer
Total FA		−0.37		−0.29	−0.43	0.50	0.47	0.51	0.49
SFAs		−0.32			−0.33	0.49	0.46	0.50	0.48
Myristic acid	(14:0)					0.45	0.45	0.45	0.48
Palmitic acid	(16:0)	−0.35			−0.36	0.46	0.45	0.48	0.46
Stearic acid	(18:0)	−0.38			−0.37	0.55	0.43	0.53	0.48
MUFAs		−0.46		−0.36	−0.52	0.42	0.34	0.41	0.37
Palmitoleic acid	(16:1)	−0.31				0.46	0.45	0.51	0.49
Oleic acid	(18:1)	−0.46		−0.37	−0.53	0.40	0.32	0.39	0.34
PUFAs						0.52	0.49	0.51	0.52
n-6PUFAs						0.45	0.36	0.45	0.39
Linoleic acid	(18:2n-6)					0.43	0.33	0.41	0.32
γ-linolenic acid	(18:3n-6)					0.46	0.26	0.40	0.50
Dihomo-γ-linolenic acid	(20:3n-6)		0.26			0.39	0.32	0.36	0.32
Arachidonic acid	(20:4n-6)					0.27	0.32	0.38	0.37
n-3PUFAs						0.49	0.61	0.53	0.62
α-linolenic acid	(18:3n-3)			−0.26	−0.37	0.27		0.32	
Eicosapentaenoic acid	(20:5n-3)					0.46	0.58	0.47	0.58
Docosapentaenoic acid	(22:5n-3)					0.46	0.54	0.52	0.49
Docosahexaenoic acid	(22:6n-3)					0.44	0.58	0.50	0.60
n-3HUFAs						0.48	0.62	0.52	0.62
PUFAs/SFAs									
n-6PUFAs/n-3PUFAs						−0.27	−0.48	−0.37	−0.48
n-6PUFAs/n-3HUFAs			−0.23		−0.27	−0.26	−0.49	−0.35	−0.48

Only statistically significant values for $|r| \geq 0.234$ (at $p < 0.05$), $|r| \geq 0.309$ (at $p < 0.01$), and $|r| \geq 0.386$ (at $p < 0.001$) are listed.

Table 3. Comparison of age- and energy-adjusted daily intake of fatty acids by season

			Autumn Mean (SD)	Winter Mean (SD)	Spring Mean (SD)	Summer Mean (SD)
Total FA		(mg)	46,751 (9820)^{ab}	43,908 (8627)^{a}	44,484 (9543)	43,389 (9303)^{b}
SFAs		(mg)	14,554 (3598)^{a}	13,730 (3175)	13,451 (2814)	13,181 (2904)^{a}
Myristic acid	(14:0)	(mg)	1348 (510)	1239 (493)	1190 (399)	1237 (410)
Palmitic acid	(16:0)	(mg)	9461 (2211)^{a}	8920 (1943)	8850 (1781)	8601 (1815)^{a}
Stearic acid	(18:0)	(mg)	3744 (1002)^{ab}	3572 (901)	3411 (740)^{a}	3343 (810)^{b}
MUFAs		(mg)	18,547 (4455)	17,566 (3998)	17,882 (4182)	17,420 (4363)
Palmitoleic acid	(16:1)	(mg)	1048 (321)^{ab}	990 (304)	940 (243)^{a}	913 (288)^{b}
Oleic acid	(18:1)	(mg)	17,498 (4201)	16,576 (3826)	16,942 (4008)	16,507 (4163)
PUFAs		(mg)	13,651 (3182)^{a}	12,611 (2781)^{a}	13,150 (3308)	12,788 (3145)
n-6PUFAs		(mg)	11,052 (2823)	10,231 (2340)	10,805 (2804)	10,477 (2676)
Linoleic acid	(18:2n-6)	(mg)	10,878 (2815)	10,074 (2333)	10,648 (2787)	10,332 (2660)
γ-linolenic acid	(18:3n-6)	(μg)	104 (287)	145 (324)	108 (257)	117 (215)
Dihomo-γ-linolenic acid	(20:3n-6)	(mg)	26 (7)^{abc}	23 (6)^{a}	22 (6)^{b}	21 (6)^{c}
Arachidonic acid	(20:4n-6)	(mg)	148 (37)^{abc}	135 (34)^{ad}	134 (39)^{b}	124 (40)^{cd}
n-3PUFAs		(mg)	2599 (655)^{ab}	2380 (643)	2346 (704)^{a}	2311 (725)^{b}
α-linolenic acid	(18:3n-3)	(mg)	1615 (448)	1524 (477)	1591 (580)	1552 (529)
Eicosapentaenoic acid	(20:5n-3)	(mg)	320 (179)^{ab}	277 (136)	277 (13)^{a}	239 (156)^{b}
Docosapentaenoic acid	(22:5n-3)	(mg)	87 (46)^{ab}	75 (38)	67 (36)^{a}	70 (47)^{b}
Docosahexaenoic acid	(22:6n-3)	(mg)	577 (251)^{ab}	504 (204)	457 (197)^{a}	449 (235)^{b}
n-3HUFAs		(mg)	984 (467)^{ab}	856 (369)	755 (357)^{a}	758 (431)^{b}
PUFAs/SFAs			1.0 (0.3)	1.0 (0.2)	1.0 (0.2)	1.0 (0.3)
n-6PUFAs/n-3PUFAs			4.4 (1.2)	4.5 (1.2)	4.8 (1.2)	4.8 (1.3)
n-6PUFAs/n-3HUFAs			15.1 (12.1)^{ab}	15.5 (11.7)	18.6 (12.8)^{a}	19.1 (12.8)^{b}

Statistically significant according to ANOVA for repeated measures.
^{abcd} Statistically significant at least at $p < 0.05$ among the respective superscript values.

Table 4. Comparison of age-adjusted plasma concentrations of fatty acids by season

			Autumn Mean (SD)	Winter Mean (SD)	Spring Mean (SD)	Summer Mean (SD)
Total FA		(mg/dl)	299.2 (59.5)^{a}	285.1 (49.5)^{ab}	299.2 (62.8)^{b}	297.0 (57.4)
SFAs		(mg/dl)	90.2 (22.1)^{a}	84.7 (16.6)^{abc}	89.2 (21.7)^{b}	89.5 (20.3)^{c}
Myristic acid	(14:0)	(mg/dl)	2.1 (1.0)^{a}	1.9 (0.7)^{a}	2.1 (1.1)	2.1 (0.9)
Palmitic acid	(16:0)	(mg/dl)	64.4 (15.8)^{a}	59.8 (12.2)^{abc}	63.4 (16.0)^{b}	63.6 (15.1)^{c}
Stearic acid	(18:0)	(mg/dl)	23.6 (4.6)	23.0 (4.2)	23.8 (5.0)	23.8 (4.8)
MUFAs		(mg/dl)	63.3 (16.4)^{a}	58.5 (13.1)^{ab}	62.3 (17.6)	63.6 (16.1)^{b}
Palmitoleic acid	(16:1)	(mg/dl)	6.2 (2.4)^{a}	5.6 (2.1)^{ab}	6.0 (2.4)	6.4 (2.5)^{b}
Oleic acid	(18:1)	(mg/dl)	57.1 (14.2)^{a}	52.9 (11.3)^{ab}	56.3 (15.5)	57.2 (14.0)^{b}
PUFAs		(mg/dl)	145.8 (24.9)	141.9 (22.7)^{a}	147.7 (26.9)^{a}	143.9 (24.7)
n-6PUFAs		(mg/dl)	119.3 (20.5)	115.4 (17.8)^{a}	121.9 (21.3)^{ab}	117.0 (19.5)^{b}
Linoleic acid	(18:2n-6)	(mg/dl)	94.1 (17.6)	90.4 (15.2)^{a}	95.7 (18.0)^{ab}	91.7 (17.2)^{b}
γ-linolenic acid	(18:3n-6)	(mg/dl)	2.5 (1.0)	2.4 (1.0)	2.5 (1.1)	2.5 (0.8)
Dihomo-γ-linolenic acid	(20:3n-6)	(mg/dl)	3.0 (1.0)	2.8 (1.0)^{ab}	3.0 (1.2)^{a}	3.0 (1.1)^{b}
Arachidonic acid	(20:4n-6)	(mg/dl)	19.7 (3.8)^{a}	19.9 (3.5)	20.6 (3.7)^{ab}	19.6 (3.4)^{b}
n-3PUFAs		(mg/dl)	28.3 (8.0)	28.2 (8.0)	27.6 (9.2)	28.6 (9.1)
α-linolenic acid	(18:3n-3)	(mg/dl)	0.7 (0.5)	0.6 (0.5)	0.7 (0.6)	0.7 (0.6)
Eicosapentaenoic acid	(20:5n-3)	(mg/dl)	7.7 (3.3)	8.1 (3.7)	7.4 (3.9)	7.9 (4.2)
Docosapentaenoic acid	(22:5n-3)	(mg/dl)	2.1 (0.6)	2.0 (0.6)^{a}	2.0 (0.7)	2.2 (0.7)^{a}
Docosahexaenoic acid	(22:6n-3)	(mg/dl)	16.0 (4.1)	15.7 (4.0)	15.6 (4.5)	16.1 (4.3)
n-3HUFAs		(mg/dl)	25.8 (7.5)	25.8 (7.6)	25.1 (8.6)	26.1 (8.8)
PUFAs/SFAs			1.6 (0.2)	1.7 (0.1)^{a}	1.7 (0.2)	1.6 (0.2)^{a}
n-6PUFAs/n-3PUFAs			4.8 (1.4)	4.7 (1.3)^{a}	5.1 (1.4)^{ab}	4.7 (1.3)^{b}
n-6PUFAs/n-3HUFAs			4.9 (1.4)	4.8 (1.4)^{a}	5.2 (1.4)^{ab}	4.8 (1.3)^{b}

EPA
DHA

Statistically significant according to ANOVA for repeated measures.
^{abc} Statistically significant at least at $p < 0.05$ among the respective superscript values.

Table 5. Spearman's partial rank correlation coefficients between age- and energy-adjusted intake and age-adjusted plasma concentrations of fatty acids by season

		Autumn		Winter		Spring		Summer	
Total FA									
SFAs									
Myristic acid	(14:0)								
Palmitic acid	(16:0)								
Stearic acid	(18:0)								
MUFAs									
Palmitoleic acid	(16:1)								
Oleic acid	(18:1)								
PUFAs									
n-6PUFAs									
Linoleic acid	(18:2n-6)								
γ-linolenic acid	(18:3n-6)								
Dihomo-γ-linolenic acid	(20:3n-6)	−0.28	*						
Arachidonic acid	(20:4n-6)			0.30	*				
n-3PUFAs								0.38	**
α-linolenic acid	(18:3n-3)								
Eicosapentaenoic acid	(20:5n-3)	0.63	***	0.51	***	0.51	***	0.58	***
Docosapentaenoic acid	(22:5n-3)	0.27	*						
Docosahexaenoic acid	(22:6n-3)	0.33	**			0.32	**	0.30	*
n-3HUFAs		0.50	***	0.41	***	0.43	***	0.45	***
PUFAs/SFAs									
n-6PUFAs/n-3PUFAs		0.44	***	0.30	*	0.40	***	0.47	***
n-6PUFAs/n-3HUFAs		0.64	***	0.50	***	0.60	***	0.58	***

Only statistically significant values for $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ are listed.

intake but also age-adjusted plasma concentrations (Table 6). Furthermore, the values for plasma concentrations were almost consistently greater than those for consumption.

Discussion

Above all, we assume that our WDRs are accurate because they were provided by female dietitians. We formerly reported the existence of great within-individual variation in consumption of energy and 30 nutrients, including FAs [10]. The next greatest differences were caused by season, followed by sequence of days and day of week. We here demonstrated magnitude and patterns of seasonal variance in plasma concentrations as well as intake of FAs, suggesting that we should take into account seasonal variation in case–control and cohort studies.

There were discrepant patterns in seasonal variance between intake and plasma concentrations of FAs. This illustrates the fact that there is great within-individual variation in food consumption in free-living people. Furthermore, there may be time lag between 7-day diet intake and plasma concentrations of FAs. On the other hand, plasma concentrations are not entirely modified by day-to-day within-individual variation since there is a homeostatic system in action, depending on intake, absorption, metabolism and excretion [41, 42].

In the present investigation there were significant CCs between consumption and plasma concentrations of FAs related to n-3 PUFAs, EPA, DHA and n-3 HUFAs taken specifically from fish/marine foods for almost all seasons [12, 13, 15–17, 30, 43–45]. Both n-6 PUFAs and n-3 PUFAs are essential FAs; however, no significant positive/inverse CCs were observed for n-6 PUFAs partly because n-6 PUFAs ubiquitously exist in foods, including vegetable oils and plants. SFAs and MUFAs are deposited in our organs/tissues, metabolized into energy or biosynthesized from dietary nutrients including carbohydrates and proteins. Thus, it appears plausible that no direct relationship may be evident.

There were seasonal differences in age- and energy-adjusted intake as well as age-adjusted plasma concentrations of FAs, but were positive inter-seasonal age- and energy-adjusted Spearman's partial rank CCs for most FAs. Thus, ranking people according to consumption as well as plasma concentrations may be possible if we match season between cases and controls. Because the values of age- and energy-adjusted Spearman's partial rank CCs for plasma concentrations were greater than those of intake, homeostasis in plasma concentrations is indicated, as mentioned above. Furthermore, individual appetite appears to be maintained throughout the year.

While we noted here that age could be a crucial confounding factor inversely associated with intake of FAs, it has been found to be positively related to

Table 6. Inter-seasonal Spearman's partial rank correlation coefficients between age- and energy-adjusted intake and age-adjusted plasma concentrations of fatty acids

	Autumn vs. winter		Winter vs. spring		Spring vs. summer		Autumn vs. spring		Autumn vs. summer		Winter vs. summer	
	Intake	Plasma	Intake	Plasma	Intake	Plasma	Intake	Plasma	Intake	Plasma	Intake	Plasma
Total FA	0.62	0.76	0.67	0.78	0.53	0.75	0.53	0.74	0.60	0.69	0.54	0.78
SFAs	0.52	0.78	0.53	0.79	0.42	0.78	0.40	0.74	0.53	0.74	0.53	0.84
MUFAs	0.56	0.71	0.65	0.67	0.51	0.64	0.54	0.72	0.52	0.65	0.55	0.72
PUFAs	0.53	0.70	0.59	0.74	0.56	0.72	0.51	0.75	0.54	0.69	0.43	0.67
n-6PUFAs	0.56	0.66	0.56	0.72	0.55	0.60	0.48	0.74	0.55	0.63	0.43	0.61
n-3PUFAs	0.52	0.60	0.53	0.55	0.36	0.63	0.45	0.61	0.29	0.67	0.29	0.57
n-3HUFAs	0.26	0.58		0.54		0.62		0.61		0.66		0.56
PUFAs/SFAs	0.47	0.52	0.44	0.42	0.44	0.43	0.27	0.59	0.35	0.61	0.42	0.51
n-6 PUFAs/n-3 PUFAs	0.37	0.53	0.37	0.56	0.27	0.45		0.57	0.33	0.58	0.35	0.44
n-6PUFAs/n-3HUFA		0.53	0.31	0.55		0.43		0.54	0.25	0.56		0.46

Only statistically significant values for $|r| \geq 0.234$ (at $p < 0.05$), $|r| \geq 0.309$ (at $p < 0.01$), and $|r| \geq 0.386$ (at $p < 0.001$) are listed.

plasma concentrations [33, 46–50]. For the former, it may be due to the fact that consumption of animal protein/fat, except for fish/marine FAs, as well as energy is relatively low and a traditional Japanese diet is maintained by elderly people. The finding of a positive correlation between plasma concentrations and age, despite this reduction in intake according to age, is intriguing and clearly deserves further attention. The role played by the physiologic system also warrants more stress.

Since we carried out this study in women, effects of smoking and drinking were not detected because these habits are not so prevalent in Japanese female, as mentioned above. It seems to us that smokers tend to consume less energy and fats/oils. In contrast, drinkers are likely to eat fish/marine foods as appetizers/hors d'oeuvres to alcohol. These factors are important in terms of interactions and/or confounding in epidemiologic studies on diet and health [51, 52].

There may be critique that the results cannot be generalized because the study subjects were dietitians. We respond that internal validity is prerequisite for external validity (generalizability) [53]. In other words, external validity is not attained unless the method is internally valid, which is particularly the case for dietary studies because to secure dietary information from the general public is problematic. Bearing those strengths and weaknesses in mind, we proposed the JADE Study and delivered a self-administered SQFFQ and lifestyle questionnaire to dietitians to assess the relations between diet and health/disease, which is, to our knowledge, a worldwide new approach.

In summary, seasonal discrepancies among intake and plasma concentrations of FAs were found in the present study, patterns substantially differing between intake and plasma concentrations. Thus, we should keep in mind seasonal effects, although the impact did not seem so great compared with within-individual variation and there were positive inter-seasonal Spearman's partial rank CCs in intake and plasma concentrations for most FAs. Matching/adjusting of seasons is recommended when classifying the study subjects according to intake and concentrations of FAs. Nevertheless, there were significantly positive CCs between consumption and plasma concentrations in all seasons in terms of n-3 PUFAs, EPA, DHA and n-3 HUFAs, perhaps because these are essential FAs derived particularly from fish/marine foods.

Acknowledgements

This study was partly supported by A Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The authors are grateful to the members of the Aichi Prefectural Dietetics Association for their participation in this study, and to Ms Y. Kubo, Ms Y. Ito, Ms K. Nishiyama and Dr M.A. Moore for their technical and language assistance in preparing this manuscript.

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Kakutani et al. Lipids in Health and Disease 2011, 10:241
http://www.lipidworld.com/content/10/1/241

LIPIDS IN HEALTH AND DISEASE

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Supplementation of arachidonic acid-enriched oil increases arachidonic acid contents in plasma phospholipids, but does not increase their metabolites and clinical parameters in Japanese healthy elderly individuals: a randomized controlled study

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Abstract

Background: The importance of arachidonic acid (ARA) among the elderly has recently gained increased attention. The effects of ARA supplementation in the elderly are not fully understood, although ARA is considered to be associated with various diseases. We investigate whether ARA supplementation to Japanese elderly subjects affects clinical parameters involved in cardiovascular, inflammatory, and allergic diseases. We also examine the levels of ARA metabolites such as prostanoids during intervention.

Methods: We conducted a randomized, double-blind and placebo-controlled parallel group intervention trial. ARA-enriched oil (240 or 720 mg ARA per day) or placebo was administered to Japanese healthy men and women aged 55-70 years for 4 weeks followed by a 4-week washout period. The fatty acid contents of plasma phospholipids, clinical parameters, and ARA metabolites were determined at baseline, 2, 4, and 8 weeks.

Results: The ARA content in plasma phospholipids in the ARA-administrated groups increased dose-dependently and was almost the same at 2 weeks and at 4 weeks. The elevated ARA content decreased to nearly baseline during a 4-week washout period. During the supplementation and washout periods, no changes were observed in eicosapentaenoic acid and docosahexaenoic acid contents. There were no changes in clinical blood parameters related to cardiovascular, inflammatory and allergic diseases. ARA supplementation did not alter the level of ARA metabolites such as urinary 11-dehydro thromboxane B_2, 2,3-dinor-6-keto prostaglandin (PG) $F_{1\alpha}$ and 9,15-dioxo-11α-hydroxy-13,14-dihydro-2,3,4,5-tetranor-prostan-1,20-dioic acid (tetranor-PGEM), and plasma PGE_2 and lipoxin A_4. ARA in plasma phospholipids was not correlated with ARA metabolite levels in the blood or urine.

Conclusion: These results indicate that ARA supplementation, even at a relatively high dose, does not increase ARA metabolites, and suggest that it does not induce cardiovascular, inflammatory or allergic diseases in Japanese elderly individuals.

Keywords: arachidonic acid, thromboxane A_2, prostacyclin, prostaglandin E_2, cardiovascular diseases, inflammation

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Background

Arachidonic acid (ARA) is an n-6 essential fatty acid that is a major constituent of biomembranes. It is converted into lipid mediators that exert various physiological actions. ARA is synthesized in the body from dietary linoleic acid (LA) and additionally most adults consume 50-250 mg/day of ARA from foodstuffs [1-3].

The consumption of ARA in breast milk is very important for infant development since the activity from LA conversion to ARA is low in infant [4]. The conversion of LA to ARA declines with age [5], and the importance of ARA supplementation among the elderly has recently gained increased attention. It has been reported that supplementation with ARA among the elderly improves cognitive response [6] and coronary flow velocity reserve [7] and some animal studies support these findings [8-11].

However, many studies show that lipid mediators derived from ARA are associated with various diseases. For example, thromboxane A_2 (TXA_2) is associated with cardiovascular diseases via its activation of thrombogenicity and vasoconstriction, whereas prostaglandin E_2 (PGE_2) leads to inflammation and might enhance tumor growth [12-14]. Levels of the urinary TXA_2 metabolite, 11-dehydro TXB_2, have been shown to be higher in patients with heart failure (3.4-fold), ischemic heart disease (1.4-fold) [15] and essential hypertension [16]. Plasma PGE_2 levels are also high in patients with ulcerative colitis [17] and advanced periodontitis [18], and levels of its urinary metabolite, tetranor-PGEM, are epidemiologically higher in colorectal cancer [19]. The relationship between lipids mediators and diseases is speculated based on the fact that cyclooxygenase (COX) inhibitors are effective against these conditions [14,20].

Many clinical trials of ARA supplementation have been done on infants [4], but there are a few reports describe the administration of ARA or ARA-containing oil to adults [21-23]. Among healthy males who consumed 1.5 g/day of ARA (as free ARA) for 50 days [21,22] or 838 mg/day of ARA for 4 weeks [23] in randomized controlled studies, platelet aggregation did not change and adverse effects did not occur. Both urinary 11-dehydro TXB_2 and 2,3-dinor-6-keto $PGF_{1\alpha}$ slightly increased in the former study [22]. However, it remains unclear whether ARA intake evokes the clinical parameters in the speculated diseases in the elderly. It is also unclear whether ARA intake increases lipid mediators derived from ARA in the elderly.

The present study investigates the effects of 240 or 720 mg/day of ARA, which is much more than that derived from food, on Japanese healthy elderly individuals. We determined clinical parameters of cardiovascular, inflammatory, and allergic diseases in blood as well as ARA content in plasma phospholipids and urinary and plasma lipid mediators. Correlations between ARA content in plasma phospholipids and lipid mediator concentrations were also determined.

Materials and methods

Study design

This randomized, double-blind and placebo-controlled parallel group intervention trial evaluated the effects of daily ARA supplementation on cardiovascular disease and/or inflammation. The Ethics Committee on Human Experimentation of Suntory Holdings Ltd. approved the study, which conformed to the principles set forth in the Declaration of Helsinki. Written informed consent was obtained from the participants of this study. Physiological parameters and blood and urine were sampled at the time of recruitment starting in August 2010. One hundred and eighteen participants were screened and randomly assigned to placebo, low-ARA or high-ARA groups. Participants received 10 gelatin-capsules containing either ARA or a placebo every morning for 4 weeks followed by a 4-week washout period. Blood and urine were sampled, a study diary was distributed and collected and dietary intake was assessed at baseline (week 0, within 4 weeks of recruitment) and again at 2, 4 and 8 weeks later. The fatty acid composition of the oils used in this study is shown in Table 1. The placebo

Table 1 Fatty acid composition of test capsules

Fatty acids[1]	Group		
(%)	Placebo	Low-ARA	High-ARA
14:0	0.0	0.2	0.5
15:0	0.0	0.0	0.2
16:0	12.2	11.9	11.3
16:1	1.2	0.8	0.0
17:0	0.0	0.1	0.3
18:0	2.8	4.5	8.1
18:1	72.3	50.3	6.3
18:2n-6	9.6	9.4	8.9
18:3n-6	0.0	0.9	2.6
18:3n-3	0.7	0.6	0.4
20:0	0.4	0.6	0.9
20:1	0.3	0.3	0.4
20:2n-6	0.0	0.2	0.7
20:3n-6	0.0	1.4	4.0
20:4n-6	0.0	14.2	42.9
22:0	0.1	1.2	3.3
22:4n-6	0.0	0.2	0.5
24:0	0.0	2.6	7.5
Others	0.4	0.6	1.2

[1]14:0, myristic acid; 15:0, pentadecanoic acid; 16:0; palmitic acid; 16:1, palmitoleic acid; 17:0, heptadecanoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2n-6, linoleic acid; 18:3n-6, γ-linolenic acid; 18:3n-3, α-linolenic acid; 20:0, eicosanoic acid; 20:1, eicosenic acid; 20:2n-6, eicosadienoic acid; 20:3n-6, dihomo-γ-linolenic acid; 20:4n-6, arachidonic acid; 22:0, behenic acid; 22:4n-6, docosatetraenoic acid; and 24:0, lignoceric acid.

group consumed 1700 mg/day of commercially available olive oil. The high-ARA group consumed 1700 mg/day of an ARA-enriched edible oil derived from *Mortierella alpina* (SUNTGA40S; 720 mg/day of ARA) [24]. The low-ARA group received 570 mg/day of ARA-enriched oil and 1130 mg/day of olive oil (240 mg/day of ARA). Blood and urine samples were obtained after an overnight fast for > 10 hours on the morning of each assessment. Cardiovascular risk parameters included prothrombin time (PT), activated partial thromboplastin time (APTT), antithrombin III (ATIII), high-sensitivity C-reactive protein (hs-CRP) and adiponectin. Allergic parameters included nonspecific immunoglobulin E (IgE) levels and eosinophils (EO). Inflammatory parameters comprised C-reactive protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels. Urinalysis was conducted for quantitative analysis of creatinine (Cre) and qualitative analyses of protein, glucose and urobilinogen.

Participants

Healthy men and women aged 55-70 years living in Tokyo and its environs were recruited. Exclusion criteria were as follows: allergy to gelatin or olive oil; continuous consumption of drugs or supplements that affect lipid metabolism; continuous intake of non-steroidal anti-inflammatory or anti-allergic drugs; a history of serious disorders such as cardiac infarction, cerebral infarction, stroke, cancer, asthma; and clinically significant systemic diseases. Participants were randomly assigned to the three groups matched by gender, age, hs-CRP, PT and estimated ARA content (%) in plasma phospholipids. To quickly estimate the ARA content in plasma phospholipids at the recruitment, the estimated ARA content was calculated from triglycerides (TG, mg/dL), phospholipids (PL, mg/dL) and the ARA content in total plasma fatty acids as: (ARA content in total plasma fatty acids)×(TG + PL)/PL. This rough estimation is based on the fact that the ARA content is much smaller in plasma TG than in plasma PL (the ARA in plasma TG is negligibly-small for the rough estimation), and used only for quick assignment to the three groups.

Chemicals and apparatus

We purchased 11-dehydro TXB_2, 9,15-dioxo-11α-hydroxy-13,14-dihydro-2,3,4,5-tetranor-prostan-1,20-dioic acid (tetranor-PGEM), lipoxin A_4 (LXA_4), 11-dehydro TXB_2-d4, 9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostan-1,20-dioic-13,13,14,14,15,15-d6 acid (tetranor-PGEM-d6), and LXA_4-d5 from Cayman Chemical (Ann Arbor, MI, USA). Fatty acid methyl esters were chromatographically separated and detected using an Agilent 6890 GLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Supelco SP-2330 column (30 m × 0.32 mm × 0.2 μm, Sigma-Aldrich, St. Louis, MO, USA). Lipid mediators were chromatographically separated and detected using an Agilent 1200 HPLC system (Agilent Technologies) equipped with a Cadenza CD-C18 column (3 μm, 2 mm i.d. × 150 mm, Imtakt, Kyoto, Japan) and a 4000 Q TRAP with electrospray interface (AB SCIEX, Foster City, CA, USA).

Fatty acid analysis

Lipids in plasma were extracted and purified by the method of Folch et al. [25]. Fatty acid residues in lipid fractions were analyzed by the method of Sakuradani et al. [26]. In brief, each fraction was incubated with an internal standard (pentadecanoic acid) in methanolic HCl at 50°C for 3 h to transmethylate fatty acid residues to fatty acid methyl esters, which were extracted with *n*-hexane and analyzed by capillary gas-liquid chromatography. Plasma was directly transmethylated without extraction or fractionation when we calculated the estimated ARA content in plasma phospholipids.

Analysis of urinary metabolites of lipid mediators

Urine samples were stored at -80°C for 5-41 days before measurement of metabolites.

Urinary 11-dehydro TXB_2 was measured by LC-MS/MS. Urine samples (0.5 mL), to which 11-dehydro TXB_2-d4 was added as an internal standard, were diluted with 1 mmol/L HCl to a final volume of approximately 3 mL and left for 1 h at room temperature. The mixtures were applied to preconditioned SPE cartridges (Empore disk cartridge C18-SD, 3M, St. Paul, MN, USA), and the cartridges were washed with 1 mmol/L HCl, water and hexane. 11-Dehydro TXB_2 and 11-dehydro TXB_2-d4 were then eluted with 1 mL of hexane/ethyl acetate (1/1, v/v). The eluates were dried by centrifugal evaporation, re-dissolved in 0.1 mL of acetonitrile/water/formic acid (250/750/1, v/v/v) and then transferred to brown glass vials at 10°C. Portions of these elute (20 μL) were injected into LC-MS/MS. Solvent A was 5 mM ammonium acetate (pH 5.5) and solvent B was acetonitrile. The separation was performed in an isocratic mode with 35% solvent B at a flow rate of 0.2 mL/min and a column temperature of 40°C. The mass spectrometer was operated in the negative ion mode. 11-Dehydro TXB_2 and 11-dehydro TXB_2-d4 were detected in selected reaction monitoring (SRM) mode by monitoring mass transitions of *m/z* 367 → 305 for 11-dehydro TXB_2, and *m/z* 371 → 309 for 11-dehydro TXB_2-d4 at a collision energy of -23 V.

Urinary tetranor-PGEM was measured by LC-MS/MS according to the modified method of Murphey et al. [27]. Urine samples (0.1 mL) to which tetranor-PGEM-d6 was added as an internal standard were diluted with 1 mmol/L HCl to a final volume of approximately 1 mL.

The diluted samples were mixed with 0.5 mL of O-methylhydroxylamine hydrochloride in 1.5 M sodium acetate buffer pH 5 (16%, w/v) and left for 1 h at room temperature. The mixtures were applied to SPE cartridges as described above, and the cartridges were washed with 1 mmol/L HCl. Tetranor-PGEM and tetranor-PGEM-d6 were then eluted with 1 mL of ethyl acetate. The eluates were dried, re-solved and transferred as described above. Portions of these elute (20 μL) were injected into LC-MS/MS. Solvent A was water/formic acid (100/0.2, v/v) and solvent B was acetonitrile/methanol/formic acid (95/5/0.2, v/v/v). The separation was performed at a flow rate of 0.2 mL/min and a column temperature of 60°C using the following linear gradient: 0-4.8 min, 30 to 94% solvent B; 4.8-5.75 min, 94 to 30% solvent B; 5.75-14.8 min, 30% solvent B. The mass spectrometer was operated in the negative ion mode. Tetranor-PGEM and tetranor-PGEM-d6 were detected in SRM mode by monitoring mass transitions at *m/z* 385 → 336 for tetranor-PGEM, and *m/z* 391 → 342 for tetranor-PGEM-d6 at a collision energy of -25 V.

Urinary 2,3-dinor-6-keto $PGF_{1\alpha}$ was measured using an enzyme-linked immunosorbent assay (EIA) kit (2,3-dinor-6-keto Prostaglandin $F_{1\alpha}$ EIA Kit, Cayman Chemical Company). Urine samples (0.5 mL) were diluted with 1 M sodium citrate buffer pH 4 to a final volume of approximately 1 mL and then vigorously mixed with 4 mL of ethyl acetate. The mixtures were separated by centrifugation and the upper phases were collected. The liquid-liquid extraction with ethyl acetate was performed three times and three upper phases of one urine sample were pooled. The pooled extracts were dried by centrifugal evaporation and re-dissolved in assay buffer for analysis.

Analysis of plasma lipid mediators

Blood samples for lipid mediators were collected in vacuum blood collection tubes containing EDTA-2Na and a final concentration of approximately 18 μM of sodium indomethacin. Plasma separated within 1 h was then stored at -80°C for 10-93 days before measurement of mediators.

Plasma PGE_2 was measured using an EIA kit (Prostaglandin E_2 EIA Kit - Monoclonal, Cayman Chemical Company). Plasma samples (1 mL) to which 5 μL of formic acid was added were diluted with 1 mmol/L HCl to a final volume of approximately 3 mL. The mixtures were applied to preconditioned SPE cartridges (BondElut C18, Agilent Technologies), and the cartridges were washed with 1 mmol/L HCl and hexane. PGE_2 was then eluted with 1 mL of ethyl acetate/methanol (99/1, v/v). The eluates were dried by centrifugal evaporation and re-dissolved in assay buffer for analysis.

Plasma LXA_4 was measured by LC-MS/MS. Plasma samples (1 mL) to which LXA_4-d5 was added as an internal standard were diluted with water/acetic acid (1000/5, v/v) to a final volume of approximately 3 mL and applied to preconditioned SPE cartridges (FOCUS, 20 mg/3 mL, Agilent Technologies). The cartridges were washed with water/acetic acid (1000/5, v/v) and water. LXA_4 and LXA_4-d5 were then eluted with 1 mL of methanol/acetonitrile/acetic acid (600/300/1, v/v/v). The eluates were dried, re-solved and transferred as described above. Portions of these elute (20 μL) were injected into LC-MS/MS. Solvent A was water/formic acid (100/0.2, v/v) and solvent B was acetonitrile/methanol/formic acid (95/5/0.2, v/v/v). The separation was performed at a flow rate of 0.2 mL/min and a column temperature of 60°C using the following linear gradient: 0-3.75 min, 50 to 98% solvent B; 3.75-5 min, 98% solvent B; 5-5.75 min, 98 to 50% solvent B; 5.75-14.8 min, 50% solvent B. The mass spectrometer was operated in the negative ion mode. LXA_4 and LXA_4-d5 were detected in SRM mode by monitoring mass transitions at *m/z* 351 → 115 for LXA_4, and *m/z* 356 → 115 for LXA_4-d5 at a collision energy of -22 V.

Dietary assessment and study diary

Dietary habits during the preceding month were assessed using the brief self-administered diet history questionnaire (BDHQ) [28]. Dietary intake was estimated using an ad hoc computer algorithm for the BDHQ based on the Standard Tables of Food Composition in Japan [29,30]. Participants were asked to keep a record throughout the study about intake of the test capsules, the presence of symptoms, amount of exercise, amount of food and alcohol consumed and the use of medication.

Statistical analysis

Results are expressed as means ± SD. Hs-CRP values that exceeded the upper limit of detection (1000 μg/dL) were rounded down to 1000 μg/dL and comprised of one measurement in the low-ARA group at baseline, one in the placebo group, one in the low-ARA group at 2 weeks and two in the high-ARA group at 4 weeks.

For physiological parameters, blood biochemical parameters except hs-CRP, hematological parameters, fatty acid composition of plasma phospholipids, urinary metabolites of lipid mediators and plasma lipid mediators, intra-group comparisons at 2, 4 or 8 weeks versus baseline were analyzed by repeated ANOVA and Dunnett's test using the actual values; inter-group comparisons at 2, 4, or 8 weeks were analyzed by ANOVA and the Tukey-Kramer test using the changes from baseline values. For hs-CRP, intra-group comparisons were analyzed by Friedman test and Steel test; inter-group

comparisons were analyzed by Kruskal-Wallis test and Steel-Dwass test. For dietary intake of nutrients, intra-group comparisons at 4 weeks versus baseline were analyzed by a paired student t-test; inter-group comparisons at 4 weeks were analyzed by ANOVA and Tukey-Kramer test. For compliance rate, inter-group comparisons were analyzed by ANOVA and Tukey-Kramer test. For adverse events, inter-group comparisons were analyzed by Kruskal-Wallis test. All p values were two-tailed, and a p value of < 0.05 was considered statistically significant.

Results

Characteristics of the participants

One participant in the placebo group withdrew for personal reasons and another was excluded due to meeting one of the exclusion criteria. Thus, we analyzed data generated from 64 participants in three groups (placebo, n = 20; low-ARA, n = 22; and high-ARA, n = 22) (Figure 1). The mean compliance rate was > 95% across the three groups and did not differ significantly among them. Side effects did not arise. The numbers of adverse events that developed were six among five participants in the placebo group, eight among six in the low-ARA group and twelve among six in the high-ARA group. None of these adverse events were severe and their frequency did not significantly differ among the groups. The adverse events were common cold (all groups), eczema (placebo and low-ARA groups), diarrhoea (high-ARA group), toothache (low-ARA group), and bone fracture (placebo group).

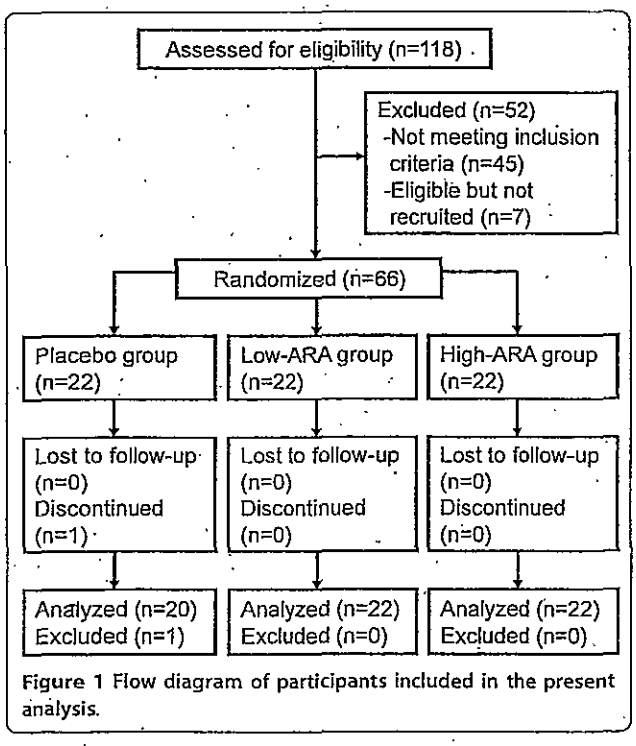

Figure 1 Flow diagram of participants included in the present analysis.

Baseline characteristics of the three groups are shown in Table 2. All groups were balanced with respect to gender, age, BMI, alcohol consumption, smoking status and exercise. Neither hs-CRP, PT nor cardiovascular risk parameters differed significantly among the groups. The mean ARA content in plasma phospholipids ranged from 8.2-8.8% among the three groups and other polyunsaturated fatty acids also did not differ among them. Macronutrient intake during the preceding month at baseline or at 4 weeks later did not differ among the groups or at any time point (Table 3). The daily ARA intake derived from food ranged from 170-200 mg/day in all three groups with no significant differences. The daily intakes of DHA and EPA were 300-500 mg/day and 500-800 mg/day, respectively.

Fatty acid profiles of plasma phospholipids

The ARA content in plasma phospholipids in the high-ARA group increased from 8.77 ± 1.32% (means ± SD) at baseline to 14.02 ± 1.50% at 2 weeks, and was almost the same at 4 weeks (14.33 ± 2.14%). The elevated ARA content declined almost to the initial level during

Table 2 Baseline characteristics of the participants[1]

Characteristics[2]		Group		
		Placebo (n = 20)	Low-ARA (n = 22)	High-ARA (n = 22)
Gender (Female)	n	12	13	12
Age	y	63.1 ± 3.8	62.8 ± 4.3	62.9 ± 4.2
BMI	kg/m^2	21.5 ± 2.4	22.5 ± 2.0	22.5 ± 3.1
Alcohol consumption				
Positive	n	11	13	9
Negative	n	9	9	13
Smoking status				
Positive	n	4	3	3
Negative	n	16	19	19
Exercise				
Habitual	n	11	15	14
Nonhabitual	n	9	7	8
hs-CRP	μg/dL	113 ± 98	106 ± 153	104 ± 116
PT	%	91.4 ± 8.5	91.4 ± 8.6	91.5 ± 6.5
HbA1c	%	4.9 ± 0.2	4.9 ± 0.3	4.9 ± 0.4
FA composition				
18:2n-6	%	18.33 ± 2.64	18.91 ± 2.16	17.78 ± 2.62
20:4n-6	%	8.27 ± 1.26	8.61 ± 0.92	8.77 ± 1.32
20:5n-3	%	2.85 ± 1.30	3.17 ± 1.42	3.59 ± 2.16
22:6n-3	%	7.83 ± 1.42	8.10 ± 1.27	8.41 ± 2.29

[1]Values are means ± SD. No significance among the groups (ANOVA and Tukey-Kramer or Kruskal-Wallis tests).

[2]hs-CRP, high-sensitivity CRP; PT, prothrombin time; HbA1c, hemoglobin A1c; FA, fatty acid; 18:2n-6, linoleic acid; 20:4n-6, arachidonic acid; 20:5n-3, eicosapentaenoic acid; 22:6n-3, docosahexaenoic acid.

Table 3 Calculated daily nutrient intake at baseline and after four weeks of supplementation [1]

Nutrient		Baseline (week 0)			Supplementation (week 4)		
		Placebo	Low-ARA	High-ARA	Placebo	Low-ARA	High-ARA
Energy	kcal/d	1871 ± 483	1779 ± 439	2017 ± 636	1973 ± 550	1748 ± 410	1945 ± 497
Protein	g/d	73.4 ± 21.6	67.1 ± 17.2	78.0 ± 22.2	74.6 ± 21.4	68.9 ± 17.8	77.0 ± 21.4
Carbohydrate	g/d	256 ± 68	234 ± 82	260 ± 91	278 ± 76	227 ± 63	259 ± 74
Total fat	g/d	56.0 ± 19.1	52.8 ± 16.6	61.0 ± 21.9	56.5 ± 22.3	52.4 ± 14.8	57.5 ± 16.8
SFA	g/d	16.2 ± 6.3	14.7 ± 5.0	16.3 ± 6.7	15.5 ± 8.1	14.1 ± 4.4	15.2 ± 5.2
MUFA	g/d	19.3 ± 7.0	18.4 ± 6.4	21.8 ± 8.2	19.6 ± 7.6	18.4 ± 5.7	20.4 ± 6.1
PUFA	g/d	13.1 ± 4.6	12.7 ± 3.6	14.8 ± 4.7	13.8 ± 4.3	12.8 ± 3.4	14.1 ± 4.0
18:2n-6	g/d	9.96 ± 3.52	9.76 ± 2.85	11.12 ± 3.60	10.59 ± 3.34	9.71 ± 2.56	10.74 ± 3.05
20:4n-6	mg/d	174 ± 56	170 ± 71	199 ± 74	172 ± 67	174 ± 73	189 ± 66
18:3n-3	g/d	1.61 ± 0.61	1.52 ± 0.48	1.79 ± 0.62	1.67 ± 0.57	1.51 ± 0.41	1.71 ± 0.51
20:5n-3	mg/d	377 ± 215	315 ± 172	447 ± 163	366 ± 197	367 ± 190	399 ± 202
22:6n-3	mg/d	616 ± 323	538 ± 267	729 ± 261	600 ± 289	611 ± 301	658 ± 306

[1]Values are means ± SD (n = 20, placebo group; n = 22, low-ARA; n = 22, high-ARA). No significant differences between time points (Student's t-test) or groups (ANOVA and Tukey-Kramer test).

[2]SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; 18:2n-6, linoleic acid; 20:4n-6, arachidonic acid; 18:3n-3, α-linolenic acid; 20:5n-3, eicosapentaenoic acid; and 22:6n-3, docosahexaenoic acid.

the 4-week washout period (10.00 ± 1.39%) (Figure 2A). The time course of the ARA increase tended to be similar in the low-ARA group. The ARA content in the low-ARA group was 8.61 ± 0.92% at baseline, 11.30 ± 1.55% at 2 weeks, and 11.15 ± 1.52% at 4 weeks; the value declined during the washout period. The ARA content in the placebo group remained unchanged throughout the study.

In the high-ARA group, the LA content in plasma phospholipids inversely declined from 17.78 ± 2.62% at baseline to 14.10 ± 2.36% at 4 weeks and then increased almost to baseline levels during the 4 week washout period (17.41 ± 2.73%) (Figure 2B). The EPA and DHA contents in plasma phospholipids were unchanged throughout the study period in all groups (Figure 2C and 2D).

Clinical parameters of cardiovascular diseases and inflammation

Changes in clinical parameters associated with cardiovascular risk, allergy and inflammation are shown in Figure 3. Although some parameters the groups or time points differed significantly, all values were within the normal ranges and did not change according to ARA supplementation. Physiological, blood biochemical and hematological parameters were also within normal ranges and were unaffected by dose of ARA (Additional file 1, Table S1). Urinary findings were normal in all groups (data not shown).

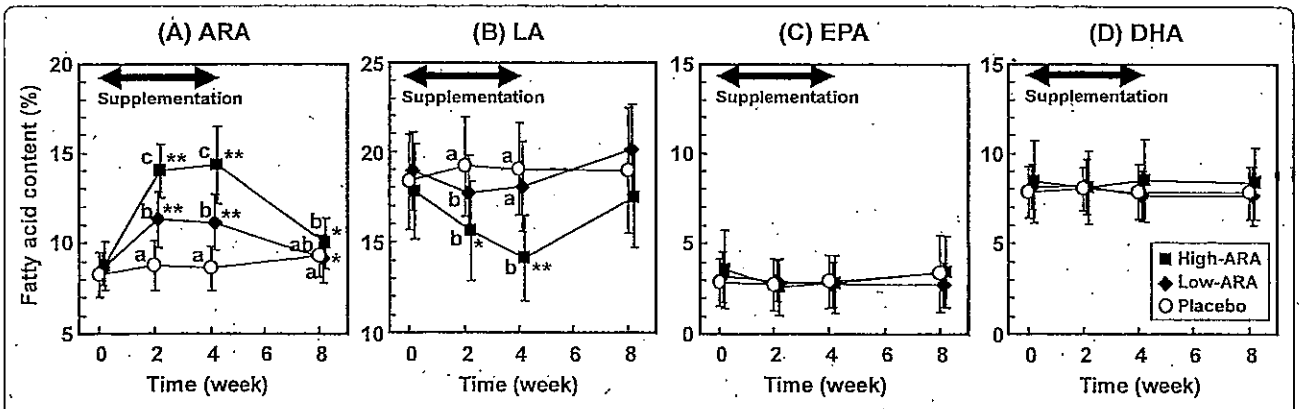

Figure 2 Fatty acid content in plasma phospholipids ((A) ARA, (B) LA, (C) DHA and (D) EPA) during 4-week supplementation and 4-week washout. Placebo, low-ARA and high-ARA groups are indicated by open circles, closed diamonds and closed squares, respectively. Values are means ± SD (n = 20 (placebo) and n = 22 (in each low- and high-ARA group)). *$p < 0.05$, **$p < 0.01$ vs. baseline in group (repeated ANOVA and Dunnett's test). $^{\#}p < 0.05$ vs. placebo group and $^{\$}p < 0.05$ vs. low-ARA group when amount of change from baseline significantly differed at each time point (ANOVA and Tukey-Kramer test).

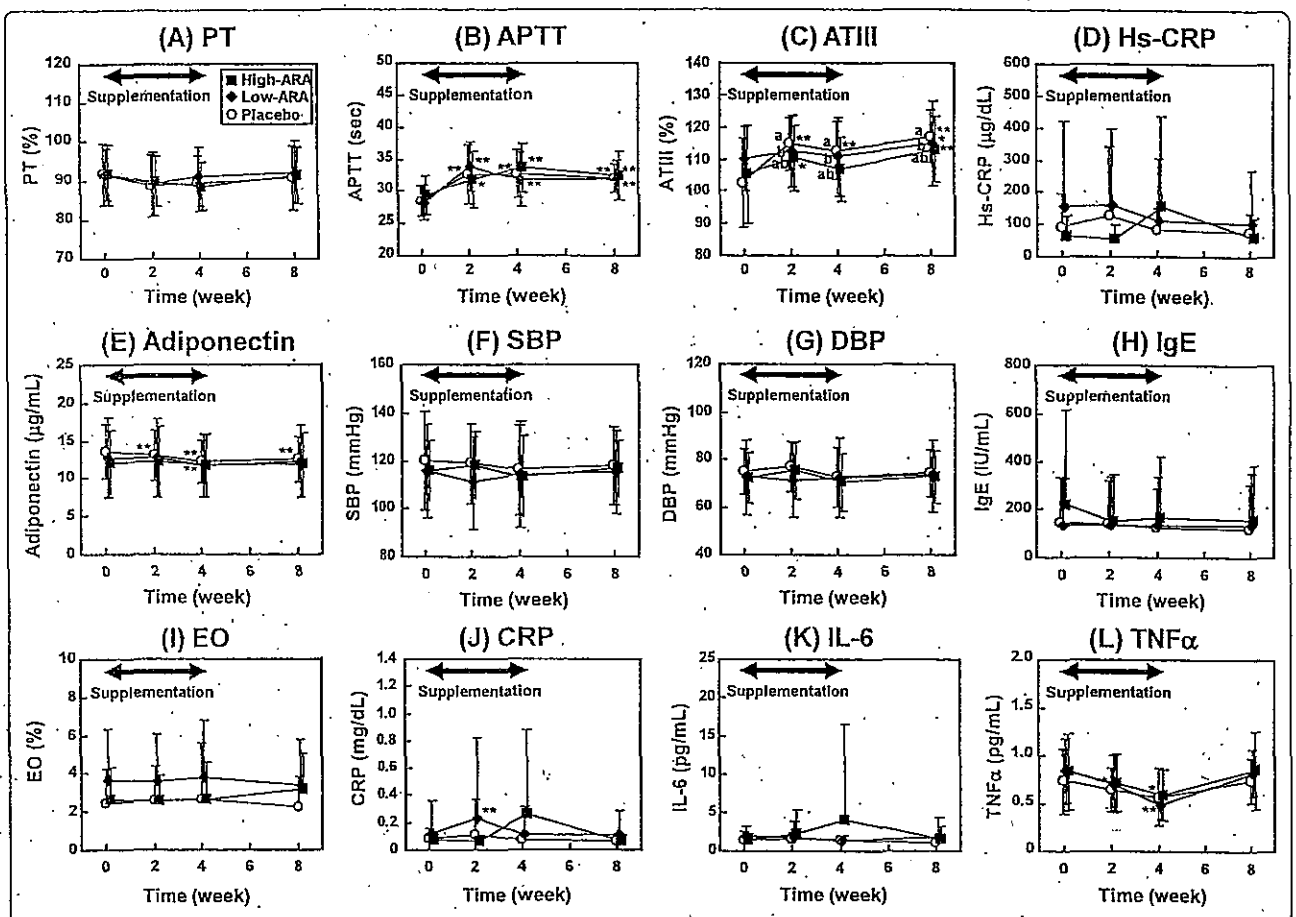

Figure 3 Plasma parameters for cardiovascular (A-G), allergic (H-I) and inflammatory (J-L) diseases during 4-week supplementation and 4-week washout. Placebo, low-ARA and high-ARA groups are indicated by open circles, closed diamonds and closed squares, respectively. Values are means ± SD (n = 20 (placebo) and n = 22 (in each low- and high-ARA group)). *$p < 0.05$, **$p < 0.01$ vs. baseline in group (ANOVA and Dunnett's test). Values without a common letter are significantly different at $p < 0.05$ (ANOVA and Tukey-Kramer test).

Levels of ARA metabolites in urine and blood

Concentrations of ARA metabolites in urine and blood are shown in Figure 4. Initial concentrations of urinary metabolites in the placebo, the low-ARA and the high-ARA groups were not significantly different. After the supplementation for 4 weeks, none of the urinary metabolites in the low-ARA and high-ARA groups significantly increased from initial levels; there were no differences among the groups at 2 and 4 weeks. At 4 weeks, the concentrations of 11-dehydro TXB_2, 2,3-dinor-6-keto $PGF_{1\alpha}$ and tetranor-PGEM in the low-ARA group were 0.160 ± 0.156 (Figure 4A), 20.6 ± 16.7 (Figure 4B) and 8.41 ± 3.63 ng/mg Cre, respectively (Figure 4C); those in high-ARA group were 0.222 ± 0.215 (Figure 4A), 16.4 ± 14.1 (Figure 4B) and 16.4 ± 14.1 ng/mg Cre, respectively (Figure 4C).

Plasma ARA-metabolites were also unchanged from the initial levels during the supplementation and showed no differences at 2 and 4 weeks among the three groups. The initial concentrations of PGE_2 in the placebo, the low-ARA and the high-ARA groups were 8.12 ± 1.96, 9.52 ± 2.48 and 10.9 ± 3.79 pg/mL, respectively (Figure 4D), and those of LXA_4 were 11.9 ± 10.8, 25.4 ± 50.0 and 19.9 ± 18.7 pg/mL, respectively (Figure 4E), respectively. After the supplementation for 4 weeks, the concentrations of PGE_2 and LXA_4 in the low-ARA group were 8.37 ± 2.40 (Figure 4C) and 20.4 ± 17.1 pg/mL, respectively (Figure 4E); those in the high-ARA group were 8.44 ± 2.33 (Figure 4C) and 17.7 ± 10.7 pg/mL, respectively (Figure 4E).

None of the ARA metabolites measured in urine and blood measured was correlated with ARA contents in plasma phospholipids at 4 weeks of ARA supplementation (Figure 5).

Discussion

The present study clarified that in spite of the increase in ARA levels in plasma phospholipids, plasma clinical parameters of cardiovascular, inflammatory and allergic diseases and levels of ARA metabolites in urine and

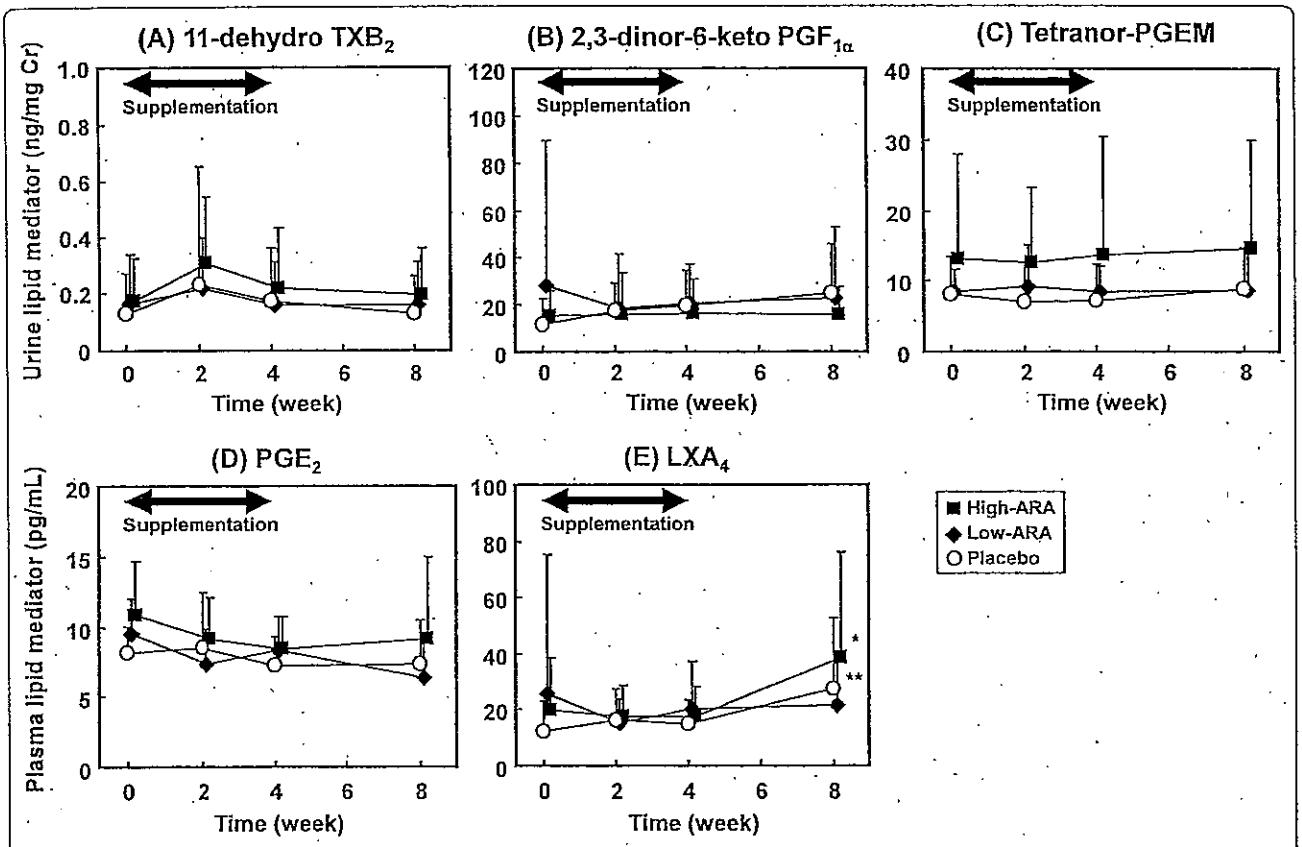

Figure 4 Change in ARA metabolites during 4-week supplementation and 4-week washout. (A) Urinary 11-dehydro TXB_2, (B) urinary 2,3-dinor-6-keto- $PGF_{1\alpha}$, (C) urinary tetranor-PGEM, (D) plasma PGE_2 and (E) plasma LXA_4. Placebo, low-ARA and high-ARA groups are indicated by open circles, closed diamonds and closed squares, respectively. Values are means ± SD (n = 20 (placebo) and n = 22 (in each low- and high-ARA group)). *$p < 0.05$, **$p < 0.01$ vs. baseline in group (ANOVA and Dunnett's test). Values without a common letter are significantly different at $p < 0.05$ (ANOVA and Tukey-Kramer test).

blood were not altered among the healthy elderly participants whose diet was supplemented with an ARA-enriched oil (240 or 720 mg/day ARA) for 4 weeks.

In previous studies, ARA content in plasma phospholipids increased by 0.7% in young females supplemented with 80 mg/day of ARA for 3 weeks [31], 2.0% in elderly males given 240 mg/day of ARA for 4 weeks [6], and by 4.3% in adult males given 838 mg/day of ARA for 4 weeks [23]. In the present study, supplementation with 240 and 720 mg/day of ARA increased ARA content by 2.54% and 5.56%, respectively (Figure 2), which was consistent with these findings. These results suggest that age or gender of participants has little effect of ARA supplementation on the increase in plasma ARA content. The results also suggest that the plasma ARA content increases dose-dependently with ARA up to at least around 800 mg/day. The ARA content in plasma phospholipids increased at 2 weeks and was almost the same between at 2 weeks and 4 weeks. The elevated ARA content decreased to almost the initial level during the 4-week washout period regardless of an intake of 240 mg/day or 720 mg/day of ARA. These results were similar to findings seen during intake of 838 mg/day of ARA [23]. The intake of ARA caused a rapid increase in plasma ARA levels but more than 2 weeks of supplementation did not result in any further increases. This is different from DHA, because the velocity of both increases and decreases in plasma DHA content seems slower when fish oil is administered and discontinued during a washout period [32]. The changes in the other fatty acids were also characteristic since plasma DHA and EPA contents remained unchanged throughout the period. The relatively high intake of DHA+EPA (approximately 1 g/day) in the present study (Table 3) might also have contributed to maintaining plasma DHA and EPA levels. The plasma LA content changed in parallel with the plasma ARA content (Figure 2). A previous study also noted this phenomenon [23]. Although ARA and DHA are considered to compete against each other, ARA intake does not reduce plasma DHA content, whereas intake of DHA and EPA reduces both plasma ARA and LA contents [32,33]. The

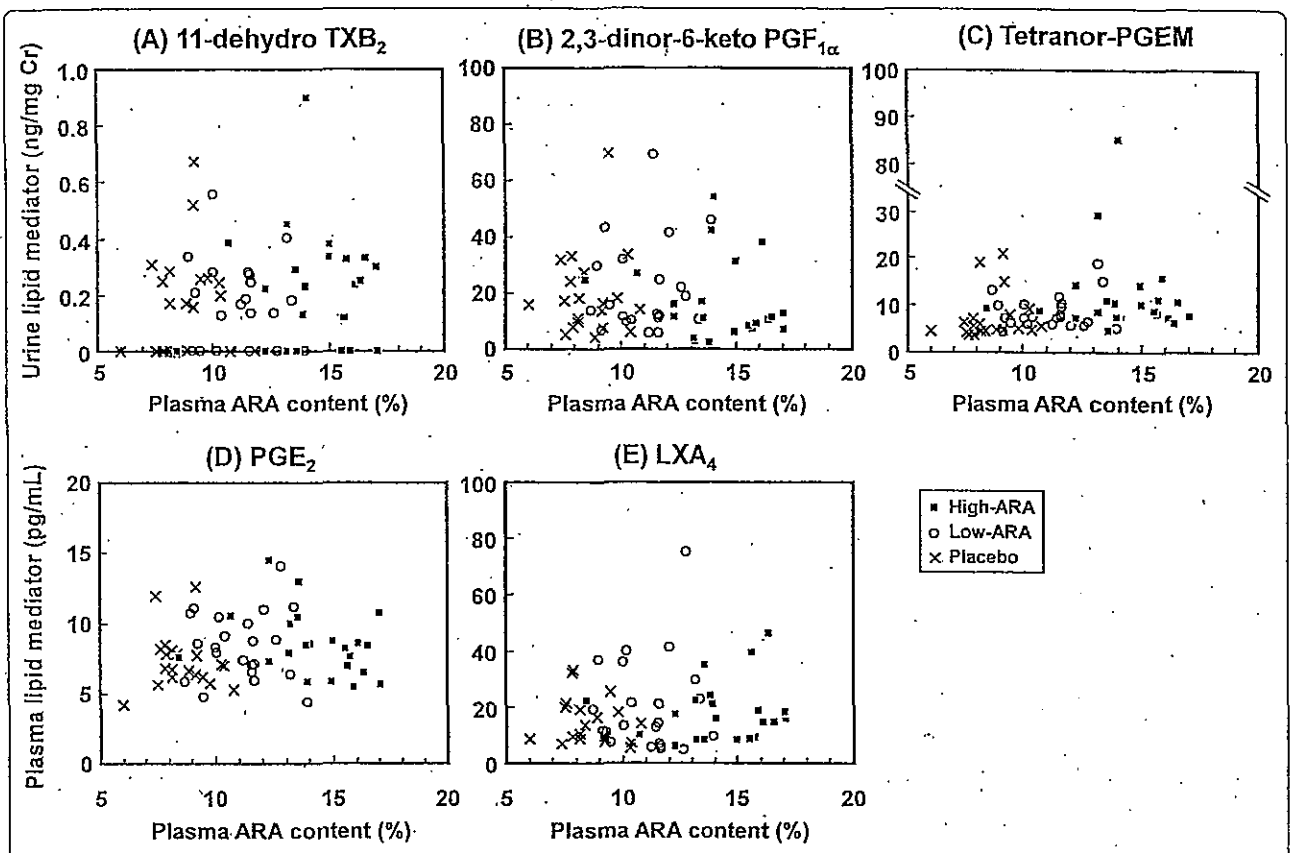

Figure 5 Correlation between plasma ARA content and ARA metabolites in blood and urine. (A) Urinary 11-dehydro TXB_2, (B) urinary 2,3-dinor-6-keto- $PGF_{1\alpha}$, (C) urinary tetranor-PGEM, (D) plasma PGE_2 and (E) plasma LXA_4 after 4-week supplementation. Placebo, low-ARA and high-ARA groups are shown as crosses (n = 20), open circles, (n = 22) and closed squares (n = 22). No correlations were identified in any of these analyses (A)-(E).

specificity of incorporation into plasma phospholipids from dietary fatty acids seems to be in the order of DHA, EPA > ARA > LA. The reason for the difference between ARA and DHA incorporation is unclear, but the specificity of some enzymes associated with acylation and/or deacylation of each fatty acid might be involved.

Platelet aggregation remains unaffected by an ARA intake of 1.5 g/day or 838 mg/day in randomized controlled studies [21,23]. The present study found that parameters of the coagulation system (PT, APTT and ATIII) remained unaltered and within the normal range. Parameters of chronic inflammation such as plasma hs-CRP, TNFα and IL-6, which are risk factors for cardiovascular disease, remained unchanged, as did plasma adiponectin, which is thought to reduce the risk for cardiovascular diseases. These results suggest that ARA intake does not affect the risk for cardiovascular disease. The parameters for inflammatory diseases (CRP, TNFα and IL-6) and allergic diseases (IgE and eosinophil) were similarly unchanged, suggesting that ARA intake does not evoke inflammatory or allergic diseases. Furthermore, general blood biochemical and hematological parameters remained within normal ranges (Additional file 1, Table S1). Thus, ARA intake appears to be safe under the conditions described here.

In this study, we measured TXA_2, PGI_2, PGE_2 and LXA_4. TXA_2 causes platelet aggregation and vasoconstriction, which are considered to lead to cardiovascular disease, PGI_2 competes against TXA_2 and suppresses cardiovascular disease and PGE_2 has various physiological roles, one of which is an inflammatory trigger in addition to possible involvement in cancer growth. LXA_4 has effects opposite to PGE_2, and reduces inflammation and cancer growth [34]. Considering their association with diseases, we estimated TXA_2 and PGI_2 production as urinary 11-dehydro TXB_2 and 2,3-dinor-6-keto $PGF_{1\alpha}$, respectively. The reported urinary concentration of 11-dehydro TXB_2 is 1.489 ng/mg Cre in patients with heart failure, 0.632 ng/mg Cre in those with ischemic heart disease, 0.44 ng/mg Cre in healthy controls [15] and around 0.6 ng/mg Cre in patients with essential hypertension and retinopathy [16]. The mean concentration of 11-dehydro TXB_2 throughout the

present study was < 0.4 ng/mg Cre, which was lower than the levels in these patients and did not significantly differ among the groups. Urinary 2,3-dinor-6-keto $PGF_{1\alpha}$ was similarly unchanged and did not differ significantly among the groups. These results show that an increase in the ARA content of plasma phospholipids from 8% to 14% did not affect TXA_2 and PGI_2 contents. This is consistent with the finding that parameters for cardiovascular disease did not change. However, these findings seem to differ from those of a previous study in which both urinary 11-dehydro TXB_2 and 2,3-dinor-6-keto $PGF_{1\alpha}$ were slightly increased by intake of 1.5 g ARA/day for 50 days [22]. The larger dose and longer study duration might explain the discrepancies between that study and ours, but the actual reason for the difference remains unclear.

Several reports have described that plasma PGE_2 increases in inflammatory diseases. For instance, the plasma PGE_2 concentration increases to > 40 pg/mL in patients with ulcerative colitis [17], and to 54.5 pg/mL in patients with advanced periodontitis [18] compared with about 10 pg/mL in controls. The plasma PGE_2 concentration in the present study was about 10 pg/mL, which was below the values associated with inflammatory diseases. This value did not change or significantly differ throughout the study. Urinary concentrations of tetranor-PGEM, another marker of PGE_2 production, are higher in patients with cancer. The reported level is 11.6 ng/mg Cre in patients with colorectal cancer and 7.0 ng/mg Cre in matched controls [19]. Another study indicated a urinary tetranor-PGEM concentration of 15.0 ng/mg Cre in patients with colorectal cancer and 7.17 ng/mg Cre in polyp-free controls [35]. The mean concentration of tetranor-PGEM in the present study was 8 ng/mg Cre in the placebo and the low-ARA group, and 14 ng/mg Cre in the high-ARA group which seems relatively higher compared with normal levels reported previously. The high level of tetranor-PGEM in the high-ARA group was due to the three participants with levels > 30 ng/mg Cre. The higher levels in these participants were not changed by ARA supplementation or during the washout period. Thus, tetranor-PGEM concentration was not significantly changed by ARA supplementation, indicating that ARA intake does not affect a candidate marker of colorectal cancer. Plasma LXA_4 in the high-ARA groups was significantly increased after the washout period (Figure 4E). It was considered to be unrelated to ARA supplementation because the increase was also observed in the placebo group. Plasma LXA_4 level may be more variable compared to the other metabolites, but the details are unclear. The increase was slight and considered not to affect the state of the participants.

Next, we analyzed correlations between plasma ARA content and ARA metabolites concentrations after 4 weeks of ARA administration (Figure 5). Concentrations of each ARA metabolite were distributed across a wide range, although all participants were healthy volunteers. None of the values correlated with plasma ARA content or ARA dose. Several individual values were high, but these values seemed normal for these patients, because the high levels in those participants were not changed at baseline and at 2 and 8 weeks. These results indicate that the increase in plasma ARA content from 8% to 14% does not increase the production of TXA_2, PGI_2, PGE_2 and LXA_4. The production of lipid mediators is not determined primarily by ARA content and seems to be controlled by other factors.

Diet was assessed to ensure that dietary intake of ARA and related fatty acids did not differ among the groups or as a result of the intervention. Participants consumed 170-200 mg/day of ARA from daily meals (Table 3), which is within the normal reported range [1-3] and values did not differ among the groups or with time. The intakes of DHA and EPA were 300-500 mg/day and 500-800 mg/day, respectively, and also did not differ among the groups. Although these values are within the common range in Japan, they are much more than those in Western countries ([2], [36]). Energy and macronutrient intake did not differ among the groups (Table 3), and did not seem to affect the present data.

The effects of ARA supplementation on healthy elderly were clarified here, but studies of patients with specific diseases are needed. In summary, blood parameters of cardiovascular, inflammatory and allergic diseases, as well as urinary and plasma ARA metabolites did not change in Japanese healthy elderly participants who consumed ARA-enriched oil (240 or 720 mg/day of ARA) for 4 weeks although plasma ARA levels significantly increased.

Additional material

Additional file 1: Supplemental Table S1. Physiological parameters and blood biochemical and hematological parameters

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Authors' contributions
SK participated in the study design, measured lipid mediators and drafted the manuscript. YI and NT participated in the study design and acquired data. CH carried out the nutrition survey. HT and MK measured blood fatty acids. HK participated in the study design and drafted the manuscript. YS, YK and HS participated in the study design and helped to interpret the

findings. IM participated in the study design and reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests

SK, YI, NT, CH, HT, MK, HK, YS, YK and HS are employees of Suntory Wellness Ltd. or Suntory Business Expert Ltd., which is a manufacturer of foods including ARA-enriched edible oil. IM has consultancy relationships with Suntory Wellness Ltd.

Received: 6 December 2011 Accepted: 22 December 2011
Published: 22 December 2011

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doi:10.1186/1476-511X-10-241
Cite this article as: Kakutani *et al.*: Supplementation of arachidonic acid-enriched oil increases arachidonic acid contents in plasma phospholipids, but does not increase their metabolites and clinical parameters in Japanese healthy elderly individuals: a randomized controlled study. *Lipids in Health and Disease* 2011 10:241.

Table S1 Physiological parameters and blood biochemical and hematological parameters[1]

Parameters[2]		Group	Supplementation 0w	2w	4w	Washout 8w
Physiological parameters						
Body weight	kg	Placebo	55.7±9.7	55.7±9.7	55.7±9.6	56.0±9.5
		Low-ARA	56.7±8.4	56.9±8.5	56.9±8.7	57.2±8.6*
		High-ARA	58.8±11.6	59.0±11.4	58.9±11.4	59.0±11.4
BMI	kg/m^2	Placebo	21.6±2.5	21.6±2.5	21.6±2.4	21.7±2.3
		Low-ARA	22.5±2.0	22.6±2.1	22.6±2.1	22.7±2.1
		High-ARA	22.5±3.2	22.6±3.2	22.6±3.1	22.6±3.2
Pulse rate	bpm	Placebo	70±12	73±10	72±10	72± 9
		Low-ARA	71±11	73±13	73±11	71±10
		High-ARA	72±9	76±10	72±10	73±10
Blood biochemical parameters						
TP	g/dL	Placebo	7.1±0.4	7.1±0.3	7.1±0.4	7.1±0.4
		Low-ARA	7.2±0.3	7.1±0.2	7.1±0.3	7.1±0.3
		High-ARA	7.2±0.3	7.3±0.3	7.1±0.3*	7.2±0.4
ALB	g/dL	Placebo	4.5±0.2	4.4±0.2	4.3±0.3**	4.4±0.3
		Low-ARA	4.5±0.2	4.4±0.2	4.3±0.2**	4.4±0.2*
		High-ARA	4.5±0.1	4.4±0.1**	4.3±0.2**	4.4±0.2**
T-BIL	mg/dL	Placebo	0.79±0.29	0.78±0.29	0.79±0.30	0.73±0.22
		Low-ARA	0.87±0.36	0.88±0.32	0.85±0.35	0.83±0.26
		High-ARA	0.82±0.29	0.81±0.28	0.90±0.29	0.79±0.24
ALP	IU/L	Placebo	227±82	213±68**	218±70*	227±70
		Low-ARA	226±54	215±56	224±56	238±51
		High-ARA	212±61	204±55	208±60	215±57
AST	IU/L	Placebo	21±4	20±4	20±3	21±4
		Low-ARA	21±4	21±4	21±3	23±8
		High-ARA	22±3	20±3	22±4	22±3
ALT	IU/L	Placebo	18±7	17±6	16±4*	17±6
		Low-ARA	19±10	20±11	18±10	22±13
		High-ARA	20±6	20±5	21±7	19±5
LDH	IU/L	Placebo	186±32	190±33	186±30	184±24
		Low-ARA	196±24	193±26	193±25	190±25
		High-ARA	203±26	197±25	198±20	192±21*
γ-GTP	IU/L	Placebo	28±26	27±26	26±21*	26±18*
		Low-ARA	29±25	30±27	28±24	32±25
		High-ARA	25±12	23±10	29±21	24±14
CPK	IU/L	Placebo	111±39	112±40	103±33	109±35
		Low-ARA	118±55	123±54	118±50	131±70
		High-ARA	131±64	116±40	130±61	118±53
T-CHO	mg/dL	Placebo	223±28	227±30	218±29	220±25
		Low-ARA	218±34	220±31	214±34	216±33
		High-ARA	218±29	219±26	215±30	216±33
TG	mg/dL	Placebo	120±41	112±45	97±36*	87±29**
		Low-ARA	111±58	96±44	99±50	96±57
		High-ARA	85±35	91±70	74±34	86±52
HDL-CHO	mg/dL	Placebo	59±14	62±18*	62±16*	64±19**
		Low-ARA	62±15	64±16*	64±17*	66±16**
		High-ARA	65±15	67±16	67±15	67±14
LDL-CHO	mg/dL	Placebo	134±24	137±22	133±24	128±23
		Low-ARA	128±27	129±25	123±24	122±26
		High-ARA	127±29	121±27*	121±28*	120±30*
GLU	mg/dL	Placebo	93±9	93±9	93±10	90±9**
		Low-ARA	94±6	94±7	94±6	93±9
		High-ARA	95±10	95±11	96±12	94±13

PL	mg/dL	Placebo	235±28	239±29	228±29	231±26
		Low-ARA	236±31	230±30	228±31	232±33
		High-ARA	229±25	233±23	230±30	229±28
BUN	mg/dL	Placebo	13.7±3.5	13.8±3.3	13.8±3.3	13.9±3.0
		Low-ARA	15.0±3.5	14.6±3.6	13.3±3.1**,#	13.9±3.0*
		High-ARA	14.4±2.5	14.6±3.8	13.1±2.9*	14.1±2.5
CRE	mg/dL	Placebo	0.72±0.17	0.71±0.17	0.68±0.16*	0.69±0.17
		Low-ARA	0.68±0.14	0.67±0.18	0.65±0.15*	0.67±0.15
		High-ARA	0.69±0.15	0.69±0.16	0.66±0.16*	0.70±0.17
UA	mg/dL	Placebo	4.9±1.4	5.1±1.5	5.0±1.3	4.7±1.4
		Low-ARA	4.9±1.3	5.0±1.6	4.8±1.2	4.7±1.3
		High-ARA	4.8±0.9#	4.6±0.9	4.6±0.9	4.7±0.9
Na	mEq/L	Placebo	140±2	141±1**	141±1**	141±1**
		Low-ARA	139±3	141±1**	141±2**	141±1**
		High-ARA	139±3	141±1**	141±1**	141±1**
K	mEq/L	Placebo	4.44±0.50	4.38±0.33	4.55±0.43	4.51±0.37
		Low-ARA	4.41±0.35	4.50±0.37	4.56±0.44*	4.63±0.48**
		High-ARA	4.37±0.32	4.40±0.30	4.49±0.40	4.70±0.56**
Cl	mEq/L	Placebo	102±1	102±1*	103±1**	103±1**
		Low-ARA	102±2	103±1	103±2*	103±1*
		High-ARA	101±2	102±1*	103±1**	103±1**
Ca	mg/dL	Placebo	9.62±0.34	9.36±0.45**	9.53±0.36	9.45±0.30*
		Low-ARA	9.50±0.27	9.39±0.23	9.45±0.25	9.45±0.28
		High-ARA	9.59±0.23	9.39±0.29**	9.49±0.25	9.51±0.29
Hematological parameters						
WBC	$10^3/\mu L$	Placebo	4.92±1.49	4.81±1.13	5.22±1.74	5.19±1.34
		Low-ARA	4.80±1.13	4.94±1.57	5.17±1.44	5.14±1.27
		High-ARA	4.73±1.18	5.01±1.42	5.75±1.90**	5.26±1.36
RBC	$10^4/\mu L$	Placebo	437±39	433±35	433±39	442±45
		Low-ARA	434±29	420±41**	426±32	436±37
		High-ARA	445±45	433±49**	434±45**	444±47
HGB	g/dL	Placebo	13.1±1.4	13.1±1.3	13.1±1.5	13.4±1.6**
		Low-ARA	13.1±0.8	13.0±1.1	13.1±0.9	13.4±0.9*
		High-ARA	13.4±1.3	13.3±1.4	13.3±1.3	13.6±1.3
HCT	%	Placebo	40.5±4.2	40.5±3.4	40.2±4.0	41.1±4.2
		Low-ARA	41.1±2.6	40.0±3.7*	40.4±2.7	41.5±2.9
		High-ARA	41.5±3.7	40.9±4.1	40.9±4.2	41.7±4.0
MCV	fL	Placebo	92.9±6.6	93.7±6.7*	93.3±7.3	93.4±7.2
		Low-ARA	94.9±4.7	95.3±4.4	95.1±4.1	95.6±4.1*
		High-ARA	93.4±3.9	94.8±4.0**,$	94.4±4.1**	94.3±3.8**
MCH	pg	Placebo	30.1±2.5	30.4±2.5*	30.3±2.7*	30.5±2.6**
		Low-ARA	30.3±1.4	31.1±1.6**,#	30.8±1.4**	30.8±1.4**
		High-ARA	30.2±1.6	30.7±1.5**	30.6±1.6**	30.6±1.5**
MCHC	%	Placebo	32.4±0.9	32.4±0.8	32.5±0.7	32.6±0.8
		Low-ARA	32.0±0.6	32.7±0.5**,#	32.4±0.5**	32.3±0.6**
		High-ARA	32.3±0.8	32.4±0.7$	32.5±0.7	32.5±0.8
PLT	$10^4/\mu L$	Placebo	22.5±4.2	23.4±4.3	23.4±4.6	24.7±5.3**
		Low-ARA	22.5±4.1	22.8±4.3	22.8±4.8	24.5±5.4**
		High-ARA	21.8±3.7	22.2±4.0	22.2±4.3	23.6±3.9**
NEUT	%	Placebo	61±8	64±6	63±8	62±10
		Low-ARA	60±10	62±8	62±7	61±9
		High-ARA	57±9	61±9	60±9	59±9
BASO	%	Placebo	0.3±0.6	0.4±0.6	0.4±0.8	0.3±0.5
		Low-ARA	0.5±0.7	0.6±0.9	0.3±0.6	0.3±0.6
		High-ARA	0.4±0.6	0.4±0.7	0.1±0.3	0.3±0.6
MONO	%	Placebo	4.8±1.2	4.9±1.6	4.3±1.2	5.3±1.4

		Low-ARA	4.8±1.3	4.9±1.2	4.7±1.0	5.0±1.4
		High-ARA	5.2±1.5	5.4±1.4	5.5±1.0	5.1±1.5
LYMPH	%	Placebo	31±8	29±6	30±7	31±9
		Low-ARA	31±9	29±8	29±6	30±7
		High-ARA	35±9	31±9	31±8	32±8

[1]Values are means ± SD (n = 20, placebo group; n = 22, low-ARA; n = 22, high-ARA). * $p < 0.05$, ** $p < 0.01$ versus baseline in the group (ANOVA and Dunnett's test). [#] $p < 0.05$ versus placebo group and [$] $p<0.05$ versus low-ARA group in the case that the amount of change from baseline is significantly different in each time point (ANOVA and Tukey-Kramer test).

[2]TP, total protein; ALB, albumin; T-Bil, total bilirubin; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; γ-GTP, γ-glutamyl transpeptidase; CPK, creatine phosphokinase; T-CHO, total cholesterol; TG, triglycerides; LDL-CHO, low-density lipoprotein cholesterol; GLU, glucose; PL, phospholipids; BUN, blood urea nitrogen; CRE, creatinine; UA, uric acid; WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit. MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; PLT, platelets; NEUT, neutrophils; BASO, basophils; MONO, monocytes; LYMPH, lymphocytes.

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Regulatory Toxicology and Pharmacology 49 (2007) 271–284
Regulatory Toxicology and Pharmacology
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Genotoxicity and subchronic toxicity studies of DHA-rich oil in rats

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Received 10 January 2007
Available online 29 August 2007

Abstract

Polyunsaturated fatty acids, including docosahexaenoic acid (DHA), are natural constituents of the human diet. DHA-algal oil is produced through the use of the non-toxigenic and non-pathogenic marine protist, *Ulkenia* sp. The safety of DHA-algal oil was assessed in a subchronic toxicity study and in genotoxicity studies. In a 90-day study, rats were orally administered water or DHA-algal oil at concentrations of 0, 500, 1000, and 2000 mg/kg in combination with 2000, 1500, 1000 or 0 mg/kg DHA-containing fish oil, respectively. Additional animals were administered water, 2000 mg/kg DHA-algal oil, or 2000 mg/kg fish oil for 90 days, followed by a 4-week recovery phase. No treatment-related effects were observed in clinical observations, food and water consumption, mortality, gross pathology, and histopathology. Increased body weights and liver weights in oil-treated groups were attributed to the large lipid load and were not regarded as toxicologically significant. Furthermore, no treatment-related differences in the measured parameters between the DHA-algal oil and fish oil groups were detected. In genotoxicity experiments, DHA-algal oil exerted no mutagenic activity in various bacterial strains, nor did it induce chromosomal aberrations in Chinese hamster fibroblast cells. These results support the safety of DHA-algal oil as a dietary source of DHA.

Keywords: DHA-rich oil; ω-3 polyunsaturated fatty acid; Docosahexaenoic acid; DHA; Toxicity; Safety; Subchronic; Genotoxicity; Food ingredient; Marine protist

1. Introduction

Polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA), are natural components of the diet, but dietary consumption of these fatty acids is generally below recommended values. The dietary sources of PUFAs include fatty fish, fish oils, shellfish, marine mammals, and organ meats (Connor, 1997). Recently, various marine microalgae have also been identified as source organisms of DHA-containing oils.

DHA-rich oil, hereafter referred to as DHA-algal oil is a refined food grade oil that is derived from *Ulkenia* sp. SAM2179, a thraustochytrid microalgae. It is manufactured through a multi-step fermentation process and a standard edible oil refining method. DHA-algal oil contains approximately 45% (w/w) of DHA, as well as smaller amounts of palmitic acid (~35%) and the PUFA docosapentaenoic acid (DPA) (~11%). The PUFAs identified in DHA-algal oil are similar to those in fish oils and other microalgal oils already consumed by the human population, such as menhaden oil and docosahexaenoic acid-rich microalgal oil from *Schizochytrium* sp., respectively (U.S. FDA, 1997; Hammond et al., 2001a). Palmitic acid is also a natural constituent of the diet, and is found primarily in meat, poultry, fish, grain products, milk, and milk products (Jonnalagadda et al., 1995).

In the United States, DHA-algal oil is intended for use as a nutritional food ingredient and as a dietary source of DHA in various food products. The mean and 90th percentile intakes of DHA-algal oil under the conditions of

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0273-2300/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.yrtph.2007.08.005

intended use are estimated to be 1.3 and 3.0 g/person/day, corresponding to 0.7 and 1.5 g DHA/person/day (Kroes et al., 2003).

Several studies have been conducted in animals to evaluate the potential toxicity of fish oils that have a similar composition and DHA content as DHA-algal oil. Generally, oral administration of these oils to mice, rats, and pigs at dietary concentrations up to 15% is well tolerated, with no treatment-related adverse effects on mortality, body weight gains, food consumption or clinical observations (Danse and Verschuren, 1978a; Ruiter et al., 1978; Willumsen et al., 1993; Hempenius et al., 1997; Rabbani et al., 1999; Hempenius et al., 2000; Oarada et al., 2000).

In some studies, microscopic evidence of yellow fat disease in animals treated with DHA-containing fish oil was reported; however, this mainly occurred in cases where the dietary vitamin E content was low (Danse and Verschuren, 1978a,b; Ruiter et al., 1978; Charnock et al., 1987; Verschuren et al., 1990; Farwer et al., 1994). Yellow fat disease is a disorder that is known to occur naturally in wildlife and domestic species, as well as in various laboratory animals administered diets rich in ω-3 PUFAs combined with a vitamin E deficient state (Jones et al., 1969; Helgebostad and Ender, 1973; Danse and Steenbergen-Botterweg, 1978; Danse et al., 1979).

Organ weight changes in the liver and spleen of rats treated with high doses of fish oil containing DHA have also been reported (Danse and Verschuren, 1978a; Hempenius et al., 1997, 2000; McGuire et al., 1997; Rabbani et al., 1999; Oarada et al., 2000). However, no histopathological effects were observed in these organs, and the increases in organ weights were hypothesized to be physiological adaptations to accommodate the large dietary lipid load.

In addition to studies conducted on DHA-containing fish oils, a number of acute, subchronic, reproductive, and developmental toxicity studies have been conducted with DHA-containing oils produced by marine algae, including *Crypthecodinium cohnii* and *Schizotrichium* sp. (Boswell et al., 1996; Wibert et al., 1997; Burns et al., 1999; Arterburn et al., 2000a,b; Hammond et al., 2001a,b,c). Overall, no adverse effects attributable to DHA-containing oil treatment were reported. Increased spleen (Boswell et al., 1996) and liver (Burns et al., 1999) weights in animals treated with DHA-containing oil were reported in some studies, as well as increased hepatic vacuolization due to accumulation of lipids and increased cardiomyopathy primarily in male rats (Duthie et al., 1988; Burns et al., 1999). These changes are considered adaptive rather than adverse as they are commonly observed in rats fed vegetable and fish oils (Burns et al., 1999).

In the present study, the safety of DHA-algal oil, in terms of subchronic toxicity and potential genotoxic activity, was assessed. A 90-day toxicity study comparing the effects of DHA-algal oil, a fish oil containing 27% DHA (DHA-fish oil), or combinations of the two oils was conducted in rats, and an Ames assay and chromosomal aberration test were employed to evaluate genotoxicity.

2. Materials and methods[1]

2.1. Materials

DHA-algal oil (99.9% pure lipid) was composed of DHA (45% w/w), palmitic acid (~35%), DPA (ω-6) (~11%), and contained 0.3–2.9% each of tetradecanoic acid (myristic acid) (14:0), pentadecanoic acid (15:0), heptadecanoic acid (17:0), octadecanoic acid (18:0), eicosatetraenoic acid (20:4, ω-6), eicosatetraenoic acid (20:4, ω-3), and DPA (ω-3). The unsaponifiable fraction of DHA-algal oil consisted primarily of sterols at a level below 1%. The three main sterols identified in DHA-rich oil were: cholesterol, 24-ethyl-cholesta-5,7,22-trien-3-ol, and 4-methyl-24-ethyl-cholesta-7,22-dien-3-ol. Vitamin E was present in the oil at a level of 0.1%. Purity was determined using The American Oil Chemists' Society Official Methods.

The source of food-grade DHA-fish oil in the subchronic toxicity study was tuna, produced by Maruha Co. (Tokyo, Japan). It was composed of the following fatty acids: 26.7% DHA (22:6n-3), 11.4% EPA (20:5 ω-3), 10.7% palmitic acid (16:0), 3.3% myristic acid (14:0), 2.5% arachidonic acid (20:4 ω-6), 2.1% DPA (22:5 ω-3), 1.4% DPA (22:5 ω-6), and 41.9% other fatty acids. The oil also contained 0.3% vitamin E.

In the subchronic toxicity study, DHA-algal oil was packed in vials at acquisition and stored at −20 °C (and at −80 °C for 4 days at the beginning of treatment) as the oil is semi-solid at room temperature and is easily oxidized in contact with air. The oil was analyzed for peroxide value and DHA content using gas chromatography and iodometry methods at the beginning and completion of the study. Levels remained constant over the duration of the study (data not shown).

2.2. Subchronic toxicity study[2]

2.2.1. Animals

Male and female Sprague–Dawley Crj:CD (SD) IGS rats were obtained from Atgusi Breeding Center, Charles River Japan (Kanagawa-Ken, Japan) at 5 weeks of age, and quarantined and acclimatized for 12 days. Animals were assessed for health conditions and allocated to groups by a stratified random sampling method based on body weight. Rats were housed individually in stainless bracket cages and provided food (AIN-76A compound feed, Oriental Yeast Co., Ltd., Tokyo, Japan) and water (household tap water) *ad libitum*. AIN-76A compound feed contained 55.0% (w/w) corn starch, 20.0% casein, 10.0% sucrose, 4.0% cellulose powder, 5.0% corn oil, 3.5% AIN-76A mineral mix, 1.0% AIN-76A vitamin mix [containing 0.5% vitamin E], 0.3% DL-methionine, and 0.2% choline bitartrate. During the study, animals were housed in a facility designed to maintain appropriate environmental conditions (19–25 °C, 12-h light/dark cycle, 30–70% humidity, ventilation frequency of ⩾10 times/h).

2.2.2. Animal treatment

Groups of 15 male and 15 female rats were administered, by daily oral gavage for 90 days, distilled water or various combinations of DHA-algal oil and DHA-fish oil (99.7% purity). DHA-fish oil was included as a source of DHA (control for the higher level of PUFA/DHA level in the diet), as well as a control for the higher level of fat consumed by the rats, while the water control was used as a normal fat control. The combinations of DHA-algal oil/DHA-fish oil were 0/2000 (DHA-fish oil group), 500/1500 (500 mg/kg group), 1000/1000 (1000 mg/kg group), and 2000/0 mg/kg body weight/day (2000 mg/kg DHA-algal oil group). These dosing regimens provided daily doses of 540, 630, 720, and 900 mg DHA/kg

[1] Studies were conducted in accordance with the OECD Principles of Good Laboratory Practice (GLP) (Organisation for Economic Co-operation and Development, OECD Principles of Good Laboratory Practice (as revised in 1997)), Paris. ENV/MC/CHEM(98)17 (OECD, 1998).

[2] The subchronic toxicity study was conducted at Nippon Experimental Medical Research Institute Co., Ltd., Japan.

body weight, respectively. Additional groups of 10 rats (5/sex/group) were administered distilled water or 2000 mg DHA-algal oil or DHA-fish oil/kg body weight/day for 90 days and were allowed a 4-week recovery period. All dose groups received a total DHA-algal oil plus DHA-fish oil dose of 2000 mg/kg body weight/day to equalize lipid content. The estimated increase in fat intake in the oil-treated animals compared to water-treated controls was approximately 67% in males and 58% in females. The dosing regimens and corresponding doses are summarized in Table 1.

2.2.3. Animal observations

Animals were observed for clinical signs, including mortality and morbundity, twice daily during the treatment period (once before and once after treatment), and once daily during the recovery period. Body weights were measured prior to treatment on the first day of treatment and once weekly during the treatment and recovery periods. Food and water consumption measurements were taken once at pre-treatment and once weekly during the treatment and recovery periods.

Neurotoxicological observations were made once before treatment commenced and once weekly during the treatment period. Functional tests (reactivity to stimuli, grip strength, and spontaneous movement) were conducted on 10 rats of each sex in week 13 of treatment. Spontaneous movement was assessed using an automated activity box (Animex-auto MK 110, Muromachi Kikai Co., Ltd., Tokyo, Japan). Due to the lack of treatment-related abnormalities observed, neurotoxicological observations and functional tests were not performed for the recovery groups.

Ophthalmoscopic examinations of the anterior portion of the eye, the optic media and the ocular fundus following dilation of the pupil with a mydriatic agent were performed once before treatment commenced, in week 13 of treatment, and in week 4 of recovery.

2.2.4. Urinalysis

At the end of the treatment or recovery periods, animals were administered 5 ml of water by oral gavage and transferred to metabolic cages. Fresh urine was collected for approximately 3 h with food and water withheld. Urine was also continuously collected for approximately 17 h (cumulative urine) with food withheld but water allowed. For fresh urine, occult blood, ketone bodies, glucose, protein, pH, urobilinogen, and bilirubin were assayed with Multistix reagent strips (Beyer-Sankyo Co., Ltd.). Sediment (optical microscope), and color (color cards, standard color cards 230, Nippon Shiki-ken Jigyo KK) were also examined. For cumulative urine, urine volume (measured in graduated cylinders), specific gravity (clinical reflectometer A-405 A, Elma Co., Ltd.), sodium, potassium, and chloride (ion-selective electrode, 7070 autoanalyzer, Hitachi Co., Ltd.) were examined.

Table 1
Summary of dosing regimen in subchronic toxicity study

Group No.	Number animals/ group	Dose of DHA-fish oil (mg/kg)	Dose of DHA-algal oil (mg/kg)	Total dose of DHA (mg/kg)
90-day dosing period only				
Control	30	0	0	0
DHA-fish oil	30	2000	0	540
500 mg/kg DHA-algal oil	30	1500	500	630
1000 mg/kg DHA-algal oil	30	1000	1000	720
2000 mg/kg DHA-algal oil	30	0	2000	900
90-day dosing period and additional 4-week recovery period				
Control	10	0	0	0
DHA-fish oil	10	2000	0	540
DHA-algal oil	10	0	2000	900

2.2.5. Hematology and serum/plasma chemistry

Prior to necropsy, while the animals were under ether anesthesia, blood samples for hematology and clinical chemistry were collected from the abdominal aorta. In EDTA-2K-treated blood, hematology parameters measured using an Automated Multiple Hematoanalyzer K-4500 (Sysmex Co., Ltd.) included erythrocyte counts (RBC), white blood cell counts (WBC), platelet counts, hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCH), and mean corpuscular hemoglobin concentration (MCHC). Differential leukocyte percentage and reticulocyte ratio were measured using an automated hematocyte analyzer (HEG-120 A, Omron Co., Ltd.). In sodium citrate-treated blood, prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured using an automated hematocoagulation analyzer (KC-4A, Amelung Co., Ltd.).

Blood collected for serum chemistry was treated with heparin sodium or untreated and centrifuged at 3000 rpm at 15 °C for approximately 10 min. The serum obtained was examined for the following parameters using a 7070 automatic analyzer (Hitachi Co., Ltd.): glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), total protein, albumin, glucose, total cholesterol, triglyceride, phospholipids, free fatty acid, total bilirubin, blood urea nitrogen (BUN), creatinine, total bile acids, inorganic phosphorus, calcium, sodium, potassium, and chloride. Protein fraction was assayed by electrophoresis and analyzed with a densitometer (Cliniscan 2, Herena Research Institute Co., Ltd.). Albumin/globulin (A/G) ratio was calculated from total protein and albumin. The plasma obtained from centrifugation was analyzed for lactate dehydrogenase (LDH) and creatine phosphokinase using a Monarch 2000 automatic biochemical analyzer (IL Co., Ltd.).

2.2.6. Clinical pathology and histopathology

At the end of the treatment or recovery periods, animals were exsanguinated by transecting the abdominal aorta. The body surface, and the intracranial, intrathracic, and intra-abdominal organs and tissues were observed macroscopically. Organ weights were obtained for the brain, pituitary gland, thyroid (including parathyroid), thymus, salivary gland (submandibular and sublingual glands), heart, lung, liver, spleen, kidneys, adrenal glands, testes, epididymis, prostate, seminal vesicles, ovaries, and uterus. The left and right kidneys, adrenal glands, testes, epididymis, and ovaries were weighed separately. Relative organ weights were calculated based on body weights measured on the day of sacrifice. In addition to the above-mentioned organs, the trachea, bronchus, tongue, esophagus, stomach, duodenum, small intestine including Peyer's patches (jejunum and ileum), large intestine (cecum, colon, and rectum), pancreas, urinary bladder, bone and bone marrow (sternum and femur including joint), skeletal muscle (femoral muscle), thoracic aorta, spinal cord (cervical, thoracic, and lumbar regions), sciatic nerve, deferent duct, vagina, mammary gland, skin, and lymph node (submandibular and mesenteric lymph node) were resected and fixed in 10% neutral buffered formalin. Both eyeballs (including optic nerve) and Harderian glands were pre-fixed in glutaraldehyde/formalin fixative, while the testes were fixed in Bouin's fixative. Whole organs were dipped in fixative with the exception of the lung, which was fixed by instillation through the trachea.

All stored organs and tissues from the control, DHA-fish oil and 2000 mg/kg DHA-algal oil male and female groups were sectioned, embedded in paraffin, sliced and stained with Hematoxylin and Eosin, and subjected to microscopic examination.

2.2.7. Statistical analysis

For quantitative data, means and standard deviations were calculated and statistical tests were performed as described below. All statistical tests were performed at the $p < 0.05$ and $p < 0.01$ levels of significance.

Data distribution was tested by Bartlett's test for homogeneity of variance, and if positive, one-way layout analysis of variance was applied. If a significant difference was observed between groups, means of each treatment group were compared pair-wise to those of the control and DHA-fish oil groups by Dunnett's method. In cases of non-homogenous variance, Kruskal–Wallis *H*-test was applied, and if a significant difference

was observed between groups, means of the treatment groups were compared pair-wise with those of the control and DHA-fish oil groups by Dunnett's test of rank order.

For comparison of the DHA-fish oil and 2000 mg/kg DHA-algal oil treatments in the recovery groups, data distribution was evaluated by the *F*-test. In the case of homogenous variance, the Student's *t*-test was performed, and in the case of heterogeneous variance, Aspen–Welch's *t*-test was applied.

Statistical analyses were not performed for clinical observations, neurotoxicological observations, qualitative values in urinalysis, ophthalmology, necropsy or histopathology.

2.3. Genotoxicity studies

2.3.1. Ames assay[3,4]

In the first mutagenicity experiment, the histidine-requiring *Salmonella typhimurium* (*S. typhimurium*) strains TA97, TA98, TA100, and TA102 were cultured in nutrient broth at 37 °C overnight. The Ames assay (pre-incubation method) was performed with or without metabolic activation by the S9 fraction from the livers of Aroclor-induced rats. The test sample (0.1 ml) was mixed with 0.5 ml of S9 mix or 0.1 M sodium phosphate buffer (pH 7.4) and 0.1 ml of bacterial culture. The mixture was incubated at 37 °C for 20 min with shaking, followed by the addition of soft agar. The mixture was then poured onto a minimal glucose agar plate. The number of revertants was counted after incubation at 37 °C for 2 days. The test sample was assayed at five doses (0.5, 1.25, 2.5, 3.75, and 5 mg DHA-algal oil/plate), with two plates for each dose. Negative (vehicle) and positive control experiments were conducted simultaneously. 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, *tert*-butylhydroperoxide, 2-aminofluorene, and benzo[*a*]pyrene were used as positive controls.

A twofold or greater increase in the mean number of revertants in the test sample compared to the number of revertants on negative control plates was considered a positive result for mutagenicity.

In the second mutagenicity experiment using the histidine-requiring *S. typhimurium* strains TA1535, TA1537, TA98, and TA100, and the tryptophan-requiring *Escherichia coli* (*E. coli*) mutant WP2 *uvrA*, the Ames assay (plate incorporation method) was performed with or without metabolic activation by the S9 fraction from the livers of Aroclor-induced rats. Just before use, the test substance was suspended in dimethylsulfoxide (DMSO) vehicle at 50 mg/ml, warmed to 37 °C and mixed by shaking for 1 h. This suspension was used to prepare serial dilutions in water. The test substance suspension (0.1 ml), 0.1 ml of fully grown bacterial culture and 0.5 ml S9-mix or 0.5 ml of 100 mM sodium phosphate (pH 7.5) were added to 2 ml molten top agar (containing 0.6% agar, 0.5% sodium chloride and 0.05 mM L-histidine·HCl/0.05 mM biotin for the *S. typhimurium* strains and supplemented with 0.05 mM tryptophan for the *E. coli* strain) maintained at 46 °C. The ingredients were thoroughly mixed and immediately poured onto minimal glucose agar plates (1.5% agar in Vogel and Bonner medium E with 2% glucose). Following incubation at 37 °C for 3 days, the number of his^+ and trp^+ revertants were counted. The test sample was assayed in triplicate at five concentrations (0.062, 0.185, 0.556, 1.667, and 5 mg DHA-algal oil/plate). Negative (solvent) and positive control experiments were also conducted. Positive control mutagens included sodium azide, 9-aminoacridine, 2-nitrofluorene, *N*-ethyl-*N*-nitrosurea, 2-aminoanthracene, and benzo[*a*]pyrene.

If a twofold or greater increase in the mean number of revertants was observed compared to the number of revertants on negative control plates, the test substance was considered to be mutagenic.

2.3.2. Chromosomal aberration experiments[5]

The ability of DHA-algal oil to induce chromosomal aberrations with or without metabolic activation was evaluated in Chinese hamster fibroblast cells. The culture medium was prepared by dissolving 9.6 g Eagle's minimum essential medium powder in pure water containing 2.2 g $NaHCO_3$, adjusting the pH to 7.2 by adding 0.1 N hydrochloric acid (HCl), sterilization by passing through Millipore of pore size 0.2 μM, and adding 100 ml of heat inactivated calf serum to 900 ml of sterilized medium. Subcultured cells were adjusted to 1×10^4 cells/ml of cell culture medium, placed into 60 mm sterile tissue culture dishes at a volume of 5 ml in each dish, and cultured in a CO_2 incubator for 3 days.

For the continuous treatment, the test substance (dissolved in 1% CMC–Na solution) or negative control (solvent) were added to the plates at a volume of 0.1 ml/ml, while the positive control (10 μg/ml mitomycin C dissolved in distilled water and diluted in physiological saline) was added to the plates at a volume of 0.01 ml/ml, and incubated for 24 or 48 h.

For the short-time treatment, the test substance or negative control was added to the plates at a volume of 0.01 ml/ml. Mitomycin C, which was used as a positive control for treatment with S9, was added at a volume of 0.01 ml/ml, and benzo[*a*]pyrene (dissolved in DMSO to a concentration of 4 mg/ml), which was used as a positive control for treatment without S9, was added to the plates at a volume of 0.005 ml/ml. S9 mix (final concentration of 5%) or physiological saline was then added to the plates and incubated for 6 h, after which the S9 and test substance were washed out with physiological saline, followed by the addition of fresh culture medium and further incubation for 18 h. Similar procedures as the short-time treatment with S9 were also followed to perform a confirmation test with an incubation period of 24 h.

To arrest the cells in metaphase, colcemid at a final concentration of 0.2 μg/ml was added to all dishes 2 h before the end of the incubation period. Cells were then treated with 0.25% trypsin solution (adjusted to 37 °C), harvested by centrifugation at 1000 rpm for 5 min, and treated with hypotonic potassium chloride (0.075 M) at 37 °C for 10–15 min. Supernatants were then removed and the cells were fixed by suspension in Carnoy's fixative. Fixed cell suspensions were dropped on clean glass slides and stained for 15 min with 1.5% Giesma solution prepared in phosphate buffer (pH 6.8). Slides were then rinsed in water, air-dried and analyzed under microscope (BHS-323N, Olympus Optical Co., Ltd.) at a magnification of 750. Blind analysis was conducted for one hundred well-spread metaphases per specimen. All experiments were conducted in duplicate at concentrations of 1.25, 2.5, and 5 mg DHA-algal oil/ml.

For the classification of chromosomal aberrations, the metaphases with numerical aberration were recorded as "polyploidy" and the metaphases with chromatid break, chromatid exchange, chromosome break, chromosome exchange, and others were classified as "structural aberrations". The metaphases with a number of gaps and breaks were recorded as "others". The chromatic area that was narrower than the width of a chromatid was identified as gap, whereas chromatid-type and chromosome-type gaps were recorded separately and not included in the judgment.

A cell having any one of the mentioned aberrations was recorded as an aberrant cell. The mean incidences (%) of chromosomal aberrations were recorded with and without gaps. Cells were evaluated based on either structural or numerical mean (%) incidence of chromosomal aberrations. The test substance was considered positive when the incidence of chromosomal aberrations increased dose-dependently as compared to those of the negative control, or when there was a reproducible increase in the incidence of chromosomal aberrations at one or more concentrations.

[3] The first mutagenicity experiment was conducted at the Institute for Fundamental Research, Suntory Ltd., Japan. The second mutagenicity experiment was conducted at TNO Nutrition and Food Research, The Netherlands.

[4] The second mutagenicity experiment was conducted according to the OECD guideline 471, Genetic Toxicology: Bacterial Reverse Mutation Test, adopted 21 July 1997.

[5] The chromosomal aberration study was conducted at the Nippon Experimental Medical Research Institute Co., Ltd., Japan, in compliance with the OECD Principles of GLP (Organisation for Economic Co-operation and Development, OECD Principles of Good Laboratory Practice (as revised in 1997), Paris).

2.3.3. Statistical analysis

For the chromosomal aberration experiment, the Fisher procedure was used to evaluate the significance of a dose-related increase.

3. Results

3.1. Subchronic toxicity study

Compared to the water control group, treatment with DHA-algal oil alone or in combination with DHA-fish oil did not affect mortality, clinical signs, responses to stimuli, ophthalmology examination, or food and water consumption, with the exception of a significant decrease in food consumption on day 79 in males from the 1000 mg/kg group. One female of the 2000 mg/kg DHA-fish oil group had an ulcer in the neck on days 12–23 and incrustation in the neck on days 24–28.

A tendency for increased body weights was observed in males in the DHA-fish oil and 500 mg/kg DHA-algal oil groups and in all oil-treated females during the 90-day treatment phase (Figs. 1 and 2). During the recovery period, a tendency to increased body weight was observed in

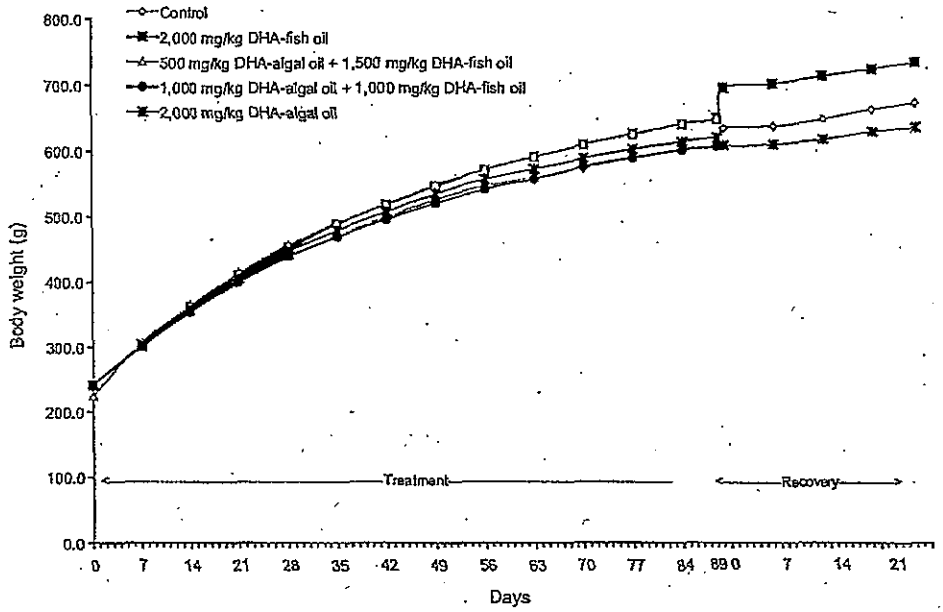

Fig. 1. Body weights of male rats treated with DHA oil for 90 days.

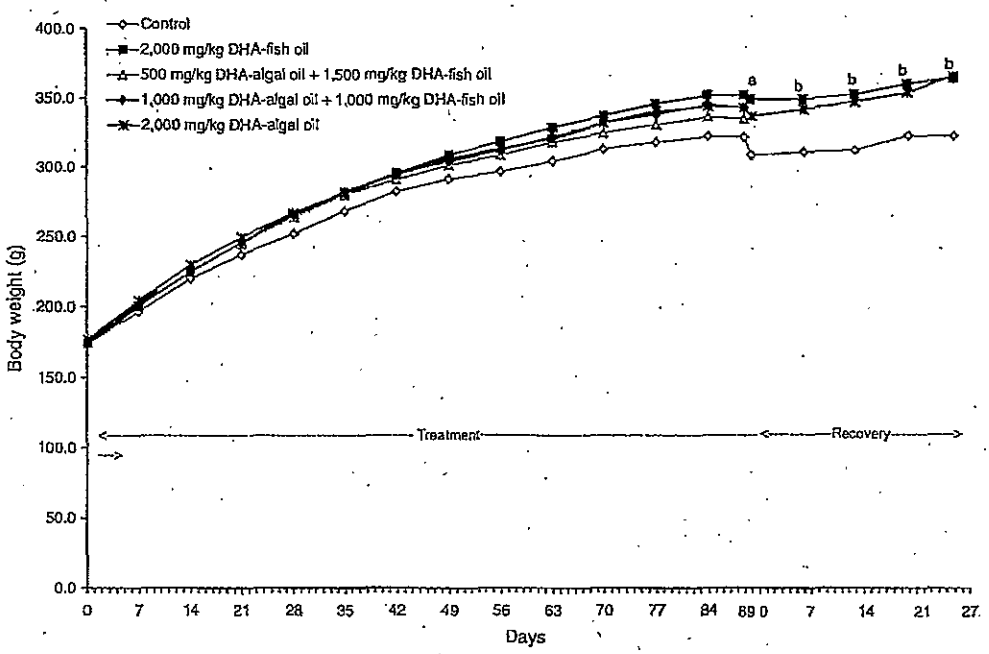

Fig. 2. Body weights of female rats treated orally with DHA oil for 90 days. (a) Significantly different from control, $p < 0.01$; (b) significantly different from control, $p < 0.05$.

males in the DHA-fish oil group. In females, a significant increase in body weight was detected for the DHA-fish oil group and a tendency to increased body weight was observed for the DHA-algal oil group compared to the control group (Fig. 2).

Urinalysis results following the 90-day treatment period indicated a significantly decreased or a tendency to lower sodium excretion values in males from all oil treatment groups and decreased potassium excretion in males from the 1000 mg/kg group and 2000 mg/kg DHA-fish oil group compared with the water control group (data not shown). In comparison to the DHA-fish oil-treated animals, no parameters were affected in animals from any of the DHA-algal oil treatment groups.

After the recovery period, females treated with DHA-algal oil had higher total excretion values of sodium and potassium compared to controls, and higher urine volume and potassium excretion compared to DHA-fish oil-treated females (data not shown).

Hematology results after the treatment period revealed a significantly higher lymphocyte ratio and a significantly lower segmented neutrophil ratio in males in the DHA-fish oil group and 2000 mg/kg DHA-algal oil group, while a significantly lower RBC count was observed in females in the DHA-fish oil and 1000 mg/kg groups (Table 2a). No hematology parameters were affected in males or females from any of the DHA-algal oil groups in comparison to the DHA-fish oil group. Furthermore, following the recovery period, a significantly prolonged prothrombin time was observed in males in the DHA-fish oil group, and a significantly decreased activated partial thromboplastin time was detected in females in the DHA-fish oil and DHA-algal oil

Table 2a
Hematology (90-day toxicity)

	Control	DHA-fish oil	500 mg/kg	1000 mg/kg	DHA-algal oil
Males					
RBC ($\times 10^4/\mu L$)	860 ± 27	825 ± 52	829 ± 51	850 ± 40	823 ± 34
Ht (%)	47.6 ± 1.6	45.8 ± 2.3	46.3 ± 2.6	46.4 ± 1.6	45.4 ± 2.3
Hb (g/dL)	16.1 ± 0.5	15.6 ± 0.7	15.7 ± 0.7	15.8 ± 0.5	15.5 ± 0.7
MCV (fL)	55.4 ± 2.5	55.6 ± 2.2	55.9 ± 1.6	54.6 ± 2.0	55.1 ± 1.5
MCH (pg)	18.7 ± 0.9	18.9 ± 1.0	18.9 ± 0.6	18.6 ± 0.9	18.8 ± 0.6
MCHC (%)	33.8 ± 0.5	34.0 ± 0.7	33.8 ± 0.5	34.0 ± 0.6	34.1 ± 0.5
Reticulocyte (%)	20 ± 7	20 ± 6	21 ± 5	20 ± 5	23 ± 6
Platelet ($\times 10^4/\mu L$)	92.8 ± 9.8	88.4 ± 8.3	87.3 ± 8.5	92.8 ± 10.1	90.5 ± 11.5
PT (s)	14.9 ± 0.5	15.3 ± 0.5	15.3 ± 0.4	15.3 ± 0.6	15.2 ± 0.4
APTT (s)	19.8 ± 1.5	21.1 ± 1.7	21.4 ± 1.8	21.3 ± 1.5	20.6 ± 1.3
WBC ($\times 10^2/\mu L$)	116 ± 23	107 ± 16	125 ± 27	113 ± 24	122 ± 22
Lymphocyte (%)	76.1 ± 7.1	82.4 ± 5.2[a]	78.9 ± 5.5	79.8 ± 5.7	83.7 ± 6.1[b]
Eosinophil (%)	1.7 ± 1.2	1.6 ± 1.2	1.5 ± 0.8	1.7 ± 1.4	1.9 ± 1.1
Monocyte (%)	6.6 ± 2.6	5.8 ± 2.5	6.5 ± 2.2	6.0 ± 1.9	6.4 ± 1.8
Basophil (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Stab (%)	0.6 ± 0.6	0.4 ± 0.3	0.4 ± 0.6	0.5 ± 0.5	0.3 ± 0.4
Seg (%)	15.1 ± 6.2	9.7 ± 3.3[a]	12.6 ± 5.5	12.0 ± 3.6	7.6 ± 5.4[b]
Females					
RBC ($\times 10^4/\mu L$)	816 ± 33	788 ± 26[a]	796 ± 18	783 ± 22[b]	795 ± 37
Ht (%)	46.0 ± 2.0	45.1 ± 1.4	45.3 ± 2.0	44.8 ± 1.5	45.1 ± 1.5
Hb (g/dL)	15.8 ± 0.7	15.5 ± 0.5	15.6 ± 0.6	15.5 ± 0.4	15.5 ± 0.4
MCV (fL)	56.4 ± 1.6	57.2 ± 1.9	56.9 ± 2.0	57.2 ± 1.2	56.8 ± 1.6
MCH (pg)	19.3 ± 0.6	19.7 ± 0.7	19.6 ± 0.7	19.8 ± 0.4	19.5 ± 0.7
MCHC (%)	34.3 ± 0.5	34.5 ± 0.6	34.4 ± 0.5	34.7 ± 0.5	34.3 ± 0.6
Reticulocyte (%)	18 ± 8	16 ± 4	14 ± 5	13 ± 4	16 ± 5
Platelet ($\times 10^4/\mu L$)	87.2 ± 10.3	86.0 ± 8.9	83.8 ± 6.5	86.7 ± 8.7	86.8 ± 13.0
PT (s)	15.1 ± 1.1	15.0 ± 1.0	15.3 ± 0.9	15.1 ± 1.0	15.2 ± 1.1
APTT (s)	17.2 ± 1.1	17.2 ± 1.2	17.7 ± 1.0	17.3 ± 1.0	17.2 ± 0.9
WBC ($\times 10^2/\mu L$)	63 ± 25	76 ± 19	73 ± 20	65 ± 16	77 ± 28
Lymphocyte (%)	78.0 ± 5.3	80.9 ± 8.0	82.4 ± 5.1	82.5 ± 5.5	82.2 ± 6.2
Eosinophil (%)	2.0 ± 1.2	1.6 ± 1.1	1.9 ± 1.0	2.0 ± 0.9	1.8 ± 1.2
Monocyte (%)	6.0 ± 2.7	4.4 ± 1.7	5.1 ± 2.8	4.3 ± 1.9	6.0 ± 2.1
Basophil (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Stab (%)	0.7 ± 0.6	0.5 ± 0.7	0.6 ± 0.5	0.6 ± 0.6	0.4 ± 0.3
Seg (%)	13.2 ± 3.6	12.7 ± 6.8	10.0 ± 3.2	10.7 ± 4.8	9.6 ± 6.0

All values presented as means ± SD.
Males: $N = 15$ for control and groups 1–3 (i.e., DHA-fish oil, 500 mg/kg, and 1000 mg/kg groups, respectively); $N = 14$ for group 4 (DHA-algal oil).
Females: $N = 15$ for every dose group.
[a] Significantly different from control, $p < 0.05$.
[b] Significantly different from control, $p < 0.01$.

groups (Table 2b). Compared to the DHA-fish oil-treated animals, females in the DHA-algal oil group displayed a significantly higher reticulocyte ratio.

Clinical chemistry results revealed significantly lower or tendency to lower serum concentrations of total cholesterol, phospholipid and free fatty acid levels in males and females of each treatment group in comparison to water-treated controls at the end of the treatment period (Table 3a). In males, additional changes included significantly higher alkaline phosphatase levels in all treated groups, significantly higher A/G ratio and albumin fraction ratio in the DHA-fish oil, 500 and 1000 mg/kg groups, a tendency to higher A/G ratio and albumin fraction ratio in the 2000 mg/kg DHA-algal oil group, significantly lower BUN levels in the 500 and 2000 mg/kg DHA-algal oil groups, a significantly lower α2-globulin fraction in the DHA-fish oil group, 500 mg/kg group, and the 2000 mg/kg DHA-algal oil group, and a significantly lower β-globulin fraction ratio in the DHA-fish oil and 500 mg/kg groups. In females additional changes included a significantly lower total bilirubin level in the DHA-fish oil, 500 and 1000 m/kg groups, and a tendency to lower bilirubin levels in the 2000 mg/kg DHA-fish oil group. Compared to the DHA-fish oil group, males treated with 2000 mg/kg DHA-algal oil had significantly lower BUN levels and albumin fraction ratio, as well as a significantly higher β-globulin fraction ratio.

At the end of the recovery period, females treated with DHA-algal oil had significantly lower total cholesterol and phospholipid levels compared to controls, while females in the DHA-fish oil group had significantly lower BUN levels (Table 3b). Compared to controls, significantly higher CPK, β-globulin fraction and inorganic phosphate levels were detected in males from the DHA-fish oil group. Further changes included significantly higher sodium levels in the DHA-fish oil and DHA-algal oil groups, and significantly higher chloride levels in the DHA-algal oil group compared to controls. In comparison with the DHA-fish oil group, significantly higher total bilirubin and chloride levels and significantly lower β-globulin fraction ratio and inorganic phosphate levels were observed for males in the DHA-algal oil group, and significantly elevated urea nitrogen and sodium levels were recorded for females in the DHA-algal oil group.

Following treatment, a tendency to increased absolute and relative liver weights was recorded in males treated with DHA-fish oil compared to controls (Table 4a, data not shown for absolute weight). Weight changes in other organs were not dose-dependent (i.e., no significant differences at the 1000 and 2000 mg/kg doses) and were limited to relative weights only. In females, a significant increase in absolute and relative liver weights was found in each treated group in comparison to controls. Other weight changes were limited to absolute weights only. In comparison with the DHA-fish oil group, relative liver weight was reduced in males from the 1000 mg/kg group and 2000 mg/kg DHA-algal oil group, while absolute and relative liver weights were decreased in females from the 2000 mg/kg DHA-algal oil group. Similarly, at the end of the recovery period, significant changes were recorded for absolute or relative organ weights only, and these were not dose-dependent (Table 4b, data not shown for absolute weight).

Gross necropsy findings after treatment included: liver discoloration in one control male, diaphragmatic hernia of the liver in one male of the 500 mg/kg group, yellow-gray plaque in one male of the 2000 mg/kg DHA-algal oil group, yellow-gray nodule in the epididymis for two males of the 2000 mg/kg DHA-algal oil group, atrophy in the salivary gland for one female of the DHA-fish oil group, and swelling of the uterus for one female of the 1000 mg/kg group and 2000 mg/kg DHA-algal oil group, respectively. At the end of the recovery period, swelling

Table 2b
Hematology (90-day toxicity + 28-day recovery)

	Control	DHA-fish oil	DHA-algal oil
Males			
RBC ($\times 10^4/\mu L$)	812 ± 21	802 ± 45	841 ± 33
Ht (%)	44.7 ± 1.2	44.4 ± 2.4	46.1 ± 3.2
Hb (g/dL)	15.2 ± 0.4	15.0 ± 0.7	15.5 ± 1.0
MCV (fL)	55.1 ± 1.3	55.4 ± 0.9	54.8 ± 2.2
MCH (pg)	18.8 ± 0.5	18.7 ± 0.3	18.4 ± 0.7
MCHC (%)	34.1 ± 0.3	33.8 ± 0.6	33.6 ± 0.5
Reticulocyte (%)	19 ± 8	29 ± 9	27 ± 18
Platelet ($\times 10^4/\mu L$)	92.2 ± 12.7	103.7 ± 9.3	100.1 ± 21.3
PT (s)	15.0 ± 0.2	15.4 ± 0.2[a]	15.2 ± 0.7
APTT (s)	19.3 ± 1.5	18.9 ± 1.7	20.1 ± 1.8
WBC ($\times 10^2/\mu L$)	106 ± 21	115 ± 10	99 ± 23
Lymphocyte (%)	84.6 ± 2.8	79.5 ± 7.3	85.1 ± 8.8
Eosinophil (%)	1.2 ± 0.8	0.9 ± 0.5	1.7 ± 1.1
Monocyte (%)	3.3 ± 3.9	2.6 ± 1.2	4.4 ± 2.4
Basophil (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Stab (%)	0.3 ± 0.3	0.8 ± 0.8	0.2 ± 0.4
Seg (%)	10.6 ± 1.7	16.2 ± 7.1	8.7 ± 9.5
Females			
RBC ($\times 10^4/\mu L$)	807 ± 18	816 ± 26	829 ± 36
Ht (%)	45.8 ± 1.1	46.2 ± 2.4	46.4 ± 2.2
Hb (g/dL)	15.6 ± 0.4	15.5 ± 0.7	15.7 ± 0.8
MCV (fL)	56.8 ± 1.8	56.5 ± 2.1	56.0 ± 1.3
MCH (pg)	19.4 ± 0.4	19.0 ± 0.6	19.0 ± 0.7
MCHC (%)	34.1 ± 0.3	33.6 ± 0.5	33.8 ± 0.5
Reticulocyte (%)	20 ± 7	13 ± 1	18 ± 3[b]
Platelet ($\times 10^4/\mu L$)	84.6 ± 4.1	80.3 ± 11.7	87.5 ± 8.2
PT (s)	15.6 ± 0.3	15.5 ± 0.3	15.9 ± 0.3
APTT (s)	18.4 ± 0.8	17.1 ± 0.6[c]	17.1 ± 0.7[c]
WBC ($\times 10^2/\mu L$)	52 ± 12	59 ± 16	53 ± 12
Lymphocyte (%)	85.0 ± 3.4	82.6 ± 7.2	89.6 ± 2.7
Eosinophil (%)	2.3 ± 0.9	1.9 ± 1.3	1.3 ± 0.6
Monocyte (%)	2.8 ± 2.4	2.6 ± 0.9	2.6 ± 1.5
Basophil (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Stab (%)	0.4 ± 0.3	0.3 ± 0.4	0.4 ± 0.4
Seg (%)	9.6 ± 2.9	12.7 ± 5.7	6.1 ± 3.2

All values presented as means ± SD.
Males: $N = 5$ for all groups.
Females: $N = 4$ for control; $N = 5$ for groups 1 and 2 (DHA-fish oil and 500 mg/kg groups, respectively).
[a] Significantly different from control, $p < 0.01$.
[b] Significantly different from group 1 (DHA-fish oil), $p < 0.05$.
[c] Significantly different from control, $p < 0.05$.

Table 3a
Blood biochemistry (90-day toxicity)

	Control	DHA-fish oil	500 mg/kg	1000 mg/kg	DHA-algal oil
Males					
GOT (IU/L)	169 ± 181	98 ± 12	104 ± 19	107 ± 16	119 ± 36
GPT (IU/L)	55 ± 73	23 ± 3	23 ± 4	26 ± 6	28 ± 8
LDH (IU/L)	171 ± 157	111 ± 33	110 ± 38	102 ± 27	99 ± 31
γ-GTP (IU/L)	0.48 ± 0.61	0.38 ± 0.45	0.32 ± 0.35	0.26 ± 0.43	0.31 ± 0.44
ALP (IU/L)	164 ± 27	207 ± 38[a]	210 ± 57[a]	219 ± 44[b]	205 ± 39[a]
CPK (IU/L)	113 ± 29	102 ± 22	112 ± 38	110 ± 19	114 ± 22
Total bilirubin (mg/dL)	0.08 ± 0.05	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.02
NEFA (μEq/L)	525 ± 97	432 ± 74[a]	440 ± 77	423 ± 84[a]	486 ± 127
Total cholesterol (mg/dL)	92.8 ± 36.6	64.7 ± 20.2[a]	62.5 ± 10.7[a]	66.5 ± 17.8[a]	68.6 ± 21.7
Phospholipid (mg/dL)	137.9 ± 43.8	101.5 ± 24.8[a]	99.4 ± 11.5[a]	102.7 ± 19.5[a]	106.0 ±25.0
Triglyceride (mg/dL)	89.0 ± 55.0	62.0 ± 23.5	60.9 ± 16.1	58.6 ± 22.1	68.6 ± 32.0
BUN (mg/dL)	12.4 ± 2.1	11.9 ± 1.4	10.7 ± 1.3[a]	11.4 ± 1.5	10.4 ± 1.3[b,c]
Creatinine (mg/dL)	0.29 ± 0.05	0.28 ± 0.03	0.29 ± 0.02	0.30 ± 0.03	0.30 ± 0.04
Glucose (mg/dL)	149.8 ± 22.8	151.2 ± 20.8	154.9 ± 21.0	150.8 ± 18.1	151.1 ± 14.3
Total protein (g/dL)	6.3 ± 0.3	6.3 ± 0.4	6.2 ± 0.2	6.0 ± 0.3	6.1 ± 0.3
Albumin (g/dL)	4.7 ± 0.2	4.9 ± 0.4	4.8 ± 0.2	4.7 ± 0.2	4.7 ± 0.3
A/G (ratio)	2.9 ± 0.4	3.7 ± 0.7[b]	3.6 ± 0.5[b]	3.7 ± 0.7[b]	3.4 ± 0.6
Albumin (%)	56.5 ± 1.7	60.7 ± 2.6[b]	59.7 ± 2.1[b]	59.6 ± 2.0[b]	57.9 ± 2.3[d]
α1-Globulin (%)	15.3 ± 1.7	14.2 ± 2.3	14.8 ± 1.7	14.1 ± 2.0	14.8 ± 2.1
α2-Globulin (%)	6.6 ± 1.0	5.5 ± 0.6[b]	5.7 ± 0.7[b]	6.0 ± 0.8	5.8 ± 0.6[a]
β-Globulin (%)	17.4 ± 1.5	15.7 ± 1.7[b]	16.0 ± 1.1[a]	16.6 ± 1.0	17.2 ± 1.1[d]
γ-Globulin (%)	4.1 ± 0.8	3.9 ± 1.2	3.8 ± 0.6	3.8 ± 0.7	4.3 ± 0.8
Ca (mg/dL)	10.5 ± 0.4	10.5 ± 0.4	10.4 ± 0.3	10.2 ± 0.3	10.3 ± 0.2
Inorganic phosphorus (mg/dL)	6.4 ± 0.8	7.0 ± 0.6	6.7 ± 0.3	6.8 ± 0.6	6.6 ± 0.6
Na (mEq/L)	143.9 ± 1.1	144.6 ± 1.0	143.9 ± 0.9	144.2 ± 1.5	144.4 ± 1.1
K (mEq/L)	4.50 ± 0.37	4.55 ± 0.22	4.55 ± 0.21	4.71 ± 0.34	4.54 ± 0.39
Cl (mEq/L)	107.0 ± 1.1	106.7 ± 1.6	106.3 ± 1.1	107.0 ± 1.4	107.0 ± 1.1
TBA (μmol/L)	16.9 ± 20.3	11.7 ± 11.3	9.0 ± 5.7	10.5 ± 8.2	7.4 ± 9.5
Females					
GOT (IU/L)	84 ± 14	78 ± 23	78 ± 14	78 ± 13	85 ± 14
GPT (IU/L)	19 ± 3	20 ± 6	20 ± 5	19 ± 3	20 ± 4
LDH (IU/L)	57 ± 16	60 ± 20	53 ± 11	54 ± 14	58 ± 15
γ-GTP (IU/L)	1.01 ± 0.47	1.06 ± 0.60	1.04 ± 0.40	1.01 ± 0.61	1.23 ± 0.36
ALP (IU/L)	91 ± 27	117 ± 36	119 ± 38	112 ± 31	118 ± 44
CPK (IU/L)	77 ± 13	69 ± 14	67 ± 11	71 ± 10	70 ± 12
Total bilirubin (mg/dL)	0.10 ± 0.03	0.07 ± 0.02[b]	0.07 ± 0.02[a]	0.07 ± 0.02[a]	0.08 ± 0.02
NEFA (μEq/L)	684 ± 154	538 ± 102[b]	498 ± 79[b]	539 ± 81[b]	565 ± 128[a]
Total cholesterol (mg/dL)	78.7 ± 17.2	57.9 ± 11.4[b]	57.3 ± 13.2[b]	62.4 ± 13.9[b]	68.2 ± 12.7
Phospholipid (mg/dL)	151.4 ± 28.9	115.1 ±17.9[b]	113.6 ± 22.8[b]	121.9 ± 24.8[b]	130.1 ±21.1
Triglyceride (mg/dL)	46.0 ± 21.9	39.3 ± 26.2	29.5 ± 15.3	50.6 ± 45.3	41.8 ± 28.3
BUN (mg/dL)	12.4 ± 2.1	12.4 ± 2.0	11.0 ± 1.3	12.4 ± 2.3	13.1 ± 2.3
Creatinine (mg/dL)	151.1 ± 19.4	139.5 ± 23.8	138.2 ± 16.4	134.3 ± 17.7	133.6 ± 6.3
Glucose (mg/dL)	151.1 ± 19.4	139.5 ± 23.8	138.2 ± 16.4	134.3 ± 17.7	133.6 ± 6.3
Total protein (g/dL)	6.7 ± 0.4	6.8 ± 0.2	6.5 ± 0.4	6.7 ± 0.3	6.7 ± 0.4
Albumin (g/dL)	5.7 ± 0.5	5.8 ± 0.3	5.7 ± 0.5	5.8 ± 0.5	5.6 ± 0.5
A/G (ratio)	6.7 ± 2.7	6.0 ± 2.0	7.4 ± 2.9	8.3 ± 5.0	5.7 ± 2.1
Albumin (%)	65.5 ± 2.7	64.7 ± 3.4	65.7 ± 2.5	66.5 ± 3.0	64.6 ± 3.0
α1-Globulin (%)	10.6 ± 1.3	11.2 ± 1.5	10.5 ± 1.3	10.2 ± 2.1	10.5 ± 2.0
α2-Globulin (%)	5.1 ± 0.6	5.0 ± 0.9	5.2 ± 0.5	4.8 ± 0.4	5.3 ± 0.6
β-Globulin (%)	13.7 ± 1.4	14.1 ± 2.0	13.8 ± 1.1	13.5 ± 1.2	14.6 ± 0.8
γ-Globulin (%)	5.0 ± 1.2	4.9 ± 1.1	4.8 ± 1.1	5.0 ± 1.3	5.1 ± 1.1
Ca (mg/dL)	10.6 ± 0.4	10.5 ± 0.3	10.4 ± 0.5	10.5 ± 0.3	10.5 ± 0.4
Inorganic phosphorus (mg/dL)	5.2 ± 1.4	5.7 ± 0.7	5.8 ± 1.0	5.4 ± 0.7	5.2 ± 0.6
Na (mEq/L)	144.0 ± 1.4	143.7 ± 1.3	143.6 ± 1.6	144.0 ± 1.3	144.5 ± 0.8
K (mEq/L)	4.37 ± 0.61	4.15 ± 0.37	4.34 ± 0.29	4.20 ± 0.29	4.22 ± 0.34
Cl (mEq/L)	109.0 ± 2.3	108.0 ± 1.4	108.1 ± 1.6	108.5 ± 1.5	109.2 ± 1.3
TBA (μmol/L)	12.3 ± 6.1	14.1 ± 10.0	14.8 ± 11.7	13.6 ± 10.0	11.2 ± 7.5

All values presented as means ± SD.
Males: $N = 15$ for control and groups 1–3 (i.e., DHA-fish oil, 500 mg/kg, and 1000 mg/kg groups, respectively); $N = 14$ for group 4 (DHA-algal oil).
Females: $N = 15$ for every dose group.
[a] Significantly different from control, $p < 0.05$.
[b] Significantly different from control, $p < 0.01$.
[c] Significantly different from group 1 (DHA-fish oil), $p < 0.05$.
[d] Significantly different from group 1 (DHA-fish oil), $p < 0.01$.

Table 3b
Blood biochemistry (90-day toxicity + 28-day recovery)

	Control	DHA-fish oil	DHA-algal oil
Males			
GOT (IU/L)	142 ± 53	133 ± 28	141 ± 35
GPT (IU/L)	32 ± 22	23 ± 4	40 ± 19
LDH (IU/L)	104 ± 32	122 ± 10	117 ± 31
γ-GTP (IU/L)	1.07 ± 0.65	0.99 ± 0.44	1.36 ± 0.50
ALP (IU/L)	140 ± 14	133 ± 21	128 ± 22
CPK (IU/L)	99 ± 14	136 ± 25[a]	112 ± 20
Total bilirubin (mg/dL)	0.06 ± 0.02	0.05 ± 0.01	0.07 ± 0.01[b]
NEFA (μEq/L)	647 ± 66	601 ± 107	608 ± 95
Total cholesterol (mg/dL)	119.2 ± 15.3	113.7 ± 15.1	111.1 ± 41.1
Phospholipid (mg/dL)	170.4 ± 23.7	151.7 ± 14.1	155.0 ± 49.9
Triglyceride (mg/dL)	114.5 ± 36.0	108.3 ± 28.1	109.8 ± 53.3
BUN (mg/dL)	11.8 ± 1.0	11.1 ± 1.3	10.9 ± 1.1
Creatinine (mg/dL)	0.30 ± 0.04	0.31 ± 0.06	0.30 ± 0.01
Glucose (mg/dL)	158.7 ± 26.9	141.4 ± 13.9	139.2 ± 23.1
Total protein (g/dL)	6.5 ± 0.3	6.5 ± 0.2	6.4 ± 0.3
Albumin (g/dL)	4.8 ± 0.4	4.8 ± 0.3	4.7 ± 0.3
A/G (ratio)	2.9 ± 0.5	2.9 ± 0.3	3.0 ± 0.6
Albumin (%)	58.1 ± 2.1	58.0 ± 1.5	58.8 ± 1.6
α1-Globulin (%)	13.6 ± 1.2	12.6 ± 1.4	13.3 ± 1.4
α2-Globulin (%)	6.1 ± 0.5	6.0 ± 0.7	6.0 ± 0.3
β-Globulin (%)	18.2 ± 0.9	19.5 ± 0.5[a]	17.6 ± 1.1[b]
γ-Globulin (%)	4.0 ± 0.3	4.0 ± 0.9	4.4 ± 0.7
Ca (mg/dL)	10.5 ± 0.3	10.2 ± 0.2	10.4 ± 0.2
Inorganic phosphorus (mg/dL)	5.3 ± 0.3	6.0 ± 0.2[c]	5.4 ± 0.2[d]
Na (mEq/L)	143.2 ± 0.5	144.2 ± 0.8[a]	144.8 ± 1.0[a]
K (mEq/L)	4.49 ± 0.24	4.64 ± 0.43	4.57 ± 0.18
Cl (mEq/L)	106.2 ± 0.8	106.3 ± 1.0	107.8 ± 0.9[a,b]
TBA (μmol/L)	17.3 ± 15.2	12.6 ± 6.1	17.1 ± 13.1
Females			
GOT (IU/L)	104 ± 14	99 ± 14	102 ± 22
GPT (IU/L)	20 ± 6	18 ± 2	24 ± 6
LDH (IU/L)	86 ± 7	68 ± 18	96 ± 37
γ-GTP (IU/L)	1.37 ± 0.34	1.08 ± 1.16	0.97 ± 0.53
ALP (IU/L)	97 ± 44	77 ± 15	103 ± 51
CPK (IU/L)	91 ± 17	78 ± 15	83 ± 23
Total bilirubin (mg/dL)	0.10 ± 0.04	0.08 ± 0.03	0.08 ± 0.04
NEFA (μEq/L)	684 ± 285	682 ± 105	695 ± 78
Total cholesterol (mg/dL)	91.6 ± 12.6	86.3 ± 13.0	69.9 ± 14.9[a]
Phospholipid (mg/dL)	166.4 ± 19.1	164.0 ± 28.0	138.6 ± 13.2[a]
Triglyceride (mg/dL)	32.6 ± 14.6	52.1 ± 20.0	41.2 ± 19.3
BUN (mg/dL)	12.9 ± 1.2	11.2 ± 0.8[a]	13.7 ± 1.8[b]
Creatinine (mg/dL)	0.30 ± 0.03	0.29 ± 0.03	0.27 ± 0.03
Glucose (mg/dL)	120.1 ± 17.3	140.0 ± 16.2	137.4 ± 9.2
Total protein (g/dL)	7.0 ± 0.6	7.0 ± 0.5	6.5 ± 0.3
Albumin (g/dL)	5.8 ± 0.9	5.9 ± 0.3	5.5 ± 0.6
A/G (ratio)	5.4 ± 2.2	5.7 ± 1.0	6.5 ± 2.8
Albumin (%)	64.0 ± 2.6	64.8 ± 1.3	64.2 ± 2.9
α1-Globulin (%)	11.1 ± 0.7	10.7 ± 0.7	10.6 ± 1.2
α2-Globulin (%)	5.0 ± 0.7	4.8 ± 0.7	5.2 ± 0.3
β-Globulin (%)	13.8 ± 1.3	14.2 ± 1.1	14.2 ± 0.7
γ-Globulin (%)	6.1 ± 0.9	5.5 ± 1.0	5.8 ± 1.7
Ca (mg/dL)	10.3 ± 0.4	10.4 ± 0.4	10.1 ± 0.4
Inorganic phosphorus (mg/dL)	5.5 ± 0.6	5.4 ± 0.8	4.8 ± 0.4
Na (mEq/L)	143.7 ± 1.0	143.6 ± 0.5	144.5 ± 0.6[b]
K (mEq/L)	4.63 ± 0.36	4.40 ± 0.16	4.53 ± 0.37
Cl (mEq/L)	109.4 ± 0.8	108.4 ± 1.7	109.9 ± 1.3
TBA (μmol/L)	13.0 ± 6.4	10.8 ± 7.7	17.5 ± 10.1

All values presented as means ± SD.
Males: $N = 5$ for all groups.
Females: $N = 4$ for control; $N = 5$ for groups 1 and 2 (DHA-fish oil and 500 mg/kg groups, respectively).
[a] Significantly different from control, $p < 0.05$.
[b] Significantly different from group 1 (DHA-fish oil), $p < 0.05$.
[c] Significantly different from control, $p < 0.01$.
[d] Significantly different from group 1 (DHA-fish oil), $p < 0.01$.

in the uterus was observed for one female in the control, DHA-fish oil, and DHA-algal oil groups, respectively.

Histopathological examination did not reveal adverse changes attributable to the dosage of DHA-fish oil and DHA-algal oil (data not shown).

3.2. Genotoxicity studies

3.2.1. Ames assay

In the first experiment, DHA-algal oil did not increase the number of revertants per plate of any *S. typhimurium* strains with or without metabolic activation in comparison to the spontaneous reversion rate in the negative control (Table 5a). No dose–response relationship was observed. The positive control mutagens induced increases in revertant colonies in each strain, as expected, while the vehicle control showed no increase in revertants per plate (Table 5b).

In the second experiment, DHA-algal oil did not cause a twofold or greater increase in the mean number of revertant colonies compared to the background spontaneous reversion rate observed with the negative control (Table 6). There was no evidence of a dose–response relationship. The mean number of revertant colonies of the negative controls was within the acceptable range, and the positive control mutagens gave the expected increase in the mean number of revertant colonies. The test substance was not toxic to any strain tested, as evidenced by the absence of a decrease in the mean number of revertant colonies.

3.3. Chromosomal aberrations

The incidence of cells with chromosomal aberration, evaluated structurally or numerically, were within the range of 0–1% and were similar to incidences observed with concurrent negative controls (Table 7). In contrast, the incidences of structurally aberrant cells were increased in the positive controls of all groups.

Because negative results were obtained in the chromosomal aberration tests, a confirmation test was conducted using the short time treatment with S9 with a 24 h recovery period instead of 18 h. Similar to negative control, incidences of cells with either structural or numerical chromosomal aberrations were within the range of 0–0.5% (Table 7).

A cell growth inhibition test revealed that the test substance did not inhibit cell growth by 50% or more at concentrations up to 5 mg/ml.

4. Conclusion

The results of the 90-day toxicity study in rats demonstrate a lack of toxicologically significant adverse effects following oral administration of DHA-algal oil at doses up to 2000 mg/kg.

Significant differences in hematology and blood biochemistry values were observed between the DHA-fish oil

Table 4a
Relative organ weights (90-day toxicity)

	Control	DHA-fish oil	500 mg/kg	1000 mg/kg	DHA-algal oil
Males					
Final body weight (g)	573 ± 71	617 ± 70	629 ± 60	589 ± 50	609 ± 57
Brain (g)	0.40 ± 0.04	0.38 ± 0.04	0.37 ± 0.04	0.38 ± 0.03	0.38 ± 0.05
Pituitary (mg)	2.2 ± 0.3	2.0 ± 0.2	2.0 ± 0.4	2.0 ± 0.3	2.0 ± 0.3
Thyroid (mg)	3.7 ± 0.6	3.6 ± 0.9	3.4 ± 0.8	3.7 ± 0.9	3.6 ± 0.8
Salivary gland (mg)	136 ± 16	130 ± 16	120 ± 22	127 ± 15	130 ± 25
Thymus (mg)	59.5 ± 15.6	48.3 ± 10.0	52.7 ± 10.4	61.1 ± 11.1[a]	58.5 ± 14.4
Heart (g)	0.28 ± 0.02	0.26 ± 0.01[b]	0.25 ± 0.02[b]	0.27 ± 0.02	0.27 ± 0.02[a]
Lung (g)	0.31 ± 0.02	0.29 ± 0.03	0.28 ± 0.02[b]	0.30 ± 0.03	0.31 ± 0.05
Liver (g)	2.34 ± 0.19	2.45 ± 0.18	2.32 ± 0.12	2.29 ± 0.14[a]	2.25 ± 0.15[c]
Spleen (mg)	160 ± 14	148 ± 21	159 ± 23	155 ± 19	151 ± 16
Right kidney (g)	0.26 ± 0.02	0.26 ± 0.02	0.25 ± 0.02	0.25 ± 0.02	0.26 ± 0.02
Left kidney (g)	0.26 ± 0.02	0.26 ± 0.02	0.025 ± 0.02	0.26 ± 0.02	0.26 ± 0.02
Right adrenal gland (mg)	5.2 ± 1.0	4.6 ± 0.7	4.5 ± 0.6	4.8 ± 0.6	5.0 ± 0.8
Left adrenal gland (mg)	5.5 ± 0.9	4.8 ± 0.5	4.9 ± 0.8	5.2 ± 0.7	5.2 ± 0.8
Right testis (g)	0.30 ± 0.04	0.28 ± 0.03	0.27 ± 0.02	0.28 ± 0.03	0.29 ± 0.03
Left testis (g)	0.29 ± 0.04	0.27 ± 0.04	0.27 ± 0.02	0.28 ± 0.03	0.28 ± 0.03
Right epididymis (g)	0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.02
Left epididymis (g)	0.11 ± 0.02	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.02	0.11 ± 0.01
Prostate (mg)	109 ± 31	106 ± 32	105 ± 34	109 ± 27	118 ± 34
Seminal vesicle (g)	0.43 ± 0.07	0.41 ± 0.09	0.40 ± 0.08	0.45 ± 0.08	0.41 ± 0.06
Females					
Final body weight (g)	313 ± 22	340 ± 47	324 ± 31	332 ± 29	330 ± 30
Brain (g)	0.66 ± 0.04	0.62 ± 0.07	0.65 ± 0.06	0.64 ± 0.06	0.65 ± 0.06
Pituitary (mg)	4.5 ± 0.7	4.3 ± 0.6	4.9 ± 0.7	4.3 ± 0.7	4.3 ± 0.8
Thyroid (mg)	5.2 ± 1.5	4.9 ± 0.9	5.5 ± 0.7	4.7 ± 1.3	5.3 ± 1.1
Salivary gland (mg)	139 ± 14	143 ± 28	152 ± 17	151 ± 18	143 ± 16
Thymus (mg)	81.6 ± 27.7	77.1 ± 13.4	77.6 ± 18.9	90.4 ± 27.9	79.9 ± 15.6
Heart (g)	0.32 ± 0.03	0.30 ± 0.03	0.31 ± 0.02	0.30 ± 0.02	0.31 ± 0.03
Lung (g)	0.40 ± 0.04	0.39 ± 0.04	0.39 ± 0.04	0.40 ± 0.03	0.41 ± 0.04
Liver (g)	2.23 ± 0.11	2.62 ± 0.18[b]	2.54 ± 0.19[b]	2.51 ± 0.12[b]	2.39 ± 0.16[c,d]
Spleen (mg)	160 ± 16	165 ± 19	173 ± 20	175 ± 17	176 ± 22
Right kidney (g)	0.29 ± 0.03	0.31 ± 0.04	0.30 ± 0.02	0.30 ± 0.02	0.30 ± 0.04
Left kidney (g)	0.28 ± 0.03	0.30 ± 0.03	0.30 ± 0.02	0.30 ± 0.02	0.30 ± 0.04
Right adrenal gland (mg)	9.4 ± 1.5	9.7 ± 1.7	10.5 ± 1.5	9.7 ± 1.4	10.3 ± 0.8
Left adrenal gland (mg)	9.7 ± 1.4	10.2 ± 1.9	10.8 ± 1.8	10.3 ± 1.3	10.3 ± 1.3
Right ovary (mg)	12.8 ± 2.5	12.3 ± 2.7	13.1 ± 2.5	11.9 ± 2.1	11.9 ± 2.1
Left ovary (mg)	13.1 ± 2.8	11.5 ± 1.8	13.5 ± 3.0	12.4 ± 1.9	12.4 ± 1.3
Uterus (g)	0.17 ± 0.03	0.17 ± 0.05	0.19 ± 0.07	0.18 ± 0.06	0.21 ± 0.07

Number of animals = 15.
All values presented as means ± SD.
[a] Significantly different from group 1 (DHA-fish oil), $p < 0.05$.
[b] Significantly different from control, $p < 0.01$.
[c] Significantly different from group 1 (DHA-fish oil), $p < 0.01$.
[d] Significantly different from control, $p < 0.05$.

and the water control group, as well as between the DHA-algal oil and the control group; however, no significant differences in hematology and blood biochemistry values were observed between the DHA-fish oil and the DHA-algal oil groups. The findings were considered to be of no toxicological significance since the changes were small, were not dose-dependent, were not observed in both sexes, and were not related to histopathological changes.

Although there was a significant increase in absolute and relative liver weights in DHA-algal oil-treated animals, no corresponding histological changes were observed. Furthermore, the magnitude of change was small. Increases in liver weights were likely the result of the large lipid load, as previously reported in several feeding and gavage studies in which rats were administered fish and marine algal-derived oils at levels of 25–9500 mg/kg body weight/day for 4–13 weeks (Boswell et al., 1996; Hempenius et al., 1997; McGuire et al., 1997; Wibert et al., 1997; Burns et al., 1999; Rabbani et al., 1999; Hempenius et al., 2000). In these studies, histopathological changes in the liver were not observed.

At necropsy and histopathology, the main microscopic observations included: liver discoloration, diaphragmatic hernia of the liver, and a yellow-gray plaque in one male in each of the control, 500, and 2000 mg/kg DHA-algal oil groups, respectively. A yellow-gray nodule was observed in the epididymis for two males of the 2000 mg/kg DHA-algal oil group, while atrophy in the salivary gland for one female of the DHA-fish oil group, and swell-

Table 4b
Relative organ weights (90-day toxicity + 28-day recovery)

	Control	DHA-fish oil	DHA-algal oil
Males			
Final body weight (g)	653 ± 23	712 ± 103	615 ± 67
Brain (g)	0.36 ± 0.01	0.33 ± 0.04	0.38 ± 0.03[a]
Pituitary (mg)	1.9 ± 0.2	2.0 ± .01	2.0 ± 0.2
Thyroid (mg)	3.4 ± 0.4	3.4 ± 0.7	3.4 ± 0.7
Salivary gland (mg)	135 ± 11	112 ± 9[b]	124 ± 10
Thymus (mg)	48.2 ± 13.4	50.7 ± 5.7	53.3 ± 16.3
Heart (g)	0.26 ± 0.01	0.25 ± 0.02	0.27 ± 0.02
Lung (g)	0.30 ± 0.01	0.26 ± 0.02[b]	0.29 ± 0.03
Liver (g)	2.55 ± 0.17	2.42 ± 0.10	2.38 ± 0.37
Spleen (mg)	136 ± 28	148 ± 12	147 ± 17
Right kidney (g)	0.26 ± 0.02	0.24 ± 0.02	0.25 ± 0.02
Left kidney (g)	0.26 ± 0.02	0.24 ± 0.02	0.25 ± 0.01
Right adrenal gland (mg)	4.8 ± 0.5	3.7 ± 0.6[c]	4.4 ± 0.9
Left adrenal gland (mg)	5.1 ± 0.5	3.9 ± 0.6[b]	4.9 ± 0.9
Right testis (g)	0.27 ± 0.01	0.24 ± 0.03	0.28 ± 0.05
Left testis (g)	0.26 ± 0.02	0.23 ± 0.02[c]	0.28 ± 0.05
Right epididymis (g)	0.11 ± 0.02	0.10 ± 0.01	0.11 ± 0.02
Left epididymis (g)	0.11 ± 0.01	0.10 ± 0.01	0.12 ± 0.02
Prostate (mg)	71 ± 20	82 ± 13	96 ± 27
Seminal vesicle (g)	0.43 ± 0.03	0.39 ± 0.04	0.43 ± 0.06
Females			
Final body weight (g)	308 ± 12	350 ± 27	346 ± 89
Brain (g)	0.69 ± 0.05	0.61 ± 0.06[c]	0.64 ± 0.14
Pituitary (mg)	5.0 ± 0.8	3.9 ± 0.7[c]	4.8 ± 0.5
Thyroid (mg)	5.2 ± 1.0	4.5 ± 0.5	5.2 ± 2.4
Salivary gland (mg)	163 ± 39	133 ± 18	147 ± 19
Thymus (mg)	78.9 ± 9.7	75.7 ± 12.6	80.5 ± 13.9
Heart (g)	0.33 ± 0.03	0.30 ± 0.01	0.31 ± 0.02
Lung (g)	0.43 ± 0.02	0.37 ± 0.04[c]	0.38 ± 0.05
Liver (g)	2.38 ± 0.11	2.33 ± 0.12	2.32 ± 0.16
Spleen (mg)	172 ± 20	154 ± 19	174 ± 24
Right kidney (g)	0.32 ± 0.02	0.28 ± 0.03[c]	0.29 ± 0.02
Left kidney (g)	0.31 ± 0.02	0.28 ± 0.03	0.28 ± 0.02
Right adrenal gland (mg)	9.4 ± 1.1	9.4 ± 0.8	8.3 ± 1.0
Left adrenal gland (mg)	10.1 ± 0.8	9.1 ± 1.1	8.3 ± 1.3[c]
Right ovary (mg)	12.0 ± 0.6	10.6 ± 2.3	12.4 ± 1.1
Left ovary (mg)	12.0 ± 3.1	9.6 ± 1.6	11.8 ± 1.0[a]
Uterus (g)	0.18 ± 0.04	0.17 ± 0.09	0.27 ± 0.12

Number of animals = 5.
All values presented as means ± SD.
[a] Significantly different from group 1 (DHA-fish oil), $p < 0.05$.
[b] Significantly different from control, $p < 0.01$.
[c] Significantly different from control, $p < 0.05$.

ing of the uterus for one female of the 1000 and 2000 mg/kg DHA-algal oil groups, respectively. Swelling in the uterus was observed at the end of the recovery period for one female in the control, DHA-fish oil, and DHA-algal oil groups, respectively. These histological effects were not reported in other studies conducted with fish oils (Danse and Verschuren, 1978a,b; Rabbani et al., 1999; Hempenius et al., 2000; Oarada et al., 2000) or algal oils rich in DHA (Boswell et al., 1996; Wibert et al., 1997; Arterburn et al., 2000a,b; Hammond et al., 2001a,b,c). The observed changes were considered unrelated to treatment because they were found only in controls or in a small number of animals, or because no differences in frequency and magnitude of changes between treated and control groups were detected.

Unlike several studies conducted on DHA-containing fish and marine-derived oils, microscopic characteristics of yellow fat disease were not observed in rats treated with DHA-algal oil and/or DHA-fish oil. The lack of effects may be attributed to lower daily-administered doses, strain and species differences, or dietary content of vitamin E. The basal diets provided sufficient levels of vitamin E (1.25 mg/day in males and 0.9 mg/day in females) to compensate for the elevated vitamin demand caused by increased consumption of PUFAs, as estimated by Muggli (1994). Furthermore, vitamin E was present in the DHA-algal and DHA-fish oils, providing additional vitamin E. Although vitamin E levels in DHA-fish oil were slightly higher than in DHA-algal oil, vitamin E levels in the diet alone were sufficient to meet minimum daily requirements. Given that the effects observed in the 90-day study were not toxic in nature and that no histopathological effects were detected, the no-observed-adverse-effects level (NOAEL) is considered to be 2000 mg DHA-algal oil/kg body weight/day, which was the highest dose tested. This value corresponds to approximately 900 mg DHA/kg body weight/day.

The safety of DHA-algal oil was further supported in studies which showed that this substance was not mutagenic under the experimental conditions for *S. typhimurium* TA97, TA98, TA100, and TA102. The second experiment

Table 5a
Ames assay (Experiment 1)

Test substance concentration (μg/plate)	Number of revertants (number of colonies/plate)							
	Base-pair substitution type				Frameshift type			
	TA100		TA102		TA97		TA98	
	S9 mix (−)	S9 mix (+)	S9 mix (−)	S9 mix (+)	S9 mix (−)	S9 mix (+)	S9 mix (−)	S9 mix (+)
Solvent control (75% Tween 80 DMSO solution)	126	142	393	453	209	250	83	88
500	126	162	399	518	214	241	72	99
1250	117	152	394	509	233	267	71	80
2500	134	143	380	507	240	287	74	98
3750	150	162	389	482	255	271	90	99
5000	186	149	382	452	260	315	93	105

Table 5b
Positive controls for Ames assay (Experiment 1)

	TA100		TA102		TA97		TA98	
With (+) or without (−) S9 mix	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Name	AF-2	B(a)P	*t*-BuOOH	ND	AF-2	2-AF	AF-2	B(a)P
Concentration (μg/plate)	0.02	5	0.5 (μL/plate)	ND	0.1	1	0.1	5
Number of colonies/plate	849	1039[a]	1109[a]	ND	1127	732	454	310

Abbreviations: AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; 2-AF: 2-aminofluorene; B(a)P: benzo(a)pyrene; *t*-BuOOH: *tert*-butylhydroperoxide; ND: not determined.
[a] Inhibition found against growth of bacteria.

Table 6
Ames assay (Experiment 2)

Dose (μg/pl)	Number of revertants (number of colonies/plate)									
	TA1535		TA1537		TA98		TA100		*E. coli*	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
0	24 ± 2	18 ± 1	18 ± 5	19 ± 9	23 ± 4	45 ± 14	154 ± 12	145 ± 4	26 ± 3	31 ± 4
62	21 ± 5	27 ± 7	18 ± 1	18 ± 1	27 ± 3	34 ± 8	145 ± 12	157 ± 11	25 ± 5	28 ± 6
185	20 ± 5	15 ± 2	12 ± 7	14 ± 2	28 ± 10	37 ± 11	150 ± 14	148 ± 6	23 ± 5	31 ± 8
556	18 ± 6	26 ± 6	13 ± 5	16 ± 6	19 ± 9	50 ± 3	124 ± 37	136 ± 18	23 ± 12	29 ± 8
1667	22 ± 6	24 ± 6	15 ± 2	16 ± 3	25 ± 4	46 ± 9	132 ± 14	147 ± 9	23 ± 7	25 ± 6
5000	29 ± 6	30 ± 2	13 ± 7	13 ± 2	21 ± 9	38 ± 7	120 ± 11	160 ± 10	30 ± 6	28 ± 2
Positive control	473 ± 82	370 ± 42	1114 ± 167	268 ± 13	1008 ± 106	652 ± 123	533 ± 21	1509 ± 59	196 ± 10	464 ± 12

All values presented as means ± SD.

verified these results and also tested additional bacterial strains, demonstrating a lack of mutagenic activity for *S. typhimurium* TA98, TA100, TA1535, and TA1537 and for *E. coli* WP2 *uvrA*. Moreover, DHA-algal oil did not induce chromosomal aberrations in Chinese hamster fibroblast cells at concentrations up to 5 mg/ml. These results are similar to those reported in studies conducted on other PUFA-rich microalgal-derived oils, in which no genotoxic activity was observed (Hammond et al., 2002; Arterburn et al., 2000c).

In addition to animal studies, information on populations who consume high levels of ω-3 PUFAs from fish and fish- and marine-based products also support the safety of DHA-algal oil. Reported dietary intakes of DHA in populations such as Japan, Norway, South Africa, and the Portuguese Island of Madeira range from 0.5 to 0.7 g/day (Bønaa et al., 1992; Schloss et al., 1997; Fluge et al., 1998; Johansson et al., 1998; Sugano and Hirahara, 2000; Torres et al., 2000). These levels are higher than estimated intake levels of DHA from DHA-algal oil under the conditions of intended use (Kroes et al., 2003).

Several clinical studies have also been conducted on DHA-containing fish and marine oils (reviewed in Kroes et al., 2003). Overall, these data do not indicate that DHA, at estimated exposures provided through the use of DHA-algal oil, would have adverse effects on low density lipoprotein cholesterol levels, glycemic control, bleeding time, platelet aggregation or other hemostatic parameters. Additionally, there is no evidence that DHA consumption would result in negative effects on immune function or response, kidney or liver function, or lipid peroxidation.

Although *Ulkenia* sp. SAM2179 has not previously been used in the production of food products or ingredients, thaustrochytrids have been found in plankton and other marine detritus and are components of the diet of filter-feeding invertebrates in the marine ecosystem (Sathe-Pathak et al., 1993; Azevedo and Corral, 1997; Naganuma et al., 1998). Thus, through consumption of fish and other marine animals, these marine microalgae are indirect components of the human diet. Moreover, it was concluded in a scientific expert evaluation pertinent to the safety of *Ulkenia* sp. SAM2179 that "the marine fungoid protest genus *Ulkenia* (i.e., *Ulkenia* sp.) is both non-toxigenic and non-pathogenic to man" (Schaumann, unpublished-a). An extended expert report stated that there was a lack of evidence for toxin formation in *Ulkenia* or related organisms, and that any analytically determined toxins that could be transferred to the oil would be destroyed under the conditions of edible oil refinement (Schaumann, unpublished-b). Analyses of DHA-algal oil and dry algal biomass confirmed the absence of algal-based biotoxins (Luckas, unpublished).

The safety of the source organism for DHA-algal oil was further supported in an Ames assay and in a short-term oral toxicity study. At concentrations of 0.5–5 mg/plate, *Ulkenia* sp. produced negative results in the Ames assay in the *S. typhimurium* strains TA97, TA98, TA100, and TA102, either with or without metabolic activation (Fujii and Suwa, 1999, unpublished). A short-term oral toxicity study in which male ICR mice were administered 2000 mg *Ulkenia* sp. suspended in distilled water by gavage for 14 days indicated no significant differences in clinical signs, body weight gains, or autopic observations compared to the control group (Celanese Ventures, 1999, unpublished).

Table 7
Chromosomal aberration test

Test substance	With (+) or without (−) S9 mix	Concentration (mg/ml)	Polyploid cells (% ±SD)	Chromosomal aberration (% ±SD)						Total	
				Gap[a]	Ctb[b]	Cte[c]	Csb[d]	Cse[e]	O[f]	−g[g]	+g[h]
Negative control (CMC)	−	0	0.0 ± 0.00	1.0 ± 0.71	1.0 ± 0.71	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 0.71	2.0 ± 0.00
	+	0	0.0 ± 0.00	1.0 ± 0.71	1.0 ± 0.71	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 0.71	2.0 ± 0.00
DHA-algal oil	−	1.25	0.0 ± 0.00	1.0 ± 0.71	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 0.71
		2.5	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 0.71	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 0.71	1.0 ± 0.71
		5	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	+	1.25	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 0.71	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 0.71	0.0 ± 0.00	2.0 ± 0.00	2.0 ± 0.00
		2.5	1.0 ± 0.71	1.0 ± 0.71	1.0 ± 0.71	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 0.71	2.0 ± 0.00
		5	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	2.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	2.0 ± 0.00	2.0 ± 0.00
Positive control [MMC]	−	0.0001	1.0 ± 0.71	2.0 ± 0.00	6.0 ± 0.00	24.0 ± 2.83	1.0 ± 0.71	5.0 ± 2.12	0.0 ± 0.00	34.0 ± 1.41	36.0 ± 1.41
Positive control [B(a)P]	+	0.02	1.0 ± 0.71	11.0 ± 0.71	26.0 ± 1.41	71.0 ± 3.54	1.0 ± 0.71	10.0 ± 0.00	1.0 ± 0.71	92.0 ± 2.83	95.0 ± 3.54

All values presented as means ± SD.
[a] Chromatid gap.
[b] Chromatid break.
[c] Chromatid exchange.
[d] Chromosome break.
[e] Chromosome exchange.
[f] Others.
[g] the percentage of aberration cells except those which have only gaps.
[h] The percentage of aberration cells including those which have gaps.

In summary, the potential toxic and genotoxic effects of DHA-algal oil were assessed. In a 90-day study conducted in rats, toxic effects attributed to DHA-algal oil were not detected. DHA-algal oil showed no mutagenic activity in the Ames assay and did not induce chromosomal aberrations. These data support the safety of DHA-algal oil as a potential dietary source of DHA.

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Food and Chemical Toxicology 38 (2000) 127–139

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Research Section

Evaluation of a Subchronic (13-Week) Oral Toxicity Study, Preceded by an *In Utero* Exposure Phase, with Arachidonic Acid Oil Derived from *Mortierella alpina* in Rats

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(Accepted 7 June 1999)

Abstract—Arachidonic acid oil (ARA-oil) derived from the fungus *Mortierella alpina* for use in infant nutrition was tested in a subchronic (13-week) oral toxicity study in rats, preceded by an *in utero* exposure phase. The ARA-oil was administered as admixture to the rodent diet at dose levels of 3000 ppm, 15,000 ppm and 75,000 ppm. An additional high-dose group received 75,000 ppm ARA-oil in combination with 55,000 ppm fish oil containing docosahexaenoic acid (DHA), at a ratio of ARA to DHA, comparable to the ratio in mother's milk of 2:1. The total levels of fat in each diet were kept constant by adding the appropriate amounts of corn oil. A concurrent control group received 130,000 ppm corn oil in the diet. An additional carrier control group was fed unsupplemented rodent diet. Administration of the test substances from 4 weeks prior to mating, throughout mating, gestation, lactation of parental (F_0) animals and weaning of the F_1 pups did not affect fertility or reproductive performance, nor the general condition of pups, viability, sex ratio or number of pups. Pup weight gain in the ARA/DHA-oil group was lower than the controls administered equal amounts of corn oil. In the subsequent subchronic study survival, clinical signs, body weight gain and food consumption were not adversely affected by the test substances. Ophthalmoscopic examination did not reveal any treatment-related changes. There were no treatment-related effects observed up to dietary test substance concentrations of 15,000 ppm. The following statistically significant differences were found in the ARA high-dose group and /or in the ARA/DHA group compared to the corn oil control group: decreased alkaline phosphatase activity, decreases in cholesterol, triglycerides and phospholipids concentrations, increased creatinine and urea concentrations. Furthermore, these groups showed increased adrenal, spleen and liver weights. The incidence of hepatocellular vacuolation was increased in females of the ARA high-dose group and the ARA/DHA group. Oil droplets were observed in the mesenteric lymph nodes and in the intestinal villi in the ARA high-dose group and the ARA/DHA group. In addition, lipogranulomas were observed in the mesenteric lymph nodes in these groups. The observed changes in the high-dose groups may be effects of the high intake of high-fat levels, rather than specific effects of the ARA-oil. The no-observed-effect level in this study was placed at 15,000 ppm ARA-oil. This level is equivalent to approximately 970 mg ARA-oil/kg body weight/day.

Keywords: arachidonic acid oil; safety; rat; reproductive indices; haematology; clinical chemistry; organ weights; histopathology.

Abbreviations: LC-PUFAs = long-chain polyunsaturated fatty acids; ARA = arachidonic acid; DHA = docosahexaenoic acid; MCHC = mean corpuscular haemoglobin concentration; MCH = mean corpuscular haemoglobin; GALT = gut-associated lymphoid tissue.

INTRODUCTION

Long-chain polyunsaturated fatty acids (LC-PUFAs) play an important role in the development of the brain in the late foetal and early postnatal period. The most abundant fatty acids in brain cellular membranes are arachidonic acid (20:4, n-6) (ARA) and docosahexaenoic acid (22:6, n-3) (DHA) (O'Brien and Sampson, 1965). The brain of the developing human child shows a massive increase in weight during the last trimester of preg-

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0278-6915/00/$ - see front matter
PII S0278-6915(99)00144-1

nancy and the first period after birth. It is obvious that this developing tissue has a high demand for these building blocks (Koletzko, 1992). In addition to the structural role, both ARA and DHA play instrumental roles in a variety of processes in the human body. They act as precursors to a class of hormone-like compounds (prostaglandins and eicosanoids), which have a function in the control of blood pressure, the regulation of growth and development, and stimulation of the immune system (British Nutrition Foundation, 1992).

The human body can synthesize ARA and DHA from the essential fatty acids linoleic (18:2, n-6), and linolenic acid (18:3, n-3), respectively, by elongation and desaturation. The human foetus initially obtains ARA and DHA by placental transfer (Koletzko and Müller, 1990). Post-natally, human milk provides a source of these preformed LC-PUFAs. The demand for these important LC-PUFAs is cut short if the infant is born prematurely or if it receives infant formula instead of being breast-fed: in contrast to breast milk, standard infant formulae contain only trace amounts of ARA or DHA (Jensen *et al.*, 1992). It has been shown in numerous studies that the infant's LC-PUFA status is maintained by breast feeding whereas the LC-PUFA concentrations decline when the child is fed a formula without LC-PUFAs (Clark *et al.*, 1992; Makrides *et al.*, 1995). This implies that the infant's capacity for synthesizing LC-PUFAs from the precursors present in the formula does not meet the demand (Demmelmair *et al.*, 1995; Sauerwald *et al.*, 1997). Therefore, there is a good rationale for including these preformed LC-PUFAs in any infant milk formula that provides the main nutrient intake of the infant. Inclusion of these compounds in infant formulae is supported by a number of expert bodies, which have recommended the addition of the preformed LC-PUFAs ARA and DHA to infant formulae. In 1992, the British Nutrition Foundation Task Force (1992) recommended that infant formulae should contain preformed ARA in an attempt to replicate the fatty acid profile of human milk. In addition, in 1994, an Expert Committee of the FAO/WHO (1994) recommended, on the basis of available data, that all infant formulae, both pre-term formulae, and full-term formulae, should be supplemented with DHA and ARA at levels normally found in human breast milk.

For these reasons, there is an increasing interest in highly purified oils rich in either ARA or DHA, thus enabling the addition of these important LC-PUFAs directly to the infant formulae. The arachidonic oil (ARA-oil) referred to in this paper is derived from the fungus *Mortierella alpina*. Extraction of the mycelium after submerged fermentation yields a triglyceride oil, containing 30–50% ARA. This triglyceride source of ARA could be used in infant formulae to provide a LC-PUFA composition, which closely matches that of human milk.

Because the ARA-oil is intended for use as an ingredient in infant formulae for pre-term and term babies, great emphasis was placed on safety testing. The starting assumption was that ARA itself is safe at the levels found in human milk. Therefore, the safety assessment was focused on the source from which the oil is derived, and on possible toxic residual levels of fermentation by-products and contaminants in the oil preparation. The safety assessment included a safety evaluation on the fungus *M. alpina*, involving an extensive literature overview and HPLC analysis of metabolites produced by the production strain under various conditions. It was concluded that the production organism, *M. alpina*, was safe for the submerged production of nutritional ingredients (Streekstra, 1997). In addition, mutagenicity tests and oral toxicity tests were carried out with the ARA-oil. The oil was not mutagenic and did not cause adverse effects when administered to rats for 4 wk at levels up to 3000 mg/kg body weight (Hempenius *et al.*, 1997). The objective of this study was to further evaluate the safety of the ARA-oil for use in infant nutrition in a subchronic (90-day) oral toxicity study. The study was preceded by an *in utero* exposure phase to mimic the intended exposure of infants to the oil, namely directly after delivery. As a result of the fact that repeated dosing of high concentrations of ARA may result in adverse effects, due to its involvement in prostaglandin synthesis, the highest concentration of ARA-oil was also tested in combination with a DHA containing fish oil. The addition of the DHA containing fish oil was intended to compensate for any adverse effects, since fish oil fatty acids are known to reduce the prostaglandin synthesis by competing with ARA for the enzyme cyclo-oxygenase (De Vries and Van Noorden, 1992). The combination ARA and DHA was tested at a similar ratio to that which is found for these compounds in human breast milk, namely 2:1 (Kohn *et al.*, 1994).

MATERIALS AND METHODS

The study was conducted at TNO Nutrition and Food Institute, Zeist, The Netherlands, in accordance with the OECD Principles of Good Laboratory Practice and met the requirements of the US Food and Drug Administration guidelines for the testing of food additives (FDA, 1982), the OECD guidelines for testing chemicals (OECD 408, 1981) and the EC Guidelines (87/302/EC, 1988).

Test materials

The ARA-oil was a triglyceride oil extracted from the fungus *Mortierella alpina* according to the procedure for commercial production. In short, after submerged fermentation the oil was solvent-

extracted from the dried biomass and purified by traditional edible oil refining techniques. Analysis of the antibiotic activity, microbial contamination, mycotoxins and heavy metals showed that the oil complied with the Joint FAO/WHO Expert Committee on Food Additives specifications (JECFA, 1992) for enzymes derived from microbial sources. The ARA-oil contained 38.6% arachidonic acid and the non-saponifiable fraction (predominantly sterols) was 1.6 g per 100 g oil. The DHA-oil was derived from fish oil (Pronova, Biocare a.s. Norway) and contained approximately 26.6% DHA. The fatty acid composition of both oils is shown in Table 1.

Animals

Wistar outbred rats (Crl:(WI)WU BR) were obtained from a colony maintained under SPF-conditions at Charles River Wiga GmbH, Sulzfeld, Germany. The animals were acclimatized for approximately 17 days to the laboratory conditions. To form the F_0 group rats were allocated to five groups (28 females and 14 males per group, except for the low-fat control group which consisted of 14 females and seven males, because this group served only as historical control) by computer randomization and proportionally to body weight. At the start of the premating period, the rats were 10–11 wk old.

To form the F1 groups, for the subchronic study, 20 males and 20 females of each group were randomly selected from the litters in such a way that no more than one animal/sex/litter was included in any group. At the start of the subchronic study the rats were 20–31 days old.

Table 1. Fatty acid profile of the ARA and DHA-oil

	Fatty acid level (% of total fatty acids)	
Fatty acid	ARA-oil	DHA-oil
C14:0	2.4	3.5
C15:0	0.2	1.0
C16:0	16.4	18.0
C16:1	0.1	5.2
C17:0	0.4	1.1
C17:1	0.0	0.6
C18:0	11.4	4.9
C18:1 (n-9)	11.7	14.4
C18:2 (n-6)	6.9	1.4
C18:3 (n-6)	3.4	0.2
C20:0	0.9	0.3
C20:1 (n-9/C18:3 (n-3)	0.4	0.9
C20:2 (n-6)	0.3	0.3
C20:3 (n-6)	3.6	0.1
C20:4 (n-6)	38.6	2.0
C20:5 (n-3)	0.1	6.8
C22:0	1.5	0.2
C22:1 (n-9)	0.0	0.0
C22:4 (n-6)	0.2	0.2
C22:5 (n-3)	0.0	1.3
C22:6 (n-3)	0.0	26.6
C24:0	1.5	0.2

Animal care

The rats were housed in suspended, stainless-steel cages fitted with wire mesh floor and front. Housing conditions were conventional. The parental rats were housed per cage during the different stages as follows:

- premating period: three or four rats from the same group per cage separated by sex;
- mating period: two females were caged with one male from the same group until pregnancy occurred;
- gestation and lactation period: dams were housed individually.

In the subchronic study rats were housed five per cage. The temperature in the animal room was generally between 20°C and 23°C. The relative humidity was generally between 40% and 70%. Lighting was artificial by fluorescence tubes and time switch controlled to a sequence of 12 hr light, 12 hr dark. Drinking water [N.V. Waterleiding bedrijf Midden-Nederland (WMN)] was provided *ad lib*. The drinking water was given in polypropylene bottles, which were cleaned weekly and filled up when necessary. The animals were fed the Institute's cereal-based rodent diet, containing crude fat 5,6%, crude protein 20.3% and crude fibre 2.7% (SDS Special Diets Services, Witham, UK). From the start of the treatment period, the Institute's rodent diet was supplemented with ARA-oil, DHA-oil and/or corn-oil.

Experimental design

The study consisted of two phases: (1) an *in utero* phase, in which the F_0 animals received the test substances during the premating, mating, gestation and lactation periods; and (2) a subchronic toxicity phase, in which the selected offspring (F_1 animals) from the *in utero* phase received the test substances for a 13-wk period.

There were two control groups incorporated in the study, namely a carrier control group, which received the standard rodent diet without additions to serve as historical reference group, and a high-fat control group, fed a diet with a fat level, provided by corn oil, similar to the highest dose of test material. Three experimental groups received low, mid and high levels of ARA-oil and one group received the highest level of ARA-oil in combination with DHA-oil at a ratio of 2:1, similar to that which is found in human milk. The addition of the DHA-oil was intended to compensate for any adverse effects due purely to the adminstration of high level of ARA-oil. From the start of the premating period (F_0 male and female rats), throughout mating, gestation and lactation, until termination of the treatment of the F_1 animals, the test substances were administrated at constant concentrations in the feed.

Experimental diet

The study comprised six groups, namely a carrier control group, a high-fat control group (130,000 ppm corn oil) control group, three groups receiving different levels of ARA-oil, namely 3000 ppm, 15,000 ppm or 75,000 ppm ARA-oil (made up with corn oil to a total of 130,000 ppm added fat), and one group receiving a mixture of ARA- and DHA-oil, namely 75,000 ppm ARA-oil and 55,000 ppm DHA-oil. The Institute's cereal based rodent diet was used as carrier. The total level of added fat in each test diet and in the corn oil control diet was kept constant (130 g/kg diet) by adding appropriate amounts of corn oil. As it is known that rats consume food to a specific caloric intake the food consumption will be reduced when fed high-fat diets. Therefore, we used for this study a standard rodent diet rich on the macro- and micronutrients to ensure adequate intake of essential nutrients from the experimental diet. The test and control diets were stored in a freezer. Daily, portions of the diets were thawed and fed to the rats.

The stability of the test substance in the diet in the animal room and in the freezer was investigated before the start of the study by quantitative determination of the levels of ARA and/or DHA. The test substances were stable in the rodent diet at all dose levels examined on storage for up to 4 days at room temperature, or for nearly 8 wk in a freezer. During the study, the stability of the diets under storage conditions in the freezer was confirmed for each batch of diets prepared by quantitative determination of ARA and/or DHA after the maximum storage period. The homogeneity and levels of ARA and/or DHA were confirmed in the first batch of diets prepared in the study, by analysing five samples per test diet.

In utero phase

After a premating period of 4 wk, during which the rats were fed their respective test diets, two females were caged with one male from the same group until pregnancy occurred, or until 2 wk had elapsed. During the mating period, vaginal smears were made daily to determine whether sperm were present. The day on which either sperm or a vaginal plug were observed was considered day 0 of pregnancy. Upon evidence of copulation, the females were caged individually for the birth and rearing of their pups and throughout lactation.

On day 4 *post partum*, litters of more than eight pups were adjusted by eliminating extra pups by random selection, to yield (as nearly as possible) four males and four females per litter. At day 21 *post partum*, the F_1 litters were weaned. 20 males and 20 females were selected randomly from 20 litters, except for the ARA-oil low-dose group, which comprised only 18 litters with live pups on day 21 of lactation. Two litters were not used because one contained only three animals and another litter was born 1 wk after the other litters. For the carrier control group, 10 males and 10 females were selected from seven litters, since this group contained only eight litters and one was not used. During the period between weaning and the start of the subchronic study, feeding of the various test diets to these F_1 rats was continued. Parental rats and the remaining pups were sacrificed.

Observations: in utero phase

Clinical signs. During premating, mating, gestation and lactation, the general condition and behaviour of all animals were checked daily.

Body weight. Body weight of each animal was recorded when starting the administration of the test substances and once every week thereafter. Mated females were weighted on days 0, 7, 14 and 21 of gestation and on days 1, 4, 7, 14 and 21 of lactation.

Food consumption. Food consumption was measured weekly on a cage basis, during the premating period in both males and females. Food consumption of mated females was recorded during pregnancy on days 7, 14, 21 and during lactation on days 4, 7, 14 and 21.

Reproductive indices. Reproductive indices with respect to fertility and reproductive performance were determined for each group:

Pup observations. Pups were checked on viability daily and observations of appearance of pups were carried out on days 1, 4, 7, 14 and 21 of lactation. The weight of the litters as a whole was recorded on days 1, 4 (before and after culling), 7 and 14 *post partum*, mean pup weight was calculated. The weight of individual pups was recorded on day 21.

Observations: subchronic study

Clinical signs. The general condition and behaviour of all animals were checked daily. Ophthalmoscopic observations were made prior to the start of the study and towards the end of the treatment period (day 84) in all rats in the corn oil control group, the ARA-oil high-dose group and the ARA/DHA-oil group.

Body weight. Body weight of each animal was recorded at the start of the subchronic study (day 0) and once every week thereafter.

Food and water consumption. Quantity of food consumed by the animals in each cage was measured weekly on a cage by cage basis and the efficiency of food utilization was calculated. The water consumption of the animals was measured per cage on 4 consecutive days in wk 2, 6 and 11 of the study.

Test substance intake. For each test group, the intake of ARA-oil or DHA-oil/kg body weight/day, was assessed on the basis of weekly food intake, body weight (mean of the body weights measured

at the start and the end of each week), and nominal dietary supplementation of the test substances.

Haematology and clinical chemistry. At autopsy under anaesthesia at the end of treatment, blood was collected from the abdominal aorta of 10 rats/sex/group and used for haematological measurements; blood plasma was used for clinical chemistry. On day 87–88 of the study, the same 10 rats/sex/group were deprived of water for 24 hr and of food during the last 16 hr of this period. Glucose in this group was determined in blood collected from the tip of the tail after the deprivation period.

Renal concentration test and urinalysis. During the last 16 hr of deprivation, the rats were kept in metabolism cages (one rat per cage) and urine was collected. The concentrating ability of the kidneys was investigated by measuring the urinary volume and density. In the urine collected from the rats in the renal concentration test, semiquantitative determinations and microscopy of the sediment were carried out.

Autopsy. After completion of the 13-wk treatment period, the animals were killed by exsanguination from the abdominal aorta under ether anaesthesia and examined macroscopically for pathological changes. Organs of all surviving animals were weighed and the relative organ weights (g/kg body weight) were calculated on the basis of the final body weights of the rats. Samples of the complete spectrum of organs and tissues as required by OECD, EC and FDA of all animals were preserved in a neutral aqueous phosphate buffered 4% solution of formaldehyde. Histopathological examination was performed on the tissues and organs of all animals of the corn oil control group and the ARA high-dose group. The kidneys, livers, lungs, small intestines, including Peyer's patches, mesenteric lymph nodes and any gross lesions were also examined microscopically in all rats of the ARA low- and mid-dose groups and the ARA/DHA group. Histopathological examination was not conducted in rats of the carrier control group, except for the examination of brain, spinal cord, small intestines, Peyer's patches and mesenteric lymph nodes in both sexes and of the liver in females.

Neurotoxicity screening. As a neurotoxicity screen the following observations were included: histopathological examination of tissue samples representative of major areas of the brain, spinal cord and peripheral nerve, a functional evaluation battery of observations and tests selected to detect signs of neurological, behavioural and physiological dysfunction. The screen was carried out in wk 1 and 12 of the study in 10 males and 10 females of each group.

Statistical analysis

Analysis of covariance was used for evaluating body weights in the subchronic study. Analysis of variance followed by Dunnett's multiple comparison tests was used for body weight and body weight changes, food consumption, food efficiency, water consumption, pup body weight, red blood cell and clotting potential, total white blood cell counts, absolute differential white blood cell counts, clinical chemistry values, volume and density of the urine and organ weights. Fisher's exact probability test was used for the evaluation of the numbers of mated and pregnant females, females with liveborn pups, females surviving delivery, females with stillborn pups or lost litters, liveborn and stillborn pups, pups lost at various stages, pups surviving 21 days and for pathological changes. Kruskal–Wallis non-parametric analysis of variance followed by the Mann–Whitney U-test was used for evaluating precoital time, duration of gestation and litter size and for evaluating reticulocytes, differential white blood cell counts (percentages) and semi-quantitative urinary observations (or the Mann–Whitney U-test only for comparison between the corn oil controls and the carrier controls). The test groups and the carrier control group were compared separately with the corn oil control group.

RESULTS

In utero and lactation phase

Clinical examinations, growth, food intake. General condition and behaviour of parental rats were not adversely affected by the test substances in any of the groups. None of the rats died prematurely. During premating and mating, overall body weight gain in females of the ARA/DHA group was statistically significantly lower than in the corn oil control group. Body weight gain in females of the carrier control group was lower than in the "high-fat" groups. During the gestation period, body weight gain in females of the ARA high-dose group and the ARA/DHA group was statistically significantly lower than in corn oil control group in the first week. Body weight gain in the carrier control group was lower than in the "high-fat" groups. On day 1 of lactation, mean body weights were decreased in the ARA/DHA group and in the carrier control group. At the end of the lactation period, however, these differences had disappeared and maternal weights were similar in all groups. Food consumption was decreased in the ARA/DHA group in both sexes during the first week of the premating period. Mean food consumption in the carrier control group was higher than in the "high-fat" groups during mating, gestation and lactation.

Reproduction indices. There were no treatment-related differences in fertility or reproductive performance among the ARA groups, the ARA/DHA group and the corn oil control group. Mating, fertility, gestation, birth and viability indices, as well as precoital and gestation times, were comparable

among the test and control groups, and no statistically significant differences in these variables occurred. All these reproduction variables were normal for rats of this strain. None of the pregnant females died. In the carrier control group, fertility was relatively low but the difference in relation to the corn oil control group was not statistically significant. In addition, the total number of pups delivered and the number of pups per litter before culling was decreased in the carrier control group (Table 2).

Observations in pups. Viability check did not reveal any treatment-related differences. The number of live pups per litter was comparable between the ARA groups, the ARA/DHA group and the corn oil control group. Sex ratio was similar in all groups (Table 2). At delivery, pups in the carrier control group were heavier than pups in the "high-fat" groups, probably as a result of the smaller size of the carrier control litters. At the end of the lactation period, pup weight in the carrier control group was comparable to that in the corn oil control group. Pup weight gain in the ARA/DHA group was decreased from day 7 of lactation. Pup weight in this group was statistically significantly lower than in corn oil control group from day 14 of lactation, namely 10% lower. General condition of pups was not adversely affected by ARA-oil or the combination ARA/DHA-oil.

Subchronic study (F_1 rats)

Clinical examinations, growth, food and water intake. General condition and behaviour were not adversely affected by the test substance in any of the groups. One male rat of the ARA high-dose group was killed on day 67 of the study because of deterioration of condition. Upon microscopic examination, this rat showed severe pyelonephritis. As similar findings were not observed in any other rats in this group, the death of this male was not considered to be treatment related. Ophthalmoscopic examination did not reveal any treatment-related changes.

At the start of the study, mean body weight in the ARA/DHA group tended to be lower than the corn oil control group, but the differences were not statistically significant. There were no dose-related differences in body weight gain between the test groups and the corn oil control group. Males of the carrier (low-fat) control group showed statistically significant increased body weights when compared to the corn oil control group throughout the study. A similar tendency was observed in females of this group in the first few weeks of the study.

Mean food intake and food conversion efficiency did not show consistent differences between the test groups and the corn oil control group. Food intake tended to be higher and food conversion efficiency tended to be lower in males and females of the carrier control group as compared to the corn oil control group. The intake of the test substance per kg body weight per day decreased as the study progressed. For each group the mean intake and the intake during the first and the last week are presented in Table 3.

Water intake in wk 2 and 6 was comparable among the test groups and the corn oil controls. In week 11, water consumption was slightly increased in the ARA/DHA group, but the differences with the corn oil control group were generally not statistically significant. In the carrier control group, water consumption tended to be higher than in the corn oil control group.

Haematology. Packed cell volume was decreased and mean corpuscular haemoglobin concentration (MCHC) increased in males of the ARA/DHA group compared to the corn oil control group. In males of the ARA high-dose group, MCHC was increased. The above changes were only slight and other values obtained for red blood cell variables did not show any significant changes. In the carrier control group, increases were observed in prothrombin time in males and in haemoglobin concentration and mean corpuscular haemoglobin (MCH) in females (Tables 4 and 5). There were no statistically significant changes in total white blood cell

Table 2. Natural delivery and litter data[1]

	Control		Dietary concentration test substance (ppm)			
			ARA-oil		ARA/DHA-oil	
Parameter	Low-fat	High-fat	3000	15,000	75,000	75,000/55,000
Litter size at birth (total pups)	9.00 ± 1.21	11.41 ± 0.64	10.21 ± 0.808	11.24 ± 0.417	11.00 ± 0.451	11.04 ± 0.366
Live born index[2]	100	99	99	99	99	99
Litter size at day 4 pre-cull	9.00 ± 1.21	11.71 ± 0.426	10.61 ± 0.647	10.96 ± 0.422	10.82 ± 0.425	10.73 ± 0.398
Viability index day 4-21[3]	100	100	99	99	100	100
Sex ratio[4]						
birth	50	55	47	58	51	53
day 21	52	52	47	53	52	50

[1]Results are mean per litter ± standard deviation with between 18 and 26 litters per group, except for the low-fat control group (eight litters). [2]Liveborn index: number of pups born alive *100/total number of pups born. [3]Viability index days 4–21: number of pups surviving 21 days/number of liveborn pups are culling at day 4. [4]Sex ratio: number of male pups date n *100/total number of pups.

Table 3. Mean intake of ARA-oil or DHA-oil over a 13-wk period

	Intake of ARA-oil or ARA/DHA-oil (g/kg bw/day)			
	Male rats		Female rats	
	Mean	First week–last week	Mean	First week–last week
Low-fat controls	NA[1]	NA	NA	NA
High-fat controls: 130,000 ppm corn oil	NA	NA	NA	NA
Low-dose: 3,000 ppm ARA-oil + 127,000 ppm corn oil	0.19[2]	0.38–0.12[2]	0.19[2]	0.38–0.13[2]
Mid-dose: 15,000 ppm ARA-oil + 115,000 ppm corn oil	0.96[2]	1.96–0.56[2]	0.98[2]	1.88–0.64[2]
High-dose: 75,000 ppm ARA-oil + 55,000 ppm corn oil	4.74[2]	9.39–2.93[2]	4.86[2]	9.17–3.29[2]
High-dose: 75,000 ppm ARA-oil + 55,000 ppm DHA-oil	4.88[2]/3.58[3]	9.42–2.94[2]/6.91–2.16[3]	5.00[2]/3.67[3]	9.17–3.31[2]/6.72–2.43[3]

[1]NA: not applicable. [2]ARA-oil. [3]DHA-oil.

counts or in differential white blood cell counts in any group.

Clinical chemistry. A number of statistically significant differences between the test groups and the corn oil control group were observed in the clinical chemistry variables, as shown in Table 6 (males) and Table 7 (females). Alkaline phosphatase activity was decreased in males and females of the ARA high-dose group. Cholesterol concentration was decreased in the ARA high-dose group in females and in the ARA/DHA group in both sexes. Triglycerides were decreased in males of the ARA high-dose group and the ARA/DHA group. A similar tendency was observed in females of these groups. Phospholipids were decreased in the ARA high-dose group and in the ARA/DHA high-dose group in both sexes. Creatinine concentration was increased in males of the ARA high-dose group and the ARA/DHA group. Urea concentration was increased in males of the ARA/DHA group. In the carrier group, decreases were observed in alkaline phosphatase activity and urea concentration in both sexes, while the albumin/globulin ratio was decreased in males, and the bilirubin, triglyceride and phospholipid concentration and alanine aminotransferase activity were increased in females, as compared to the corn oil control group.

Renal concentration test and urinalysis. The renal concentration test showed an increased volume and decreased density in the ARA high-dose group in both sexes and in the ARA/DHA group in males as compared to the corn oil control group. The urinary volume was also higher in males and females of the carrier control group than in corn oil control group, but the density was not affected in this group. There were no significant differences in semi-quantitative observations in the urine or in the microscopy of the urinary sediment among the groups.

Organ weights. The relative weight of the spleen was increased in males and females of the ARA high-dose group and the ARA/DHA group (not statistically significant in males of the ARA high-dose group) as shown in Table 8 (males) and Table 9 (females). The relative weight of the liver was increased in females of the ARA high-dose group and the ARA/DHA group. The relative weight of the adrenals was increased in females of the ARA/DHA group. In females of the carrier control group, the weights of the kidneys and liver

Table 4. Haematological parameters in 10 male rats/group after feeding ARA-oil or ARA/DHA-oil for 13 wk (mean ±SEM)

	Control		Dietary concentration test substance (ppm)			
			ARA-oil		ARA/DHA-oil	
Parameter	Low-fat	High-fat	3000	15,000	75,000	75,000/55,000
Red blood cells (10^{12}/litre)	7.69 ± 0.05	7.86 ± 0.08	7.88 ± 0.08	8.00 ± 0.11	7.71 ± 0.07	7.67 ± 0.09
Haemoglobin (mmol/litre)	9.4 ± 0.1	9.2 ± 0.1	9.0 ± 0.1	9.2 ± 0.1	9.0 ± 0.1	8.9 ± 0.1
Packed cell volume (litre/litre)	0.421 ± 0.004	0.416 ± 0.007	0.402 ± 0.005	0.408 ± 0.003	0.397 ± 0.006	0.394 ± 0.006*
Reticulocytes/1000	12.6 ± 1.9	14.4 ± 2.8	9.8 ± 1.0	12.0 ± 1.2	14.0 ± 1.7	14.0 ± 2.2
Mean corpuscular volume (fl)	52.9 ± 0.5	52.9 ± 0.8	51.1 ± 0.4	51.0 ± 0.7	51.4 ± 0.5	51.4 ± 0.7
Mean corpuscular haemoglobin concentration (mmol/litre)	22.2 ± 0.1	22.1 ± 0.2	22.4 ± 0.1	22.5 ± 0.2	22.7 ± 0.1*	22.7 ± 0.1*
Thrombocytes (10^9/litre)	983 ± 23	921 ± 27	924 ± 26	973 ± 32	961 ± 25	896 ± 24
Prothrombin time (sec)	41.8 ± 0.5##	39.3 ± 0.5	38.8 ± 0.8	39.3 ± 0.7	39.8 ± 0.4	39.8 ± 0.8

Statistically significant differences between the test groups and the high-fat (corn oil). Control group are indicated with $^*P < 0.05$. Statistically significant differences between the low-fat (carrier) control group and the high-fat (corn oil) control group are indicated with $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

Table 5. Haematological parameters[1] in 10 female rats/group after feeding ARA-oil or ARA/DHA-oil for 13 wk (mean ±SEM)

	Control		Dietary concentration test substance (ppm)			
			ARA-oil		ARA/DHA-oil	
Parameter	Low-fat	High-fat	3000	15,000	75,000	75,000/55,000
Red blood cells (10^{12}/litre)	7.16 ± 0.08	7.16 ± 0.10	7.16 ± 0.15	7.22 ± 0.10	6.87 ± 0.21	7.04 ± 0.10
Haemoglobin (mmol/litre)	9.0 ± 0.1#	8.7 ± 0.1	8.7 ± 0.1	8.9 ± 0.1	8.5 ± 0.1	8.5 ± 0.1
Packed cell volume (litre/litre)	0.402 ± 0.005	0.388 ± 0.006	0.387 ± 0.007	0.396 ± 0.006	0.376 ± 0.010	0.372 ± 0.005
Mean corpuscular volume (fl)	56.2 ± 0.7	54.3 ± 0.9	54.2 ± 0.6	54.9 ± 0.6	54.8 ± 0.6	52.9 ± 0.7
Mean corpuscular haemoglobin (fmol)	1.26 ± 0.01##	1.21 ± 0.01	1.22 ± 0.01	1.23 ± 0.01	1.24 ± 0.01	1.22 ± 0.02
Mean corpuscular haemoglobin concentration (mmol/litre)	22.4 ± 0.2	22.4 ± 0.2	22.5 ± 0.1	22.5 ± 0.2	22.6 ± 0.1	23.0 ± 0.2
Thrombocytes (10^9/litre)	875 ± 27	858 ± 9	861 ± 24	828 ± 16	891 ± 35	834 ± 24
Prothrombin time (sec)	37.2 ± 0.7	34.9 ± 0.8	36.0 ± 0.5	34.6 ± 0.3	34.7 ± 0.6	34.7 ± 0.4

[1]Reticolocytes were not counted for females. Statistically significant differences between the test groups and the high-fat (corn oil) control group are indicated with $*P < 0.05$; $**P < 0.01$. Statistically significant differences between the low-fat (carrier) control group and the high-fat (corn oil) control group are indicated with $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

were increased as compared to the corn oil control group.

Pathology—macroscopic examination. Gross examination of the surviving rats at autopsy did not reveal any treatment-related changes.

Pathology—microscopic examination. The mesenteric lymph nodes of most males and several females of the ARA high-dose group and the ARA/DHA group contained focal aggregates of oil droplets. Oil droplets were also present in the tips of the villi of the small intestine of many animals of the ARA high-dose group and the ARA/DHA group. This histopathological change was not present in any animal of the other groups, except for one male in the corn oil group. Oil droplets were also observed in the Peyer's patches of the small intestine in several rats of all groups, including the corn oil control group, but not in the carrier control group. In addition, a number of the animals had lipogranulomas in either the Peyer's patches or in the mesenteric lymph nodes. A rare animal in the ARA high-dose group or in the ARA/DHA group had focal, slight inflammation in the Peyer's patches. Some rats of the carrier control group exhibited mineralisation of the Peyer's patches. It appeared that mineralization was almost absent in the treated animals. The significance of this condition is unknown, but it is apparently not due to the administration of ARA-oil or DHA-oil.

Several males and females of the corn oil control group and the ARA high-dose group exhibited vacuoles in the brain, especially in the white matter

Table 6. Clinical chemistry in plasma in 10 male rats/group after feeding ARA-oil or ARA/DHA-oil for 13 wk (mean ± SEM)

	Control		Dietary concentration test substance (ppm)			
			ARA-oil		ARA/DHA-oil	
Parameter	Low-fat	High-fat	3000	15,000	75,000	75,000/55,000
Glucose (mmol/litre)[1]	3.93 ± 0.09	3.87 ± 0.10	3.80 ± 0.09	3.88 ± 0.09	4.04 ± 0.13	4.13 ± 0.05
Alkaline phosphatase (U/litre)	134 ± 8##	227 ± 14	222 ± 13	192 ± 11	179 ± 6*	248 ± 9
Alanine aminotransferease (U/litre)	30 ± 3	33 ± 2	31 ± 1	28 ± 1	29 ± 1	31 ± 1
Aspartate aminotransferase (U/litre)	73 ± 4	72 ± 3	70 ± 3	68 ± 3	69 ± 6	79 ± 4
Gamma-glutamyl transferase (U/litre)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total protein (g/litre)	67 ± 0	66 ± 1	64 ± 1	66 ± 1	66 ± 1	65 ± 1
Albumin (g/litre)	35 ± 0	36 ± 0	35 ± 0	35 ± 0	35 ± 0	36 ± 1
Albumin/globulin ratio	1.13 ± 0.01#	1.19 ± 0.02	1.17 ± 0.02	1.16 ± 0.02	1.13 ± 0.01	1.21 ± 0.02
Urea (mmol/litre)	7.1 ± 0.2##	5.3 ± 0.2	5.5 ± 0.1	5.4 ± 0.2	5.6 ± 0.1	6.0 ± 0.1*
Creatinine (μmol/litre)	28 ± 1	27 ± 1	29 ± 1	30 ± 0	31 ± 1*	32 ± 1**
Total bilirubin (μmol/litre)	1.6 ± 0.1	2.0 ± 0.2	1.6 ± 0.1	1.7 ± 0.1	1.4 ± 0.1	1.6 ± 0.2
Cholesterol (mmol/litre)	1.95 ± 0.11	2.02 ± 0.07	2.10 ± 0.11	2.21 ± 0.08	1.82 ± 0.07	1.37 ± 0.08**
Triglycerides (mmol/litre)	2.73 ± 0.19	1.98 ± 0.38	1.51 ± 0.12	1.58 ± 0.11	1.00 ± 0.05**	1.00 ± 0.18**
Phospholipids (mmol/litre)	2.28 ± 0.09	2.04 ± 0.10	2.04 ± 0.10	2.13 ± 0.06	1.66 ± 0.05**	1.43 ± 0.05**
Calcium (mmol/litre)	2.79 ± 0.02	2.75 ± 0.04	2.74 ± 0.03	2.76 ± 0.03	2.74 ± 0.03	2.73 ± 0.03
Potassium (mmol/litre)	3.6 ± 0.1	3.7 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	4.1 ± 0.1
Sodium (mmol/litre)	147 ± 0	147 ± 1	146 ± 0	147 ± 0	147 ± 0	146 ± 1
Chloride (mmol/litre)	106 ± 0	107 ± 0	107 ± 0	107 ± 0	107 ± 0	106 ± 0
Inorganic phosphate (mmol/litre)	1.78 ± 0.10	1.63 ± 0.09	1.72 ± 0.12	1.61 ± 0.08	1.68 ± 0.08	1.83 ± 0.10

[1]Fasting glucose was determined in blood collected after overnight fasting in wk 12. Statistically significant differences between the test groups and the high-fat (corn oil) control group are indicated with $*P < 0.05$; $**P < 0.01$. Statistically significant differences between the low-fat (carrier) control group and the high-fat (corn oil) control group are indicated with $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

Table 7. Clinical chemistry in plasma in 10 female rats/group after feeding ARA-oil or ARA/DHA-oil for 13 wk (mean ±SEM)

	Control		Dietary concentration test substance (ppm)			
			ARA-oil		ARA/DHA-oil	
Parameter	Low-fat	High-fat	3000	15,000	75,000	75,000/55,000
Glucose (mmol/litre)[1]	3.91 ± 0.11	3.81 ± 0.15	3.83 ± 0.08	4.00 ± 0.13	3.97 ± 0.08	4.25 ± 0.13
Alkaline phosphatase (U/litre)	130 ± 6##	199 ± 19	200 ± 14	186 ± 10	145 ± 7*	172 ± 11
Alanine aminotransferease (U/litre)	41 ± 5#	30 ± 2	28 ± 1	29 ± 1	27 ± 1	27 ± 2
Aspartate aminotransferase (U/litre)	69 ± 4	64 ± 2	61 ± 2	62 ± 3	63 ± 3	67 ± 3
Gamma glutamyl transferase (U/litre)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Total protein (g/litre)	64 ± 1	64 ± 1	64 ± 1	64 ± 1	65 ± 1	66 ± 0
Albumin (g/litre)	36 ± 0	37 ± 0	37 ± 0	37 ± 0	37 ± 1	39 ± 1
Albumin/globulin ratio	1.33 ± 0.02	1.39 ± 0.02	1.38 ± 0.02	1.39 ± 0.03	1.32 ± 0.03	1.40 ± 0.03
Urea (mmol/litre)	8.3 ± 0.7##	5.7 ± 0.3	6.3 ± 0.3	7.1 ± 0.4	7.4 ± 0.5	6.8 ± 0.6
Creatinine (μmol/litre)	29 ± 1	30 ± 2	33 ± 2	35 ± 2	37 ± 2	34 ± 2
Total bilirubin (μmol/litre)	6.3 ± 2.2#	1.3 ± 0.2	2.3 ± 0.5	1.8 ± 0.4	1.3 ± 0.1	1.5 ± 0.3
Cholesterol (mmol/litre)	1.77 ± 0.09	1.76 ± 0.07	1.68 ± 0.07	1.87 ± 0.08	1.48 ± 0.07*	1.39 ± 0.08**
Triglycerides (mmol/litre)	1.42 ± 0.17##	0.80 ± 0.09	0.85 ± 0.15	0.89 ± 0.12	0.72 ± 0.08	0.58 ± 0.05
Phospholipids (mmol/litre)	2.28 ± 0.10##	1.94 ± 0.06	1.91 ± 0.06	2.09 ± 0.08	1.68 ± 0.07*	1.54 ± 0.06**
Calcium (mmol/litre)	2.66 ± 0.02	2.61 ± 0.03	2.66 ± 0.02	2.65 ± 0.03	2.64 ± 0.03	2.60 ± 0.03
Potassium (mmol/litre)	3.2 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.4 ± 0.1	3.3 ± 0.1
Sodium (mmol/litre)	146 ± 1	145 ± 0	146 ± 0	146 ± 0	146 ± 0	146 ± 0
Chloride (mmol/litre)	107 ± 0	108 ± 0	108 ± 0	108 ± 0	109 ± 0	109 ± 1
Inorganic phosphate (mmol/litre)	1.68 ± 0.14	1.30 ± 0.15	1.39 ± 0.15	1.41 ± 0.15	1.33 ± 0.19	1.34 ± 0.16

[1]Fasting glucose was determined in blood collected after overnight fasting in wk 12. Statistically significant differences between the test groups and the high-fat (corn oil) control group are indicated with $*P < 0.05$; $**P < 0.01$. Statistically significant differences between the low-fat (carrier) control group and the high-fat (corn oil) control group are indicated with $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

of the cerebellum, and in the spinal cord. On account of these findings it was decided to process and examine the brains and spinal cord of the carrier (low-fat) control group as well, but vacuolation was not seen in these organs in any rat of this group. In addition, frozen slides of the brain were made and stained with Cong Red for two animals that showed clear vacuolation in haematoxylin/eosin stained brain sections. The result was negative, implicating that the vacuoles did not contain fat. As vacuoles occurred both in the ARA high-dose group and in the corn oil (high-fat) control group, and their incidence was lower in the ARA high-dose group than in the corn oil control group, they are not ascribed to treatment with ARA-oil.

In females, a dose-dependent increase of heptocellular vacuolation in the liver was observed, being statistically significant in females of the ARA high-dose group and the ARA/DHA group. Hepatocellular vacuolation was not present in any female of the carrier control group. Vacuolation was also present in the liver of about one-third of the males of all ARA groups and the corn oil control group, but surprisingly, absent in the ARA/DHA group. The incidence of mononuclear-cell infiltration in the liver was slightly increased in

Table 8. Terminal body weights and relative organ weights in 20 male rats/group after feeding ARA-oil or ARA/DHA-oil for 13 wk (mean ±SEM)

	Control		Dietary concentration test substance (ppm)			
			ARA-oil		ARA/DHA-oil	
Parameter	Low-fat	High-fat	3000	15,000	75,000	75,000/55,000
Mean weights (g)						
Terminal body weight	445.1 ± 7.4##	395.6 ± 6.7	411.4 ± 9.6	414.1 ± 7.7	411.2 ± 7.2	400.3 ± 6.6
Testes	7.75 ± 0.15	7.82 ± 0.35	7.89 ± 0.16	7.64 ± 0.17	8.40 ± 0.15	8.16 ± 0.15
Adrenals	0.112 ± 0.004	0.117 ± 0.004	0.106 ± 0.004	0.109 ± 0.003	0.108 ± 0.003	0.113 ± 0.002
Kidneys	4.90 ± 0.12	4.73 ± 0.07	4.66 ± 0.09	4.53 ± 0.08	4.62 ± 0.07	4.67 ± 0.07
Thymus	1.45 ± 0.09	1.28 ± 0.05	1.31 ± 0.07	1.42 ± 0.07	1.26 ± 0.07	1.49 ± 0.07
Brain	4.45 ± 0.07#	4.72 ± 0.06	4.69 ± 0.10	4.56 ± 0.08	4.67 ± 0.10	4.68 ± 0.07
Spleen	1.52 ± 0.05	1.59 ± 0.04	1.50 ± 0.03	1.52 ± 0.04	1.68 ± 0.04	1.77 ± 0.04**
Heart	2.96 ± 0.09	2.82 ± 0.07	2.64 ± 0.05*	2.72 ± 0.04	2.80 ± 0.04	2.89 ± 0.04
Liver	35.3 ± 1.1	33.3 ± 0.6	32.0 ± 0.3	33.5 ± 0.6	32.7 ± 0.3	33.6 ± 0.4

Statistically significant differences between the test groups and the high-fat (corn oil) control group are indicated with $*P < 0.05$; $**P < 0.01$. Statistically significant differences between the low-fat (carrier) control group and the high-fat (corn oil) control group are indicated with $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

Table 9. Terminal body weights and relative organ weights in 20 female rats/group after feeding ARA-oil or ARA/DHA-oil for 13 wk (mean ±SEM)

	Control		Dietary concentration test substance (ppm)			
			ARA-oil		ARA/DHA-oil	
Parameter	Low-fat	High-fat	3000	15,000	75,000	75,000/55,000
Mean weights (g)						
Terminal body weight	247.5 ± 6.9	245.2 ± 5.5	244.5 ± 4.6	253.7 ± 5.0	242.9 ± 4.1	246.1 ± 3.7
Ovaries	0.296 ± 0.014	0.317 ± 0.012	0.303 ± 0.010	0.295 ± 0.011	0.317 ± 0.012	0.308 ± 0.012
Adrenals	0.232 ± 0.012	0.221 ± 0.008	0.224 ± 0.005	0.209 ± 0.008	0.244 ± 0.006	0.268 ± 0.009*
Kidneys	5.84 ± 0.14##	5.11 ± 0.09	5.22 ± 0.08	5.16 ± 0.11	5.14 ± 0.08	5.28 ± 0.09
Thymus	1.54 ± 0.07	1.57 ± 0.07	1.47 ± 0.06	1.56 ± 0.06	1.60 ± 0.06	1.50 ± 0.05
Brain	7.20 ± 0.16	7.07 ± 0.16	7.09 ± 0.11	6.75 ± 0.15	7.13 ± 0.11	7.07 ± 0.11
Spleen	1.92 ± 0.06	1.82 ± 0.05	1.80 ± 0.06	1.81 ± 0.04	2.03 ± 0.05*	2.11 ± 0.06**
Heart	3.29 ± 0.06	3.20 ± 0.07	3.04 ± 0.05	3.20 ± 0.06	3.19 ± 0.05	3.28 ± 0.06
Liver	31.5 ± 0.8#	29.0 ± 0.5	28.7 ± 0.4	29.1 ± 0.6	31.7 ± 0.4**	34.5 ± 0.5**

Statistically significant differences between the test groups and the high-fat (corn oil) control group are indicated with $*P < 0.05$; $**P < 0.01$. Statistically significant differences between the low-fat (carrier) control group and the high-fat (corn oil) control group are indicated with $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

males of the ARA/DHA group. In view of the fluctuation in incidence of this lesion, this finding is probably coincidental.

In all test groups as well as in the corn oil control group, several males showed increased hyaline droplet nephropathy. This phenomenon is characteristically found in male rats and encountered very often, and its incidence may vary considerably. In the present study, statistics show that the incidence was significantly increased in the ARA high-dose group and the ARA/DHA group. The changes were not accompanied by signs of cell damage and regenerative features. All other histopathological changes are common findings in rats of this strain and age, and were about equally distributed among the various groups, or occurred in one or a few animals only; they were, therefore, not ascribed to treatment. In Table 10 a summary is given of the incidences of the relevant microscopic findings of the target organs involved.

Neurotoxicity screening. Functional observation and motor activity assessment did not reveal any indications of neurotoxicity of the test substances. In addition, histopathological examination of tissue

Table 10. Summary of incidences of only the relevant microscopic observations in rats after feeding ARA-oil or ARA/DHA-oil for 13 wk[1]

	Incidence of lesions (numeric)											
	Males						Females					
		ARA-oil (ppm)		ARA/DHA-oil (ppm)				ARA-oil (ppm)		ARA/DHA-oil (ppm)		
Changes	Corn oil control	3000	15,000	75,000	75,000/55,000	Control	Corn oil control	3000	15,000	75,000	75,000/55,000	Control
Brain	(20)			(20)		(10)	(20)			(20)		(10)
Extracellular vaculoation	8			5		0	13			6*		0**
Kidney	(20)	(20)	(20)	(20)	(20)		(20)	(20)	(20)	(20)	(20)	
Increased hyaline droplet nephropathy	5	9	10	16**	14**		0	0	0	0	0	
Liver	(20)	(20)	(20)	(20)	(20)		(20)	(20)	(20)	(20)	(20)	(10)
Hepatocellular vacuolation	7	6	7	7	0**		2	3	7	10*	11**	0
Focal mononuclear-cell infiltrate	1	5	2	3	7		6	7	7	2	6	3
Mesenteric lymph nodes	(20)	(20)	(20)	(20)	(20)	(9)	(20)	(20)	(20)	(20)	(20)	(10)
Oil droplets	0	0	0	13***	14***	0	0	0	0	6*	7**	0
Lipogranuloma(ta)	*0*	*0*	*0*	2	6*	*0*	*0*	*0*	*0*	*1*	3	*0*
Inflammatory reaction	0	0	0	0	1	0	0	0	0	0	0	0
Peyer's Patches	(20)	(19)	(20)	(20)	(20)	(10)	(20)	(20)	(20)	(20)	(20)	(10)
Oil droplets	7	5	12	11	13	0	4	10	5	9	10	0
Histiocytic infiltration	1	1	1	1	5	1	0	0	0	0	2	0
Inflammatory reaction	0	0	0	2	1	0	0	0	0	1	1	0
Mineralisation	0	2	0	0	0	4**	0	0	1	0	0	3*
Small intestines	(20)	(20)	(19)	(20)	(20)	(10)	(20)	(20)	(20)	(20)	(20)	(10)
Oil droplets	1	0	0	14***	14***	0	0	0	0	6*	16***	0

Figures in parentheses represent the number of animals from which the tissue was examined microscopically. Asterisks indicate the statistically significance of the incidences ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$); two-sided Fisher's exact test.

samples representative of major areas of the brain, spinal cord and peripheral nerve did not reveal any changes that pointed to neurotoxicity of ARA-oil or DHA-oil.

DISCUSSION

Previous studies with ARA-oil derived from the fungus *Mortierella alpina* have shown that the lethal dose for ARA-oil is greater than 20 g/kg body weight, the maximum dose physically possible in rats (Boswell *et al.*, 1996; Hempenius *et al.*, 1996). In a 4-wk oral toxicity study in rats with ARA-oil, the no-observed-adverse-effect level (NOAEL) was 3000 mg/kg body weight /day, the highest dose level tested (Hempenius *et al.*, 1996). The study described in this paper was conducted to assess further the safety of the oil for use in infant formulae. The dose levels chosen were based on the following factors: (1) the human milk content of arachidonic acid (ARA) averaged out between the United States, Europe and Africa is 0.6% of total fatty acids (British Nutrition Foundation, 1992; Jensen *et al.*, 1992); (2) infant intake is about 100 kcal/kg body weight; (3) fat content in infant formulae is 5.56 g fat per 100 kcal (or 50% of total calories); and (4) formulae will be supplemented to attain the average ARA level in human milk. Thus, the amount of ARA-oil (containing 38.6% arachidonic acid), which should be added to infant formula to obtain the same ARA concentration as in human milk is 86 mg ARA-oil/100 kcal infant formula. Taking into account a caloric content for rat feed of 350 kcal/100 g, the mean doses of ARA-oil per 100 kcal rat feed for the low-, mid- and high-dose groups corresponds respectively to approximately 1, 5 or 25 times the anticipated intake in infant formulae. Calculated one body weight basis, the actual mean intake of the ARA-oil was in the low-, mid- and high-dose groups 2, 11 and 55 times, respectively, the anticipated exposure via infant formula.

Administration of ARA-oil as such, or in combination with DHA-oil to parental rats, did not affect health, fertility, reproductive performance or pup characteristics. The only change observed was growth retardation in parental females of the ARA high-dose group and ARA/DHA group during premating, mating and gestation periods, accompanied by a decrease in pup weight in the ARA/DHA group. The lower pup weights were, however, not reflected in significant effects on body weight of the F_1 rats in the subchronic study.

The subchronic study showed that administration of an over abundance of ARA-oil did not have any effect on food intake or growth in rats. The decreases in cholesterol, triglycerides and phospholipid concentrations in plasma of rats of the ARA high-dose group and/or the ARA/DHA group can be ascribed to the lowering effects of polyunsaturated fatty acids on blood lipids (Harris, 1989, Herzberg, 1989). Although unsaturated fatty acids of the n-3 series have been reported to be more effective than the n-6 fatty acids (Geelen *et al.*, 1995; Lu *et al.*, 1996; Saynor *et al.*, 1986), the present results indicate that high levels of both ARA-oil and DHA-oil are about equally effective in lowering blood lipids as compared to the corn oil-fed group.

In the present study, increases in spleen and liver weight were observed in the ARA high-dose group and the ARA/DHA group. The effect was most pronounced in the ARA/DHA group. Burns *et al.* (1999) also reported an increase in spleen and liver weights in rats fed diets containing high levels of ARA/DHA (131 g/kg feed). Previous studies with rats fed diets high in (n-3) polyunsaturated fatty acids also showed increases in spleen and liver weights (Alexander *et al.*, 1995; Lina, 1996). In the present study, the increase in spleen weight was not associated with morphological changes. Furthermore, although it is known that polyunsaturated fatty acids can alter the immune system, routine measurements on haematology and clinical parameters related to the immune system did not reveal any evidence of immunotoxic effects of the ARA-oil. Therefore, the increased spleen weight appears to be a physiological adaptation rather than a toxic effect.

The relevance of the vacuolation in the brain and in the spinal cord in the corn oil control group and the ARA high-dose group is not clear. Conventionally prepared paraffin embedded sections of the brain from most laboratory animal species may show moderately-sized round vacuoles in white matter. This phenomenon is not uncommon and is generally regarded an artefact (Greaves, 1990). To determine whether this was also the case in the present study, the brains and spinal cords of the carrier controls were processed and examined as well, but vacuolation was not seen in these organs in this group. Congo Red staining of frozen brain slides from two rats that showed clear vacuolation in haematoxylin/eosin sections was negative, indicating that the vacuoles did not contain fat. Furthermore, in the frozen sections it appeared to be much more difficult to recognize the vacuoles. The relevance of the presence of vacuoles in the brain and spinal cord remains unclear, but since the incidence of these findings was lower in the ARA high-dose group than in the corn oil control group, they are not ascribed to treatment with ARA-oil.

The presence of oil droplets in the mesenteric lymph nodes and intestinal villi of rats fed high doses of ARA-oil or ARA-oil in combination with DHA-oil did not have adverse physiological effects, as determined by their normal growth, weight gain and absence of significant abnormalities in their biochemical or haematologic parameters. Therefore, it is concluded that it is a harmless finding. Moreover, this conclusion is supported by the lit-

erature data (Boinott *et al.*, 1966a,b; Dincsoy *et al.*, 1982; Kelsall and Blachwell, 1969; Stryker, 1941; Wanless and Geddie, 1985), in which the frequent occurrence of oil droplets and lipogranulomas in human lymph nodes is described in the absence of any evidence of an adverse effect, such as inflammation, on the tissue.

In the present study, the dietary administration of 75,000 ppm ARA-oil, both with and without 55,000 ppm DHA-oil, resulted in a number of treatment-related changes. The majority of the changes observed are effects related to the intake of high levels of fats, rather than specific effects linked to the ARA-oil. No treatment-related changes were observed up to dietary dose levels of 15,000 ppm ARA-oil. Therefore, the no-observed-effect level (NOEL) in the present study was placed at 15,000 ppm ARA-oil in the diet. Based on this and the results of the previous studies (Hempenius *et al.*, 1997; Streekstra, 1997), it is concluded that it is safe to supplement infant formulae with ARA-oil derived from *Mortierella alpina* to provide levels of arachidonic acid similar to those in human breast milk.

Acknowledgements—The authors thank Juliet Griffiths for assistance in manuscript preparation.

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