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Subchronic (13-week) oral toxicity study, preceded by an in utero exposure phase, with arachidonate-enriched triglyceride oil (SUNTGA40S) in rats

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Abstract

Polyunsaturated fatty acids (PUFAs), such as arachidonic acid (ARA) and docosahexaenoic acid (DHA) are natural constituents found in human milk, fish oil or egg yolk. Until recently, infant formulas, though providing the essential fatty acid precursors for these PUFAs, did not contain preformed ARA or DHA.

In this study the safety of SUNTGA40S as source of ARA, not only for use in infant formulas but also for nutritional products or food supplements, was evaluated in a subchronic study in Wistar rats, preceded by a 4-week pretreatment period of parental (F_0) rats and exposure of the F_0 dams throughout mating, gestation and lactation. SUNTGA40S was administered at dietary levels of 0.5%, 1.5% and 5% (wt/wt) adjusted with corn oil to 5.76% added fat. An additional group received 3.65% (wt/wt) SUNTGA40S in conjunction with 2.11% (wt/wt) high DHA Tuna oil, providing an ARA:DHA ratio of 2.7:1. High-fat and low-fat controls received basal diet with or without 5.76% corn-oil supplement.

The content, stability and homogeneous distribution of the test substances in the diet were confirmed under study conditions. The administration of SUNTGA40S, with or without DHA oil, did not affect health, growth, fertility or reproductive performance of the parental rats, nor pup characteristics (condition, weight gain, viability, number per litter or sex ratio). In the subchronic study with the offspring (F_1) rats, no significant differences were found in condition, neurobehavioural observations, ophthalmoscopy, growth, urinalysis or macroscopic and microscopic findings between the test groups and the low-fat or the high-fat controls. In males of the 5% SUNTGA40S and the SUNTGA40S/DHA group, red blood cell counts, haemoglobin concentration and packed cell volume were lower and reticulocytes were slightly higher than in the high-fat and low-fat control groups. Cholesterol, triglycerides and phospholipids in plasma were lower than in the high-fat controls in both sexes in the 5% SUNTGA40S and the SUNTGA40S/DHA group and (for triglycerides only) in the 1.5% SUNTGA group. Due to the administration of extra dietary fat, food intake and prothrombin time (males only) were lower and alkaline phosphatase activity was higher in all the high-fat groups, including the corn-oil controls, as compared to the low-fat controls. The weight of the spleen was higher in males of the 5% SUNTGA40S and the SUNTGA40S/DHA group compared to both the low-fat and the high-fat controls. The effects noted in this study at high dose levels of SUNTGA40S are consistent with previously reported physiological responses to dietary intake of high PUFA containing oils. The present results provide evidence that SUNTGA40S is a safe source of arachidonic acid. Except during lactation when the intake in dams doubled, 5% Suntga40S in the diet was equivalent to an overall intake of approximately 3 g/kg body weight/day in F_0 and F_1 animals.

Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration.

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1. Introduction

The human body can synthesize ARA and DHA through desaturation and elongation of the 18-carbon fatty acids linoleic acid (C18:2($n-6$)) and linolenic acid (C18:3($n-3$)). The human fetus and neonate initially obtains ARA and DHA by placental transfer and via human milk. In contrast to human milk, standard infant formulas contain only trace amounts of ARA and DHA.

Many advisory bodies (e.g. ESPGAN, 1991; the British Nutrition Foundation Task Force, 1992, SCF, 1993) and other scientists have suggested that infant formulas should contain the same amounts of DHA and ARA as human milk. For these reasons, there is an increasing interest in highly purified oils rich in ARA or DHA, because they can be mixed to provide a ratio of ARA/DHA similar to that of human milk while minimizing the exposure to other fatty acids.

Furthermore other arguments stress the need for fortifying human diet with ARA. There is an age-dependent decrease in the concentration of ARA in the hippocampus, and aged rats exhibit an impaired ability to sustain long-term potentiation (LTP) (Soderberg et al., 1991; McGahon et al., 1997). The age-dependent suppression of LTP is restored by chronic supplementation of ARA or γ-linolenic acid (McGahon et al., 1997).

Many safety studies of ARA enriched triglyceride obtained from *Mortierella alpina* have been reported (Streekstra, 1997; Hempenius et al., 1997, 2000). The objective of this study was to assess the safety of SUNTGA40S, a newly extracted oil from *M. alpina* and highly purified, in a subchronic study in F_1 rats, preceded by a 4-week pretreatment period of parental (F_0) rats and exposure of the F_0 dams throughout mating, gestation and lactation. This design was used in order to mimic the intended exposure of pregnant women and infants to the oil. SUNTGA40S was administered at dietary levels of 0.5%, 1.5% and 5% (wt/wt). Because feeding high levels of SUNTGA40S might result in an imbalance between $n-6$ and $n-3$ polyunsaturated fatty acids, SUNTGA40S was also administered at a level of 3.65% in conjunction with 2.11% high DHA Tuna oil. This combination group provided a total of ARA + DHA identical to the amount of ARA in the 5% SUNTGA40S group and a ratio ARA: DHA of 2.7:1, considering the ARA and DHA ratio of human milk reported in many papers.

Average ARA and DHA contents in total fatty acids and ARA/DHA ratio of breast milk are 0.1%, 0.3%, 0.3 for European and African women (Koletzko et al., 1992); 0.36%, 0.22%, 1.6 for German women (Kohn et al., 1994); 0.6%, 0.1%, 6.0 for American women (Putnam, 1982); 0.54%, 0.59%, 0.91 for women on a balanced meat and vegetable diet (Sanders et al., 1978); 0.72%, 0.23%, 3.1 for women consuming a vegan diet in United Kingdom (Sanders et al., 1978); and 0.35%, 1.46%, 0.23 for Japanese women (Tanaka et al., 1994), respectively.

2. Materials and methods

The study was conducted in accordance with the OECD Principles of Good Laboratory Practice (OECD, 1998b), and conformed to OECD Guidelines for the Testing of Chemicals 408 (OECD, 1998a) and EEC Directive 87/302/EEC (1988).

2.1. Materials

Arachidonate-enriched Triglyceride oil (SUNTGA40S), a clear yellow oil, lot number 01030351, produced by Suntory Limited, Osaka, Japan, was extracted from a biomass of submerged fermented *M. alpina* and refined by high purification processes. SUNTGA40S contained 41.5% arachidonic acid (ARA; C20:4($n-6$)), 0.1% eicosapentaenoic acid (EPA; C20:5($n-3$)) and no docosahexaenoic acid (DHA C22:6($n-3$)). It had a peroxide value of 0.42 meq/kg, low unsaponifiable matters not more than 1.0%.

DHA-containing oil (high DHA Tuna oil), a whitish cloudy oil, lot number 030121 was obtained from Nissui, Tokyo, Japan. High DHA Tuna oil contained 26.6% docosahexaenoic acid (C22:6($n-3$)), 7.1% eicosapentaenoic acid (C20:5($n-3$)) and 0.5% total tocopherols (peroxide value 0 meq/kg). Both test materials were stored in a freezer (<−18 °C) under nitrogen. The reference substance (corn oil), lot numbers L405366 and L405195 was obtained from Oliehoorn, Zwaag, The Netherlands and stored in a refrigerator (2–10 °C).

2.2. Animals and maintenance

The welfare of the animals was maintained in accordance with the general principles of the European Communities (Directive 86/609/EEC) and the Netherlands legislation (the Experiments on Animals Act 1997), governing the use of animals in toxicity experiments. Parental (F_0) male and female rats (75 males and 150 females), Wistar outbred (Crl:(WI)WU BR), were obtained from Charles River Deutschland, Sulzfeld, Germany. At the

commencement of the treatment period, the F_0 rats were approximately 9–10-week old and their body weight variation did not exceed ±20% of the mean weight. Offspring (F_1) rats were selected from the F_0 litters at day 21 post partum. The rats were housed in one room in macrolon cages with sterilized wood shavings as bedding material. During the mating period, two females were caged with one male from the same group until they were mated. During gestation and lactation, the dams were housed one/cage. In the subchronic study, the F_1 rats were housed in groups of five of the same sex. Housing conditions were conventional; room temperature was targeted at 22 ± 3 °C and relative humidity at 30–70%; the number of air changes in the animal room was at least 10/h. Artificial light was provided by fluorescent tubes for 12 h/day continuously. The animals were fed a commercial rodent diet (RM3 diet, SDS Special Diets Services, Witham, England). Diet and tap water were provided ad libitum.

2.3. Experimental design

The study comprised two phases: (1) an in utero exposure phase in which parental (F_0) animals were fed the various test or control diets starting 4 weeks prior to mating, throughout mating, gestation and lactation until weaning of the F_1 rats; and (2) a subchronic study in which the selected offspring (F_1 rats) received the test or control diets for 13 weeks.

There were two control groups, viz. one high-fat control group fed RM3 diet supplemented with 5.76% corn oil (containing the C18 fatty acids α-linolenic acid and linoleic acid, but no C20 or C22 polyunsaturated fatty acids), and one carrier (low-fat) control group receiving the RM3 diet without additions to serve as a reference group. SUNTGA40S was administered at dietary levels of 0.5%, 1.5% and 5% (wt/wt). These test diets were adjusted with corn oil to 5.76% added fat (Table 1).

In addition, SUNTGA40S was administered at a level of 3.65% in conjunction with 2.11% high DHA Tuna oil. This combination group provided a total of ARA + DHA identical to the amount of ARA in the 5% SUNTGA40S group and a ratio ARA:DHA of 2.7:1. Fresh batches of the test and control diets were prepared approximately once every month, sealed in air-tight plastic containers under nitrogen blanket to avoid oxidation, and stored in a freezer (<−18 °C). Twice a week, containers were thawed and the feed in the animal feeders was replaced by fresh portions. The content and homogeneous distribution of the test substances in the diet, and the stability in the animal room (4 days) or freezer (5 weeks) were confirmed by quantitative determination of the level of ARA and DHA by gas chromatography with flame ionization detection after chloroform/methanol extraction, saponification and derivatisation.

2.3.1. Parental (F_0) rats

Twenty four females and 12 males were allocated randomly to each group of F_0 rats, which number was sufficient to provide at least 20 litter per group. After a premating period of 4 weeks, in which the F_0 rats were fed their respective test or control diets, the females were mated (two females with one male from the same group). Vaginal smears were made daily. The day on which sperm was observed in the smears was considered day 0 of gestation. The administration of test or control diets was continued throughout gestation and lactation. The morning after birth was considered day 1 post partum. On day 4 post partum, litters of more than eight pups were adjusted by eliminating surplus pups by random selection, to obtain (as nearly as possible) four males and four females per litter. At day 21 post partum, the F_1 litters were weaned. All surviving female parent rats were killed under CO_2/O_2 anaesthesia shortly after weaning of the pups.

The following observations were made:

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

Table 1
Treatment and overall intake of test substances

Group	Low-fat (carrier) control	High-fat (corn oil) control	0.5% SUNTGA40S	1.5% SUNTGA40S	5.0% SUNTGA40S	3.65% SUNTGA40S + 2.11% high DHA Tuna oil	
Dietary supplement (g/100 g feed)							
Corn oil	–	5.76	5.26	4.26	0.76	–	
SUNTGA40S	–	–	0.5	1.5	5.0	3.65	
High DHA Tuna oil	–	–	–	–	–	2.11	
Overall test substance intake (g/kg body weight/day)							
	SUNTGA40S	SUNTGA40S	SUNTGA40S	SUNTGA40S	SUNTGA40S	SUNTGA40S	DHA oil
F_0, pre-mating period males/females	–	–	0.3/0.3	0.9/0.9	3.0/3.0	2.2/2.2	1.3/1.2
F_0, gestation period females	–	–	0.3	0.9	3.1	2.3	1.3
F_0, lactation period females	–	–	0.7	2.0	6.7	4.6	2.7
F_1, sub-chronic study males/females	–	–	0.3/0.3	0.8/0.9	2.8/2.9	2.0/2.2	1.2/1.2

Body weight. The body weight of each animal was recorded when starting the administration of the test substance and once every week thereafter. Mated females were weighed on days 0, 7, 14 and 21 of gestation. Females that gave birth were weighed on days 1, 4, 14 and 21 post partum.

Food consumption. During the premating period, food consumption of both males and females was measured per cage weekly. Food consumption of mated females was recorded during pregnancy over successive periods (days 0–7, 7–14 and 14–21), and during lactation on days 1–4, 4–7 and 7–14. The intake of test substance per kg body weight was calculated from the nominal dietary concentration, the food consumption and the body weight.

Reproductive data and indices. For assessment of the fertility and reproductive performance, mating, fertility, fecundity, gestation, live births and viability indices were calculated, and the sex ratio of pups was determined. For each litter, the litter size (dead and live pups), number of male and female pups, and number of pups with external abnormalities were determined on post natal (PN) days 1, 4, 7, 14 and 21. The litters were weighed on PN days 1, 4 (before and after culling), 7 and 14. At weaning (PN day 21), all pups were weighed individually.

2.3.2. Offspring (F_1) rats

At day 21 post partum, F_1 rats were randomly selected from the F_0 litters (10 rats/sex/group, each selected from a different litter). During the period between weaning and the start of the subchronic study, the feeding of the respective test or control diets to the selected F_1 rats was continued. The following observations were made:

Clinical observations. The general condition and behaviour of all animals were checked daily. Neurobehavioural testing, comprising detailed clinical observations outside the home cage, was conducted on all animals once weekly. In addition, behavioural endpoints (functional observational battery) and motor activity assessment were performed during the last week of the treatment period (Moser et al., 1997). Ophthalmoscopic examinations were made in the first week and in the last week of the 13-week study in all rats of the high-fat control group, the SUNTGA high-dose group and the SUNTGA/DHA oil group, using a slit lamp after induction of mydriasis with atropine sulphate.

Body weights. The body weight of each animal was recorded at initiation of the 13-week study (day 0), and once per week thereafter.

Food consumption. The quantity of food consumed was measured per cage, over successive periods of 3 or 4 days. The intake of test substance per kg body weight was calculated from the nominal dietary concentration, the food consumption and the body weight.

Haematology and clinical chemistry. K_2-EDTA blood, collected from the abdominal aorta of all rats at necropsy was examined for haemoglobin, packed cell volume, red blood cell count, reticulocytes, total white blood cell count, prothrombin time, thrombocyte count (ABX Pentra 120 Haematology Analyzer) and differential white blood cell count (microscopic examination), and the indices mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated. Whole blood collected from the tip of the tail after overnight fasting in week 13 was examined for fasting glucose (Hitachi-911 analyzer). Clinical chemistry analyses were performed on plasma samples derived from heparinised blood at necropsy. The measurements comprised alkaline phosphatase activity (ALP), aspartate aminotransferase activity (ASAT), alanine aminotransferase activity (ALAT), gamma glutamyl transferase (GGT), total protein, albumin, ratio albumin to globulin, urea, creatinine, total bilirubin, total chlolesterol, triglycerides, phospholipids, calcium, sodium, potassium, chloride and inorganic phosphate (Hitachi-911 analyzer).

Renal concentration test and urinalysis. Urine, collected from all rats during the last 16 h of a 24 h deprivation period of food and water in week 13, was analysed for volume, density (refractometer), appearance, pH, glucose, occult blood, ketones, protein, bilirubin, urobilinogen (Combur-7 test strips) and microscopy of the sediment.

Necropsy. The animals were killed under CO_2/O_2-anaesthesia and subjected to a complete macroscopic examination. The weights of the adrenals, brain, epididymes, heart, kidneys, liver, ovaries, spleen, seminal vesicles (with coagulating glands), testes, thymus and uterus were recorded and related to the final body weight. In addition to these organs, specimens of following tissues were preserved in neutral 4% formalin, embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin: aorta, axillary lymph nodes, caecum, colon, eyes, gut associated lymphoid tissue (GALT, including Peyer's patches), lungs, mammary gland (females), mesenteric lymph nodes, oesophagus, pancreas, parathyroid, parotid salivary glands, pituitary, prostate, rectum, skeletal muscle (thigh), sciatic nerve, skin, small intestine (duodenum, jejunum and ileum), spinal cord (retained in vertebral column, at least three levels were examined microscopically), sternum with bone marrow, stomach (glandular and non-glandular part were examined microscopically), sublingual and submaxillary salivary glands, thyroid, trachea/bronchi, urinary bladder, vagina as well as any tissue showing gross lesions. Histopathological examination was performed on all tissues listed for all animals in the high-fat control group and the SUNTGA high-dose group. In addition, the kidneys, liver and relevant gross lesions were examined microscopically in all rats.

2.4. Statistical analysis

Means ± standard error of the mean (SEM) were calculated. Two separate statistical analyses were conducted, viz. the test groups were compared with the high-fat control group, and all high-fat groups were compared with the low-fat (carrier) control group. Analysis of variance (Anova) followed by Dunnett's multiple comparison tests or the least significant difference (LSD) test were used for evaluating data on body weights, food consumption, haematology, clinical chemistry, volume and density of the urine and organ weights. Body weights of F_1 rats were analysed by one-way analysis of covariance (covariate: body weight on day 0). Fisher's exact probability test was used for the evaluation of data on reproductive performance (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 the evaluation of histopathological changes. Kruskal–Wallis non-parametric Anova followed by Mann–Whitney U-tests was used for evaluating precoital time, duration of gestation and litter size, reticulocytes, relative differential white blood cell counts, and semi-quantitative urinary parameters. Functional observational battery and motor activity results were evaluated by Anova followed by Dunnett's multiple comparison tests (continuous data); by Kruskal–Wallis non-parametric Anova followed by multiple comparison tests (rank order data); or by Pearson χ^2 test (categorical data).

3. Results

3.1. Intake of the test substances

Except during lactation when the intake in dams doubled, the overall intake of SUNTGA40S in the F_0- and F_1-animals was approximately 0.3, 0.9 and 3 g/kg bw/day in the low-, mid- and high-dose group, respectively (Table 1); in the SUNTGA40S/DHA group, the overall intake was approximately 2.2 g SUNTGA40S + 1.2 g high DHA Tuna oil/kg bw/day (Table 1).

3.2. Results obtained in F_0 rats and observations in pups

Clinical observations, growth and food intake. General condition and behaviour of F_0 rats were not adversely affected by the test substances and none of the parental rats died untimely. Gross examination of the F_0 animals at sacrifice did not reveal any effect of the test substances on maternal organs and tissues. Body weights and body weight gain in F_0 rats in the premating period (both sexes), and in the gestation period and the lactation period (females) were similar among the test groups, the low-fat control group and the corn-oil control group. There were no significant differences in food intake between the treatment groups and the high-fat control group. Food intake in the low-fat control group was statistically significantly higher (overall about 10%) than in all high-fat groups during the premating and the gestation period.

Reproduction indices. There were no treatment-related differences in fertility and reproductive performance among the groups. Indices for mating, female fecundity, female fertility, male fertility and gestation, birth and viability, as well as precoital and gestation times were similar among the groups (Table 2). The number of pregnant females in the high-fat controls was statistically significantly lower than in the SUNTGA40S/DHA group, but this was considered to be a fortuitous finding.

Observations in pups. There were no relevant differences among the groups in general condition and body weights of the pups, or in viability, sex ratio or number of pups per litter (Table 2). On days 14–21, pup weight and weight gain were statistically significantly higher in the 0.5% SUNTGA40S group than in low-fat controls. Because these differences were not dose-related, they were not ascribed to treatment.

3.3. Results obtained in F_1 rats in the subchronic study

Clinical observations and mortality. None of the rats died during the study and there were no treatment-related clinical signs. Clinical observations outside the homecage, and the results of the neurobehavioural observations and motor activity assessment did not indicate any neurotoxic potential of the test substances. Ophthalmoscopic examination did not reveal any treatment-related changes.

Growth and food intake. There were no statistically significant differences in body weights between the test groups and the low-fat control group or the corn-oil control group. Food intake was significantly higher (overall about 11%) in males and females of the low-fat control group compared to all high-fat groups. Compared to the high-fat control group, food intake was occasionally increased in males of all test groups, but these changes were neither consistent nor dose-related (data not shown).

Haematology. In males of the 5% SUNTGA40S and the SUNTGA40S/DHA group, haemoglobin concentration and packed cell volume were statistically significantly lower and reticulocytes were slightly higher than in the high-fat control group and/or the low-fat control group. Red blood cell count was also slightly decreased in males of these groups (statistically significant in the 5% SUNTGA40S group only). Mean corpuscular haemoglobin concentration (MCHC) was slightly, though

Table 2
Natural delivery and litter data

Parameters	Control groups		Test groups			
	Low-fat (carrier) control	High-fat (corn oil) control	0.5% SUNTGA40S	1.5% SUNTGA40S	5.0% SUNTGA40S	3.65% SUNTGA40S + 2.11% DHA oil[a]
No. of females mated	24	23	24	24	24	23
No. of females pregnant	22	18	23	22	20	23[#]
Mean pre-coital time (days)	2.7 (0.4)[b]	2.7 (0.5)	2.6 (0.5)	3.0 (0.6)	2.9 (0.5)	2.2 (0.2)
Live birth index (%)[c]	99	100	99	100	99	100
Mean litter size at birth	10.1 (0.5)	10.2 (0.5)	10.5 (0.5)	10.8 (0.4)	10.3 (0.5)	10.3 (0.5)
Mean litter size day 4[d]	9.6 (0.5)	10.1 (0.5)	9.6 (0.6)	10.7 (0.4)	9.9 (0.5)	10.1 (0.5)
Mean litter size day 21	7.6 (0.2)	7.7 (0.2)	7.7 (0.2)	7.9 (0.1)	7.8 (0.1)	7.7 (0.2)
Viability index days 4–21 (%)[e]	100	100	98	100	100	100
Sex ratio at day 1[f]	51	51	54	55	47	49
Sex ratio at day 21[f]	49	51	54	51	51	49
Post-implantation loss (%)[g]	15.0 (2.9)	9.9 (3.8)	7.3 (2.1)	9.0 (2.2)	12.1 (5.3)	9.5 (2.7)
Mean pup weight day 1 (g)	5.3 (0.1)	5.2 (0.1)	5.3 (0.2)	5.1 (0.1)	5.2 (0.2)	5.1 (0.2)
Mean pup weight day 14 (g)	26.9 (0.6)	28.7 (0.4)	30.3 (0.5)[##]	28.2 (0.5)	28.6 (0.5)	28.1 (0.6)
Mean male pup weight day 21 (g)	44.5 (0.9)	47.6 (0.9)	50.2 (0.9)[##]	47.4 (0.7)	46.6 (0.7)	46.9 (1.0)
Mean female pup weight day 21 (g)	43.6 (0.9)	45.0 (0.7)	48.1 (0.8)[##,#]	45.4 (0.8)	46.0 (0.6)	44.8 (0.8)

[#] $p < 0.05$; fisher exact probability test; significantly different from corn oil controls.
[##] $p < 0.001$; fisher exact probability test; significantly different from carrier (low fat) controls.
[a] High DHA Tuna oil.
[b] In brackets is given the standard error of the mean (SEM).
[c] Number of pups born alive/total number of pups × 100.
[d] Preculling.
[e] Number of live weanlings/number of pups alive on day 4 post partum × 100.
[f] The number of live male pups/number of live pups.
[g] (Number of implantation sites – number of pups born alive)/number of implantation sites × 100.

statistically significantly increased in these groups in both sexes and in males of the 1.5% SUNTGA group, whereas mean corpuscular volume (MCV) was decreased in males of the SUNTGA40S/DHA group (Table 3).

Prothrombin time was not changed in all high-fat groups, but was higher in males of the low-fat control group as compared to all high-fat groups, including the corn-oil controls. There were no treatment-related changes in total or differential white blood cell counts.

Clinical chemistry. Compared to the low-fat controls, cholesterol concentration was higher in the high-fat control group and in the 0.5% and 1.5% SUNTGA40S group in both sexes (not statistically significant in females of the 0.5% SUNTGA40S group). At the higher SUNTGA40S levels (in the 5% SUNTGA40S and SUNTGA40S/DHA groups) cholesterol levels were lower than in the high-fat controls (statistically significant in females only) and were comparable to those in the low fat controls (Table 4). In both sexes, plasma triglycerides and phospholipids were statistically significantly lower in the 5% SUNTGA40S and the SUNTGA40S/DHA groups as compared to the low-fat controls and/or the high-fat controls. In addition, triglycerides were lower in the 1.5% SUNTGA40S than in high-fat controls in both sexes. Bilirubin concentration was lower in females of the 5% SUNTGA group as compared to the low-fat controls only. Alkaline phosphatase activity was statistically significantly higher in several high-fat groups, including males and females of the corn-oil controls, when compared to the low-fat controls, but there were no differences between the corn-oil controls and the test groups. A few other changes in clinical chemistry parameters reached the level of statistical significance (viz. aspartate aminotransferase activity, albumin/globulin ratio and total protein and sodium levels). These changes were not ascribed to the administration of SUNTGA40S because they did not show a dose–response relationship or did not differ significantly from the corn-oil controls.

Urinalysis. The renal concentration test did not show any significant changes in urinary volume or density, and semi-quantitative (dipstick) urinary observations and microscopy of the urinary sediment did not reveal relevant changes.

Organ weights. The absolute weight (not shown) and the relative weight (Table 5) of the spleen were higher in males of the 5% SUNTGA40S and the SUNTGA40S/DHA group both compared to the low-fat controls and the high-fat controls. Due to slight fluctuations in terminal body weight, the relative weight of the brain was decreased in males of the SUNTGA40S/DHA group and increased in females of the 1.5% SUNTGA40S group. A fortuitous finding of a decreased relative weight of the uterus was observed in the high-fat controls and the 0.5% SUNTGA40S group.

Table 3
Selected haematological parameters in blood collected from 10 rats/sex/group at necropsy

Parameters	Control groups		Test groups			
	Low-fat (carrier) control	High-fat (corn oil) control	0.5% SUNTGA40S	1.5% SUNTGA40S	5.0% SUNTGA40S	3.65% SUNTGA40S + 2.11% DHA oil[a]
Males						
Red blood cells (10^{12}/l)[A/D]	8.08 (0.09)[b]	8.14 (0.08)	8.39 (0.13)	8.33 (0.10)	7.79 (0.08)$^{\#}$	7.90 (0.08)
Haemoglobin (mmol/l)[A/D]	9.6 (0.1)	9.6 (0.1)	9.8 (0.1)	9.9 (0.1)	9.2 (0.1)*	9.1 (0.1)$^{*,\#}$
Packed cell volume (l/l)[A/D]	0.413 (0.006)	0.418 (0.005)	0.420 (0.005)	0.422 (0.004)	0.388 (0.005)$^{**,\#\#}$	0.380 (0.005)$^{**,\#\#}$
MCV[c] (fl)[A/D]	51.2 (0.7)	51.4 (0.6)	50.1 (0.6)	50.8 (0.6)	49.9 (0.3)	48.2 (0.3)$^{**,\#\#}$
MCH[d] (fmol)[A/D]	1.19 (0.01)	1.18 (0.01)	1.16 (0.01)	1.19 (0.02)	1.18 (0.01)	1.16 (0.01)
MCHC[e](mmol/l)[A/D]	23.3 (0.1)	22.9 (0.1)	23.2 (0.1)	23.5 (0.1)$^{\#}$	23.7 (0.1)$^{\#\#}$	24.0 (0.1)$^{**,\#\#}$
Reticulocytes (per 1000)[K/M]	34.4 (1.3)	32.3 (1.5)	33.6 (1.1)	31.8 (1.3)	39.0 (1.3)$^{*,\#\#}$	39.6 (2.5)$^{\#}$
Prothrombin time (s)[A/D]	39.4 (0.5)	37.0 (0.5)*	36.2 (0.5)**	37.1 (0.6)*	37.3 (0.6)*	37.0 (0.5)*
Females						
Red blood cells (10^{12}/l)[A/D]	7.60 (0.10)	7.62 (0.06)	7.73 (0.08)	7.45 (0.13)	7.57 (0.06)	7.56 (0.09)
Haemoglobin (mmol/l)[A/D]	9.3 (0.1)	9.4 (0.1)	9.3 (0.1)	9.3 (0.1)	9.4 (0.1)	9.4 (0.1)
Packed cell volume (l/l)[A/D]	0.403 (0.003)	0.404 (0.005)	0.410 (0.003)	0.399 (0.005)	0.400 (0.004)	0.396 (0.006)
MCV[c] (fl)[A/D]	53.1 (0.7)	53.1 (0.6)	53.1 (0.6)	53.6 (0.7)	52.8 (0.5)	52.4 (0.6)
MCH[d](fmol)[A/D]	1.23 (0.02)	1.23 (0.02)	1.21 (0.01)	1.24 (0.02)	1.24 (0.01)	1.24 (0.02)
MCHC[e](mmol/l)[A/D]	23.1 (0.1)	23.1 (0.1)	22.7 (0.1)$^{\#}$	23.2 (0.2)	23.6 (0.1)$^{*,\#}$	23.7 (0.1)$^{**,\#\#}$
Reticulocytes (per 1000)[K/M]	37.7 (2.2)	36.1 (1.2)	33.9 (1.3)	35.5 (2.9)	34.6 (1.4)	36.9 (1.4)
Prothrombin time (s)[A/D]	34.8 (0.6)	34.1 (0.6)	34.5 (0.6)	34.1 (0.4)	36.1 (0.4)	34.4 (0.6)

[A/D] Anova + Dunnett tests.
[K/M] Kruskal–Wallis Anova + Mann–Whitney *U*-test.
$^{*}p < 0.05$; $^{**}p < 0.01$; significantly different from carrier (low-fat) controls.
$^{\#}p < 0.05$; $^{\#\#}p < 0.01$; significantly different from corn oil controls.
[a] High DHA Tuna oil.
[b] In brackets is given the standard error of the mean (SEM).
[c] Mean corpuscular volume.
[d] Mean corpuscular haemoglobin.
[e] Mean corpuscular haemoglobin concentration.

Pathology. Macroscopic examination at necropsy and microscopic examination of organs and tissues did not reveal any treatment-related findings. All histopathological changes observed were common findings in rats of this strain and age, and their incidences were comparable amongst the examined groups.

4. Discussion

This 13-week subchronic study in F_1 rats, preceded by a pretreatment period of F_0 rats and exposure of the F_0 dams throughout mating, gestation and lactation, using SUNTGA40S at dietary levels of 0.5–5%, provides evidence that this oil is a safe source of arachidonic acid. The administration of SUNTGA40S, with or without DHA oil, did not affect health, growth, fertility or reproductive performance of the parental rats, nor pup characteristics. In the subchronic study with the offspring rats, no significant differences were found in condition, neurobehavioural observations, ophthalmoscopy, growth, urinalysis or macroscopic and microscopic findings between the test groups and the low-fat or the high-fat controls. The feeding of SUNTGA40S at high doses in the subchronic study was, however, associated with a few differences in haematology, clinical chemistry and spleen weight.

Changes in red blood cell parameters similar to those observed in the present study in F_1 rats fed high levels of SUNTGA40S, with or without DHA, have been reported in other studies with PUFAs. MCHC was elevated in rats administered a combination of ARA and DHA-containing oil for 4 weeks (Hempenius et al., 1997). Increased MCHC and decreased packed cell volume were also noted in rats fed high levels of ARA or ARA/DHA-containing oil for 13 weeks (Hempenius et al., 2000). Decreased haemoglobin and packed cell volume were found in another study in rats fed ARA/DHA (2:1)-containing oil for 13 weeks (Burns et al., 1999). As in the present study, these effects were noted especially in males. Accumulation of polyunsaturated fatty acids in red blood cell membranes may increase lipid peroxidation and red blood cell senescence. Both the administration of high levels of $n-3$ PUFA (DHA)-containing oil to rats and mice (Calviello et al., 1997; Oarada et al., 2000) and $n-6$ PUFA (ARA)-containing oil to piglets (Sarkadi et al., 2003) has been associated with increased oxidative sensitivity. In humans it has been reported that both fish oil (high in $n-3$ fatty acids) and safflower oil (high in $n-6$ fatty

Table 4
Selected clinical chemistry parameters in plasma collected from 10 rats/sex/group at necropsy

Parameters	Control groups		Test groups			
	Low-fat (carrier) control	High-fat (corn oil) control	0.5% SUNTGA40S	1.5% SUNTGA40S	5.0% SUNTGA40S	3.65% SUNTGA40S + 2.11% DHA oil[a]
Males						
Cholesterol (mmol/l)	1.83 (0.06)[b]	2.22 (0.08)*	2.36 (0.10)**	2.30 (0.10)**	1.92 (0.08)	1.94 (0.10)
Triglycerides (mmol/l)	1.92 (0.22)	2.33 (0.30)	1.97 (0.28)	1.42 (0.17)#	0.87 (0.10)**,##	1.12 (0.13)*,##
Phospholipids (mmol/l)	1.96 (0.09)	2.26 (0.08)	2.28 (0.11)	2.08 (0.10)	1.66 (0.08)##	1.73 (0.08)##
Bilirubin (μmol/l)	1.2 (0.2)	1.2 (0.1)	1.1 (0.1)	0.9 (0.2)	1.1 (0.1)	0.9 (0.1)
Alkaline phosphatase (U/l)	79 (4)	118 (6)**	116 (7)**	100 (6)	110 (7)**	115 (4)**
Aspartate aminotransferase (U/l)	85 (5)	75 (3)	88 (4)#	70 (3)*	76 (4)	82 (2)
Total protein (g/l)	70 (0)	67 (1)	70 (1)#	70 (1)#	68 (0)	68 (1)
Albumin/globulin ratio	1.82 (0.04)	1.84 (0.05)	1.79 (0.03)	1.59 (0.05)**,##	1.77 (0.05)	1.80 (0.05)
Sodium (mmol/l)	152 (1)	151 (1)	150 (0)*	150 (0)	150 (0)	149 (1)**
Females						
Cholesterol (mmol/l)	1.70 (0.06)	2.00 (0.08)*	1.93 (0.06)	2.13 (0.08)**	1.60 (0.06)##	1.57 (0.09)##
Triglycerides (mmol/l)	1.22 (0.14)	1.36 (0.18)	1.01 (0.18)	0.73 (0.06)##	0.66 (0.11)*,##	0.71 (0.09)*,##
Phospholipids (mmol/l)	2.08 (0.07)	2.28 (0.08)	2.17 (0.09)	2.15 (0.06)	1.61 (0.06)**,##	1.69 (0.09)**,##
Bilirubin (μmol/l)	0.9 (0.1)	0.7 (0.1)	1.0 (0.1)	0.8 (0.2)	0.3 (0.1)*	0.6 (0.1)
Alkaline phosphatase (U/l)	74 (8)	99 (8)*	92 (8)	78 (5)	74 (4)	87 (5)
Aspartate aminotransferase (U/l)	80 (4)	78 (4)	80 (2)	76 (1)	67 (3)*	86 (5)
Total protein (g/l)	69 (1)	68 (1)	69 (1)	66 (1)	66 (1)	67 (1)
Albumin/globulin ratio	2.34 (0.07)	2.41 (0.05)	2.30 (0.08)	2.30 (0.07)	2.19 (0.06)	2.44 (0.04)
Sodium (mmol/l)	150 (0)	149 (1)	149 (0)	149 (0)	149 (0)	150 (0)

Anova + Dunnett tests.
*$p < 0.05$; **$p < 0.01$; significantly different from carrier (low-fat) controls.
#$p < 0.05$; ##$p < 0.01$; significantly different from corn oil controls.
[a] High DHA Tuna oil.
[b] In brackets is given the standard error of the mean (SEM).

acids) increased the susceptibility of erythrocytes to oxidative damage by free radical generation (Mills et al., 1995). It may be speculated that, in the present study, modification of the fatty acid composition of the erythrocyte membrane by high dietary levels of PUFAs affected the survival time of the erythrocytes in the circulation. The effects on the red blood cell system are not specifically ascribed to the test substance, but are rather attributable to exposure to high levels of polyunsaturated fatty acids.

Plasma triglycerides and phospholipids were reduced in both sexes in the 5% SUNTGA40S and the SUNTGA40S/DHA group and (for triglycerides only) in the 1.5% SUNTGA group. Cholesterol concentration was higher in the high-fat control group and the 0.5% and 1.5% SUNTGA groups than in low-fat controls. In the high-dose (5% SUNTGA40S and SUNTGA40S/DHA) groups, however, cholesterol levels were decreased compared to the high-fat controls, showing a cholesterol lowering effect of substituting ARA- and ARA/DHA-containing oil for corn oil. The observed diminution of phospholipids, cholesterol and triglycerides with ARA- or DHA-containing oil are ascribed to the lowering effects of these PUFAs on blood lipids (Harris, 1989; Hempenius et al., 2000; Hammond et al., 2001; Kroes et al., 2003; Merritt et al., 2003).

The increased weight of the spleen in males of the 5% SUNTGA and the SUNTGA/DHA group was not accompanied by histopathological changes in this organ. Many authors reported increased spleen weights without effects on splenic morphology in mice, rats and piglets administered high PUFA diets (Danse and Verschuren, 1978; McGuire et al., 1997; Hempenius et al., 1997, 2000; Burns et al., 1999; Rabbani et al., 1999; Merritt et al., 2003). The increased spleen weight in these studies, without histopathological correlates indicative of a toxic action, is generally considered to be a physiological adaptation to high dietary levels of unsaturated fatty acids and not a manifestation of toxicity.

A few changes observed were not specifically related to the administration of PUFA-containing oil, but rather attributable to the incorporation of extra fat in the diet. The decreased food intake in all high-fat groups, including the high-fat controls, is due to the higher caloric density of these diets. The decreased prothrombin time in males of all high-fat groups as compared to the low-fat control group is also ascribed to the high-fat content of the test diets and the corn-oil control diet. A decreased prothrombin time has been reported previously in rats fed diets high in corn oil or PUFA-containing oils (Hempenius et al., 2000). The increase in alkaline phosphatase activity in several high-fat groups including the corn-oil controls is also

Table 5
Mean terminal body weight (g) and relative organ weights (g/kg body weight)

Parameters	Control groups		Test groups			
	Low-fat (carrier) control	High-fat (corn oil) control	0.5% SUNTGA40S	1.5% SUNTGA40S	5.0% SUNTGA40S	3.65% SUNTGA40S + 2.11% DHA oil[a]
Males						
Terminal body weight	408 (8)[b]	395 (7)	413 (10)	411 (9)	392 (6)	419 (8)
Adrenals	0.117 (0.004)	0.118 (0.004)	0.116 (0.005)	0.114 (0.004)	0.124 (0.004)	0.116 (0.004)
Brain	4.78 (0.08)	4.92 (0.08)	4.74 (0.10)	4.75 (0.09)	4.97 (0.07)	4.58 (0.06)$^{\#}$
Heart	2.92 (0.08)	3.07 (0.06)	2.85 (0.06)	2.88 (0.05)	3.07 (0.07)	2.92 (0.06)
Kidneys	5.26 (0.09)	5.56 (0.12)	5.15 (0.10)	5.16 (0.25)	5.23 (0.10)	5.24 (0.16)
Liver	35.6 (1.5)	37.0 (1.0)	37.0 (0.9)	33.6 (0.9)	36.4 (1.4)	36.5 (0.8)
Spleen	1.76 (0.04)	1.76 (0.05)	1.72 (0.06)	1.73 (0.06)	2.17 (0.07)$^{**,\#\#}$	1.98 (0.06)$^{*,\#}$
Thymus	0.90 (0.03)	0.98 (0.07)	0.94 (0.07)	0.97 (0.04)	1.00 (0.04)	0.90 (0.05)
Testes	8.34 (0.31)	8.72 (0.24)	8.39 (0.24)	8.48 (0.27)	9.01 (0.22)	8.42 (0.20)
Seminal vesicles	3.65 (0.23)	3.54 (0.17)	3.54 (0.17)	3.66 (0.13)	3.48 (0.15)	4.08 (0.16)
Epididymides	3.32 (0.10)	3.34 (0.10)	3.29 (0.10)	3.27 (0.10)	3.28 (0.07)	3.31 (0.08)
Females						
Terminal body weight	223 (3)	224 (4)	224 (5)	215 (3)	222 (4)	230 (5)
Adrenals	0.265 (0.009)	0.250 (0.007)	0.243 (0.015)	0.281 (0.009)	0.257 (0.007)	0.278 (0.011)
Brain	7.94 (0.14)	7.70 (0.15)	7.90 (0.21)	8.34 (0.13)$^{\#}$	7.99 (0.13)	7.65 (0.16)
Heart	3.48 (0.09)	3.50 (0.06)	3.53 (0.08)	3.62 (0.09)	3.32 (0.05)	3.43 (0.08)
Kidneys	6.06 (0.11)	6.06 (0.11)	6.14 (0.14)	6.09 (0.11)	5.74 (0.13)	6.12 (0.13)
Liver	34.6 (1.6)	34.7 (1.5)	35.2 (1.3)	34.9 (1.8)	32.0 (0.7)	35.4 (1.5)
Spleen	2.20 (0.07)	2.11 (0.07)	2.14 (0.06)	2.33 (0.10)	2.37 (0.05)	2.28 (0.09)
Thymus	1.26 (0.03)	1.18 (0.06)	1.22 (0.05)	1.27 (0.05)	1.16 (0.06)	1.24 (0.08)
Ovaries	0.334 (0.015)	0.287 (0.017)	0.314 (0.013)	0.321 (0.011)	0.302 (0.016)	0.326 (0.014)
Uterus	3.973 (0.544)	2.617 (0.271)*	2.579 (0.213)*	3.694 (0.476)	2.813 (0.268)	3.112 (0.265)

Anova + Dunnett tests.
$^{*}p < 0.05$; $^{**}p < 0.01$; significantly different from carrier (low-fat) controls.
$^{\#}p < 0.05$; $^{\#\#}p < 0.01$; significantly different from corn oil controls.
[a] High DHA Tuna oil.
[b] In brackets is given the standard error of the mean (SEM).

attributed to the administration of extra fat in the diet. Similar increases in alkaline phosphatase activity have been reported previously with high-fat diets (corn oil, canola oil as well as PUFA-containing oils) (Burns et al., 1999; Hempenius et al., 2000; Kroes et al., 2003).

The fundamental issue in this study was not the safety of ARA per se, but rather to investigate whether SUNTGA40S as a source of this fatty acid is safe. Changes observed in some blood and clinical chemistry parameters and spleen weight in the present study at high dose levels of SUNTGA40S are consistent with the previously reported physiological responses to dietary intake of high PUFA containing oils. The results of this study support the safety of SUNTGA40S under the conditions of intended use in infant formulas, traditional foods, speciality foods or food supplements.

References

The British Nutrition Foundation, 1992. Recommendations for intakes of unsaturated fatty acids. In: Unsaturated fatty acids: Nutritional and Physiological Significance. Chapman and Hall, London, pp. 152–163, 179.

Burns, R.A., Wibert, G.J., Diersen-Schade, D.A., Kelly, C.M., 1999. Evaluation of single-cell sources of docosahexaenoic acid and arachidonic acid: 3-month rat oral safety study with an *in utero* phase. Fd. Chem. Toxicol. 37, 23–36.

Calviello, G., Palozza, P., Franceschelli, P., Bartoli, G.M., 1997. Low-dose eicosapentaenoic or docosahexaenoic acid administration modifies fatty acid composition and does not affect susceptibility to oxidative stress in rat erythrocytes and tissues. Lipids 32, 1075–1082.

Danse, L.H., Verschuren, P.M., 1978. Fish oil induced yellow fat disease in rats. I. Histological changes. Vet. Pathol. 15, 114–124.

EEC Directive 87/302/EEC, Official Journal of the European Communities, no. L133, 30.5.88.

ESPGAN (European Society of Pediatric Gastroenterology and Nutrition) Committee on Nutrition, 1991. Comment on the content and composition of lipids in infant formulas. Acta Paediatr. Scand. 80, 887–896.

Hammond, B.G., Mayhew, D.A., Naylor, M.W., Ruecker, F.A., Mast, R.W., Sander, W.J., 2001. Safety assessment of DHA-rich microalgae from *Schizochytrium* sp.; I. Subchronic rat study. Regul. Toxicol. Pharmacol. 33, 192–204.

Harris, W.S., 1989. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. J. Lipid Res. 30, 785–807.

Hempenius, R.A., van Delft, J.M.H., Prinsen, M., Lina, B.A.R., 1997. Preliminary safety assessment of an arachidonic acid-enriched oil derived from *Mortierella alpine*: summary of toxicological data. Fd. Chem. Toxicol. 35, 573–581.

Hempenius, R.A., Lina, B.A.R., Haggit, R.C., 2000. Evaluation of a sub-chronic (13-week) oral toxicity study, preceded by an *in utero* exposure phase, with arachidonic acid oil derived from Mortierella alpine in rats. Fd. Chem. Toxicol. 38, 127–139.

Kohn, G., Sawatzki, G., Van Biervliet, J.P., Rossenue, M., 1994. Diet and the essential fatty acid status of term infants. Acta Paediatr. Suppl. 402, 69–74.

Koletzko, B., Thiel, I., Abiodun, P.O., 1992. The fatty acid composition of human milk in Europe and Africa. J. Pediatr. 120, S62–S70.

Kroes, R., Schaefer, E.J., Squire, R.A., Williams, G.M., 2003. A review of the safety of DHA-oil. Fd. Chem. Toxicol. 41, 1433–1446.

McGahon, B., Clements, M.P., Lynch, M.A., 1997. The ability of aged rats to sustain long-term potentiation is restored when the age-related decrease in membrane arachidonic acid concentration is reversed. Neuroscience 81, 9–16.

McGuire, S.O., Alexander, D.W., Fristche, K.L., 1997. Fish oil source differentially affects rat immune cell alpha tocopherol concentration. J. Nutr. 127, 1388–1394.

Merritt, R.J., Auestad, N., Kruger, C., Buchanan, S., 2003. Safety evaluation of sources of docosahexaenoic acid and arachidonic acid for use in infant formulas in newborn piglets. Fd. Chem. Toxicol. 41, 897–904.

Mills, D.E., Murthy, M., Galey, R., 1995. Dietary fatty acids, membrane transport, and oxidative sensitivity in human erythrocytes. Lipids 30, 657–663.

Moser, V.C., Tilson, H.A., MacPhail, R.C., Becking, G.C., Cuomo, V., Frantik, E., Kulig, B.M., Winneke, G., 1997. The IPCS collaborative study on neurobehavioral screening methods: II. Protocol design and testing procedures. Neurotoxicology 18, 929–938.

OECD, 1998a. Guideline for the Testing of Chemicals 408. Repeated dose 90-day oral toxicity studying rodents. OECD, Paris.

OECD, 1998b. OECD Principles of Good Laboratory Practice. ENV/MC/CHEM (98) 17. OECD, Paris.

Oarada, M., Furukawa, H., Majima, T., Miyazawa, T., 2000. Fish oil diet affects on oxidative senescence of red blood cells linked to degeneration of spleen cells in mice. Biochim. Biophys. Acta Mole. Cell Biol. Lipids 1487, 1–14.

Putnam, J.C., 1982. The effect of variations in dietary fatty acids on the fatty acid composition of erythrocyte phosphatidylcholine and phosphatydilethanolamine in human infants. Am. J. Clin. Nutr. 36, 106–113.

Rabbani, P.I., Sheikh, N.M., Chirtel, S.J., Jackson, R., Ruffin, G., 1999. Effects of long-term consumption of high doses of fish oil concentrates on clinical parameters in male and female rats. J. Nutr. Sci. Vitaminol. 45, 553–565.

Sanders, T.A.B., Ellis, F.R., Path, F.R.C., Dickerson, J.W.T., 1978. Studies of vegans: the fatty acid composition of plasma choline phosphoglycerides, erythrocytes, adipose tissue, and breast milk, and some indicators of susceptibility to ischemic heart disease in vegans and omnivore controls. Am. J. Clin. Nutr. 31, 805–813.

Sarkadi, N.E., Huang, M.C, Diau, G.Y., Kirwan, R., Chao, A.C., Tschanz, C., Brenna, J.T., 2003. Long chain polyunsaturated supplementation does not induce excess lipid peroxidation of piglet tissues. Eur. J. Nutr. 42, 293–296.

Report of the SCF (Scientific Committee for Food) on the essential requirements for infant formulae and follow-on formulae. Opinion expressed on 17 September 1993.

Soderberg, M., Edlund, C., Kristensson, K., Dallner, G., 1991. Fatty acid composition of brain phospholipids in aging and Alzheimer's disease. Lipids 26, 421–425.

Streekstra, H., 1997. Review: On the safety of *Mortierella alpina* for the production of food ingredients, such as Arachidonic acid. J. Biotechnol. 56, 153–165.

Tanaka, Y., Konishi, Y., Nishinume, T., 1994. Fatty acid composition of mature human milk in Osaka prefecture—relationship between the content of mother's diet and fatty acid composition of human milk. Osaka-Huritsu Koshuken Shoto, Shokunin Eiseihen 25, 21–31.

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Excessive ingestion of long-chain polyunsaturated fatty acids during developmental stage causes strain- and sex-dependent eye abnormalities in mice

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ABSTRACT

The eyes are riched in long-chain polyunsaturated fatty acids (LC-PUFAs) such as arachidonic acid [ARA; 20:4 (n–6)] and docosahexaenoic acid [DHA; 22:6 (n–3)]. Despite their abundance in the eyes, ARA and DHA cannot be sufficiently synthesized *de novo* in mammals. During gestation, eye development is exceptionally rapid, and substantial amounts of LC-PUFAs are needed to ensure proper eye development. Here, we studied the influences of dietary LC-PUFAs in dams (C57BL/6 and C3H/He) on the eye morphogenesis and organogenesis of their pups. Intriguingly, fetuses and newborn mice from C57BL/6 dams fed an LC-PUFA (particularly ARA)-enriched diet displayed a much higher incidence of eye abnormalities such as microphthalmia (small eye) and corneal opacity than those from dams fed an LC-PUFA-poor diet. The effects of LC-PUFAs on eye anomalies were evident only in the female pups of C57BL/6 inbred mice, not in those of C3H/He mice or male C57BL/6 mice. These results demonstrate a gene-by-environment (GxE) interaction in eye development in mice. Furthermore, our molecular analysis suggested the potential roles of *Pitx3* and *Pax6* in the above interaction involving ARA.

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs) have manifold effects on health and development. For instance, during gestation, large amounts of arachidonic acid [ARA; 20:4 (n–6)] and docosahexaenoic acid [DHA; 22:6 (n–3)] are deposited in the fetal eyes and brains, suggesting that these fatty acids are critical for normal visual and neuronal development [1]. Indeed, recent studies have shown that ARA and DHA may play an important role in eye and brain development during infancy [2]. The composition of LC-PUFAs influences the membrane stability, fluidity and function of many cell types through its effects on gene expression and tissue differentiation [1]. Fatty acid utilization in fetuses is largely dependent on the supply of what from the dam's blood, which is determined by the dam's fatty acid intake.

The mature eye is a complex organ that develops through a highly organized process during embryogenesis. Abnormalities in the ocular developmental program can lead to a variety of structural defects of the eyes that are present at birth [3]. At the severe end of the anomaly spectrum are anophthalmia (absence of eyes) and extreme microphthalmia (small eye) [4]. Microphthalmia has a complex aetiology including chromosomal and gene defects and environmental risk factors. Genes that have been implicated in microphthamia include those for multiple transcription factors (or regulators), e.g., *Lhx2*, *Rax*, *Otx2*, *Foxe3*, *Pitx3*, *Maf*, *Sox2* and *Pax6* [5–12]. As environmental factors, exposure to radiation, chemicals and viruses have been reported.

Other common eye malformations are congenital cataracts, and corneal opacity including sclerocornea (a congenital condition in which the sclera and cornea are considered as a single layer), and disorders of early retinal differentiation. The cornea is a transparent structure on the front of the eyeball; in corneal opacity, this tissue becomes cloudy or opacified, eliciting serious vision problems. The cornea is scarred by injury, vitamin A deficiency, measles, and viral infections including ocular or eye herpes, herpes zoster and shingles [13]. However, the genes for corneal opacity are not well understood. Much of our knowledge about the function of genes relevant to cataracts has been derived from the molecular analysis of spontaneous and induced mutations in mice; (i) the most frequent mutations in congenital cataracts are seen in genes coding for gamma-crystallins (Cryg family), (ii) some postnatal, progressive cataracts are induced by mutations in the alpha- and beta-crystallin-encoding genes (*Cryα* and *Cryb* families, respectively),

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(iii) defects of transcription factors such as Foxe3, Pitx3, Maf, Sox2 and Pax6 cause both cataracts and microphthalmia [10,14–17].

In our previous study using rats with a *Pax6* mutation, we obtained evidence that an enriched maternal dietary intake of ARA is beneficial for improving human mental illness-related phenotypes [18]. Then, we continued to assess the biological consequences of antenatal and postnatal LC-PUFA administrations in mice. In that process, we noticed the increased incidence of eye abnormalities in female C57BL/6 mice fed an LC-PUFA-enriched diet during eye development. Therefore, in this study we set out to systematically evaluate the effects of LC-PUFA-enriched diets on eye development, by examining two different inbred strains (C57BL/6 and C3H/He) of both sexes. Because of the complexity of eye abnormalities and the well-documented retinal abnormalities induced by LC-PUFAs [19,20], we mainly focus on the abnormalities of anterior portions of ocular structures of the eyes in this study.

2. Materials and methods

2.1. Animals

Inbred strains of C57BL/6NCrlCrlj (C57BL/6) and C3H/HeNCrlCrj (C3H/He) were obtained from Japan's Charles River Laboratories (Tokyo, Japan). The animals were housed in groups of four in standard cages in a temperature and humidity-controlled room with a 12-h light/dark cycle (lights on at 08:00), and had free access to chow and tap water. All the experiments were performed between 10:00 and 14:00. The sex of the fetuses at embryonic day 12.5 (E12.5) and E16.5 was checked by the PCR-amplification of the *Sry* gene. The experimental protocol was approved by the RIKEN Animal Ethics Committee.

2.2. Various LC-PUFA-containing and conventional diets

We prepared 4 different diets by altering the fatty acid compositions of AIN-76: ARA(−)/DHA(−), ARA(+)/DHA(−), ARA(−)/DHA(+) and ARA(+)/DHA(+) diets (Table S1) [18]. All the chows were stored at 4 °C and shaded from light to prevent oxidation before use. In addition, they were not treated by gamma irradiation or autoclave sterilization. As a conventional diet, we used CRF1 (Charles River formula; purchased from Oriental Yeast Co., Ltd., Tokyo, Japan) (Table S2).

2.3. Histological examination

The sections were stained with hematoxylin and eosin. See Supplementary Materials and Methods for details.

2.4. Magnetic resonance imaging (MRI)

MRI pictures of the eye portions of adult mice were acquired by MRI using a vertical-bore 9.4 T Bruker AVANCE 400WB imaging spectrometer (Bruker BioSpin, Rheinstetten, Germany). See Supplementary Materials and Methods for details.

2.5. Real-time quantitative RT-PCR

The transcript levels were determined by real-time quantitative PCR (qRT-PCR), using a TaqMan Gene Expression Master Mix, transcript-specific minor groove binding (MGB) probes (Applied Biosystems) and an ABI 7900 sequence detection system, according to the manufacturers' instructions. The *Gapdh* gene was chosen as the control (Applied Biosystems). See Supplementary Note for details.

2.6. Statistical analysis

The mean sizes of the eyes of the mice among the different diet groups were evaluated using Dunn's multiple comparison test, with the ARA(−)/DHA(−) group as the control. The incidence rates of eye abnormalities were compared using Fisher's exact test. The gene expression levels were evaluated using two-tailed unpaired Student's *t*-test.

3. Results

3.1. Effects of dietary LC-PUFAs on eye development

We raised two strains of mice (C57BL/6 and C3H/He) on either of four different LC-PUFA diets for a period spanning 2 weeks before mating to 3 weeks after the birth of pups (this age corresponds to the weaning point) (Fig. 1A, Fig. S1A). The LC-PUFA diets were ARA(−)/DHA(−), ARA(+)/DHA(−), ARA(−)/DHA(+) and ARA(+)/DHA(+) (Table S1) [18]. After weaning, the mice were raised on a conventional diet (CRF1) until 6 months of age (Table S2). We used the ARA(−)/DHA(−) (LC-PUFA-poor) diet, not the CRF1 diet, during the above period as the control group, because (1) we thought that it is important to make the contents of all components except lipids in the diets even, to strictly compare the dietary effects on embryonal development, (2) we observed no differences in the incidence rates of eye abnormalities between the ARA(−)/DHA(−) and CRF1 diets in our preliminary experiments (data not shown), and (3) to date, we have performed our study using the same combination of the ARA(−)/DHA(−), ARA(+)/DHA(−), ARA(−)/DHA(+) and ARA(+)/DHA(+) diets [18]. The prolonged administration of the LC-PUFA diets from birth to the weaning point is also for our planned next study, where we will examine the behavioral consequences of what in adulthood (See Introduction).

We examined 878 eyes (C57BL/6 mice: 196 male eyes and 188 female eyes; C3H/HE mice: 228 male eyes and 266 female eyes) at 6 months of age. All the LC-PUFA-diet-fed mice grew normally in terms of the morphologies of their bodies and various organs (data not shown), except the eyes. In the eyes, we observed that the female C57BL/6 mice fed the ARA(+)/DHA(−) or ARA(+)/DHA(+) diet show a higher incidence of microphthalmia than those treated with the ARA(−)/DHA(−) diet, whereas the male C57BL/6 mice did not display statistically significant eye size differences among the distinct diet groups (Fig. 1B). Eyeball size (longest eye axis) was measured carefully using a stereoscopic microscope (Fig. S2). Typical MRI pictures of microphthalmia in living mice are shown in Fig. 1C. Microphthalmia was already evident in an embryonic stage (E16.5) (Fig. S3). In contrast, C3H/He mice of both sexes did not show any differences in eye size among the four different diet groups (Fig. S1B). The eyes whose sizes were zero are deemed to correspond to anophthalmia (Fig. 1B).

The 878 eyes were also examined regarding other ocular abnormalities occurring in the anterior ocular segments and vitreous body. Strikingly, in the C57BL/6 female mice, the incidence of cataract and corneal opacity including sclerocornea, both of which involve the disturbances of the anterior surface of the eye globe, was significantly increased in the ARA(+)/DHA(+) group, compared with that in the ARA(−)/DHA(−) group (Table 1). Keratolenticular strands, which are aberrant tissues bridging the cornea and lens and thereby leading to the reduction in the size of the anterior chamber, were also seen, although there were no statistical differences in the incidence. Irido-lenticular/corneal synechia means the adhesion of the iris to the cornea (Table 1). This anomaly was sometimes seen in C57BL/6 mice, although the incidences were not statistically different among the diet groups. The incidence rates of all three eye abnormalities listed in Table 1 tended to be

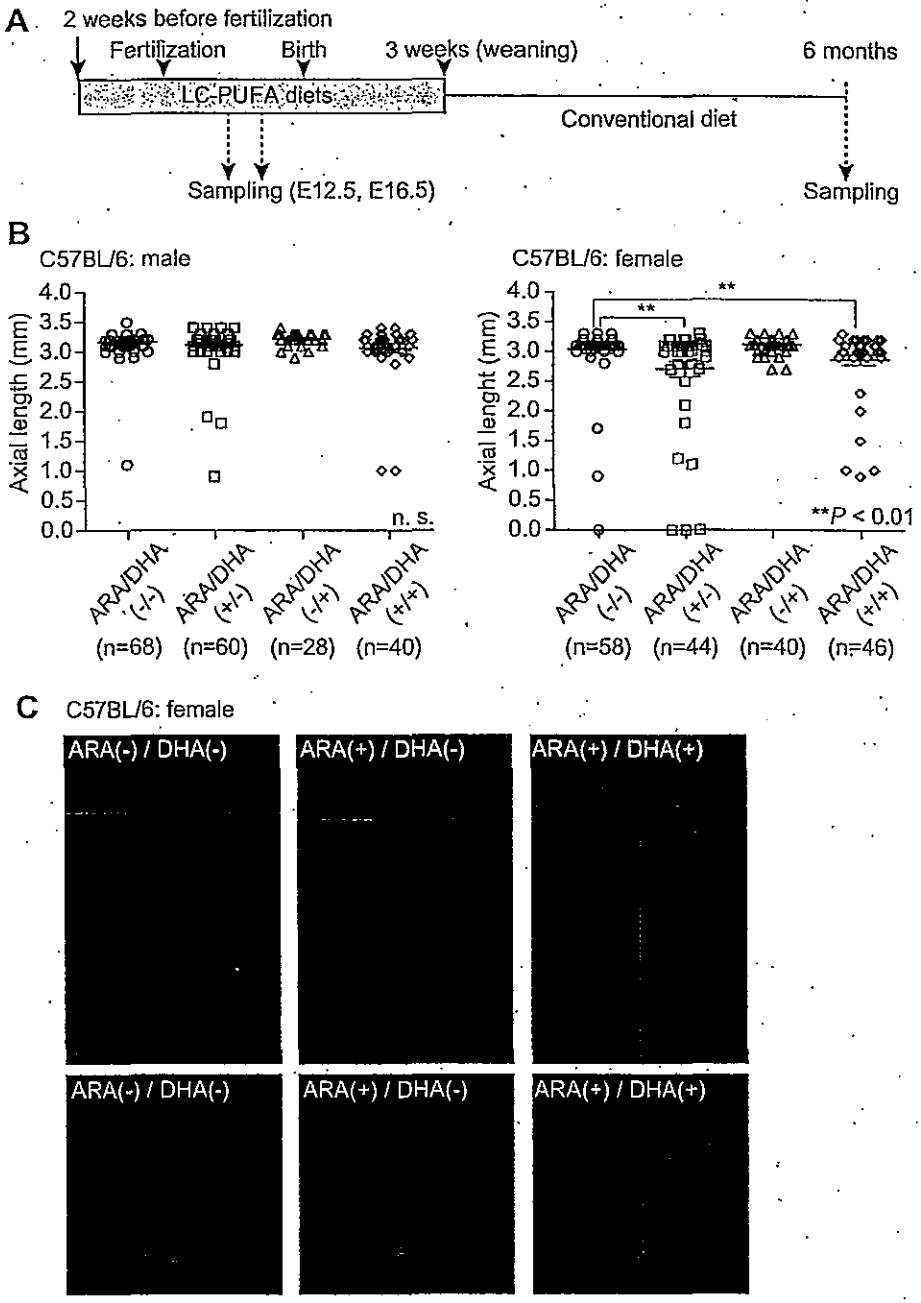

Fig. 1. Effects of LC-PUFA diets on axial length of eyes in C57BL/6 adult mice. (A) Experimental schedule of diet administration and sampling. (B) The effects of LC-PUFA diets on the axial length of eyes. Each error bar shows a mean ± SEM. $^{**}P < 0.01$ (C) Typical MRI images of abnormal eyes. The upper panels show horizontal sections and the lower coronal sections at the levels of green horizontal lines shown in the corresponding upper panels. Left panels: both eyes are normal in size; middle panels: both eyes show microphthalmia or anophthalmia; right panels: the left eye is normal in size and the right eye shows microphthalmia or anophthalmia.

Table 1
Ocular abnormalities observed in C57BL/6 mice

Sex	Ocular abnormalities	Diet			
		ARA(−)/DHA(−)	ARA(+)/DHA(−)	ARA(−)/DHA(+)	ARA(+)/DHA(+)
Male	Cataract and/or corneal opacity (including sclerocornea)	3/68 (4.4%)	8/60 (13.3%)	2/28 (7.1%)	6/40 (15.0%)
	Keratolenticular strand	4/68 (5.9%)	2/60 (3.3%)	0/28 (0%)	3/40 (7.5%)
	Irido-lenticular or corneal synechia	7/68 (10.3%)	2/60 (3.3%)	1/28 (3.6%)	4/40 (10.0%)
Female	Cataract and/or corneal opacity (including sclerocornea	8/58 (13.8%)	12/44 (27.3%)	5/40 (12.5%)	17/46 (37.0%)*
	Keratolenticular strand	5/58 (8.6%)	4/44 (9.1%)	2/40 (5.0%)	3/46 (6.5%)
	Irido-lenticular or corneal synechia	9/58 (15.5%)	4/44 (9.1%)	5/40 (12.5%)	5/46 (10.9%)

The numericals in the column show the eye numbers with indicated phenotype/total numbers of eyes examined.
* $P < 0.01$ compared to the ARA(−)/DHA(−) group.

higher in the female C57BL/6 mice than in the male C57BL/6 mice. In contrast, these abnormal phenotypes were scarcely seen in C3H/He mice (Table S3).

We also performed histological examinations of the eyes from the C57BL/6 females treated with the ARA(+)/DHA(−) and ARA(+)/DHA(+) diets. At E16.5, when the development of eye anlage is almost completed, a normally developing lens is observed, as shown in Fig. 2A. However, lenses from the ARA(+)/DHA(−) treated embryos were hypoplastic (Fig. 2B, a) or missing (Fig. 2B, b) with abundant abnormal retrolental tissue (arrowheads in Fig. 2B). There were no clear signs of corneal differentiation (Figs. 2B, a and b). In a 6-month-old mouse, normally developing eye structures including the retina and optic nerve are shown in Fig. S4A. But, in the eyes of the ARA(+)/DHA(−) treated group, retinal lamination defects and abnormal folding were clear in addition to lens dysgenesis and the presence of pigmented retrolental tissues (Fig. S4B).

3.2. qRT-PCR analysis of candidate genes underlying eye abnormalities

To address the molecular basis of abnormal eye development observed in the female C57BL/6 mice treated with the LC-PUFA-enriched diets, we examined whether there are perturbations of expressions of genes involved in eye development at E12.5 (corresponding to around the middle stage of eye development) and E16.5. As the known causative genes for microphthalmia, *Lhx2*, *Rax*, *Otx2*, *Foxe3*, *Pitx3*, *Maf*, *Sox2* and *Pax6* were selected [5–12]. For cataract-causing genes, *Foxe3*, *Pitx3*, *Maf*, *Sox2*, *Pax6*, *Cryaa*, *Cryab*, *Crybb1* and *Crygf* were examined (Fig. 3A). *Foxe3*, *Pitx3*, *Maf*, *Sox2* and *Pax6* are overlapping between the two phenotypes. We carefully removed the eyes from the embryonic bodies under a stereoscopic microscope, and extracted mRNA from them. Gene expressions were compared between the ARA(−)/DHA(−) and ARA(+)/DHA(−) groups, and between the ARA(−)/DHA(−) and ARA(+)/DHA(+) groups. Interestingly, at E12.5, the ARA(+)/DHA(−) diet evoked the downregulation of *Pitx3*, compared with the ARA(−)/DHA(−) diet (Fig. 3A, left panel). However, the ARA(+)/DHA(+) did not alter the expression levels of any genes at this developmental stage (Fig. 3B, left panel). On the other hand, at E16.5, the ARA(+)/DHA(−) diet elicited the upregulation of *Lhx2* (Fig. 3A, right panel). The ARA(+)/DHA(+) diet induced significant expressional changes in all the genes examined except *Otx2* (Fig. 3B, right panel).

Dietary PUFAs regulate a transcription factor called sterol regulatory element-binding factor 1 (*Srebf1*) [21,22]. Our database search (TFBIND: http://tfbind.hgc.jp/) showed that each of the 12 genes examined here has multiple consensus binding sites for Srebf1 in regions spanning 1000 bp upstream of the 5′-end of exon 1 to 1000 bp downstream from the 5′-end of intron 1, with a cutoff value of more than 0.72. The ARA(+)/DHA(+) group at E16.5, but not at E12.5, showed upregulated *Srebf1* mRNA levels, compared with the ARA(−)/DHA(−) diet group in the C57BL/6 female mice (Fig. 4, lower panel). There were no differences between the ARA(−)/DHA(−) and ARA(+)/DHA(−) diet groups at E12.5 or E16.5 (Fig. 4, upper panel). We did not compare the expression levels between the ARA(−)/DHA(−) and ARA(−)/DHA(+) diet groups, because the ARA(−)/DHA(+) group did not show any significant increases in the incidences of the examined abnormalities. These

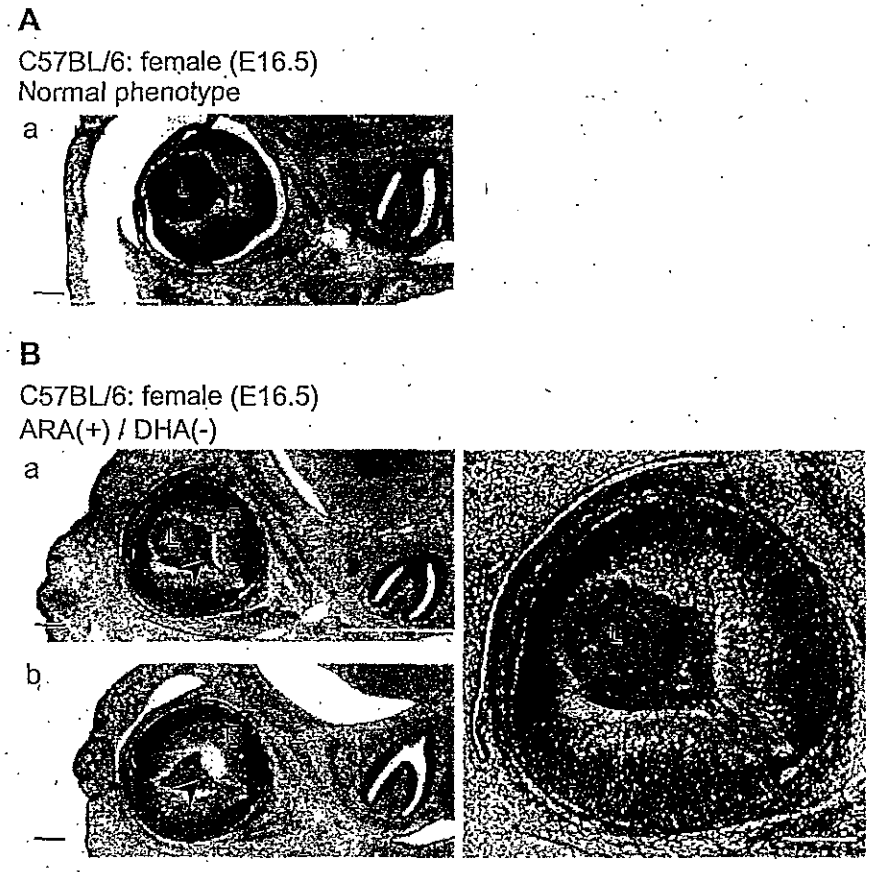

Fig. 2. Histological examinations of eyes from C57BL/6 females fed ARA(+)/DHA(−) diet. (A) Regularly formed eye. (B) The lens is abnormally small (B, a) or the lens is almost missing (only remnant is present) (B, b) in the ARA(+)/DHA(−) group at E16.5. The picture of (B, c) is a magnified one of the ocular portion shown in (B, a). Arrowheads in A, a and b show abnormal retrorental tissues. Scale bars indicate 200 μm. L, lens; R, retina.

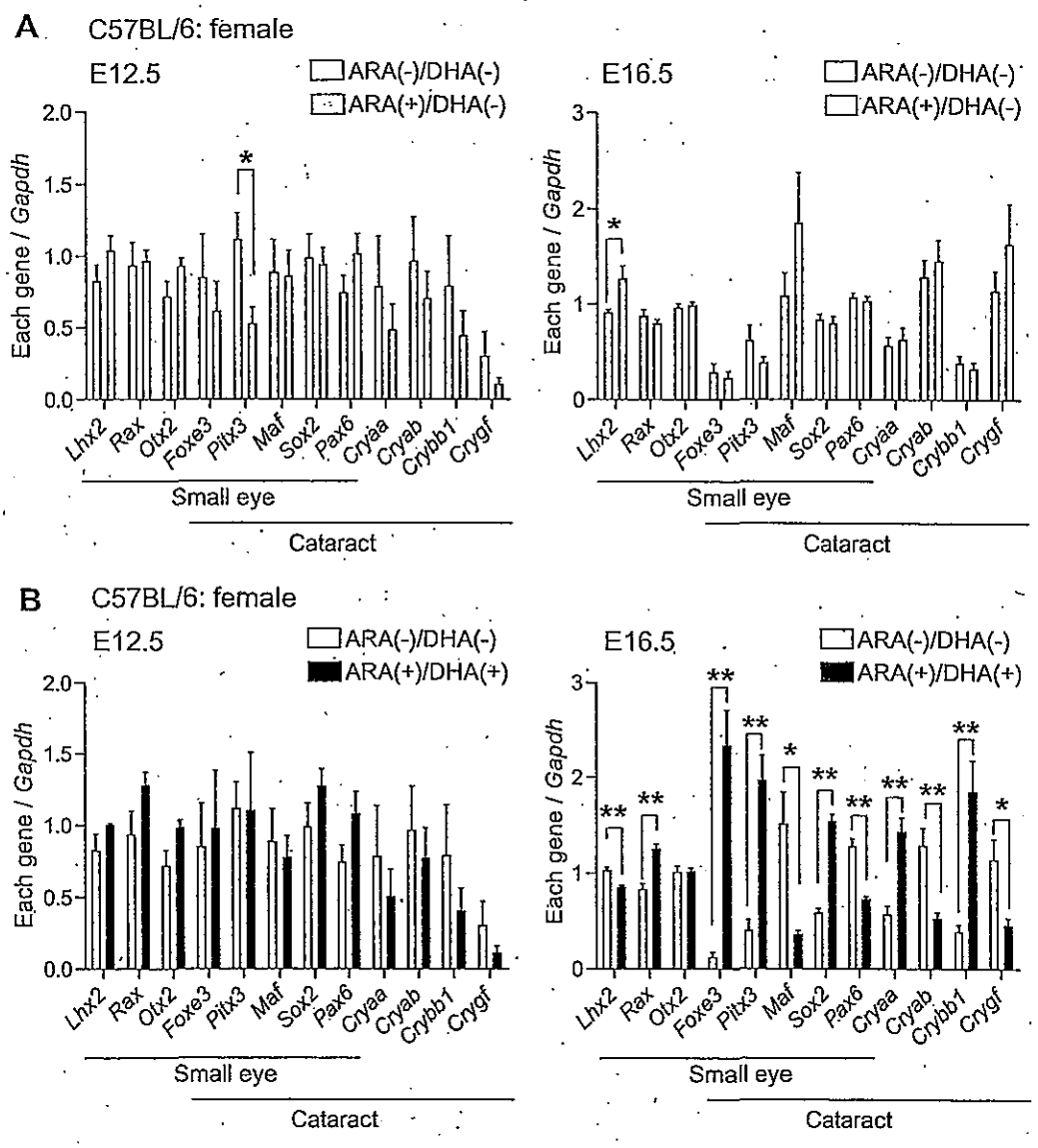

Fig. 3. qRT-PCR analysis of eye development-related genes. Each error bar shows a mean ± SEM. $n = 6$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$.

results suggest that the elevated expression levels of genes shown in Fig. 3B, right panel, may be at least partly due to the upregulation of the *Srebf1* gene.

To narrow down the candidate genes responsible for enriched-LC-PUFA-induced microphthalmia, and cataract and/or corneal opacity phenotypes in C57BL/6 female mice and to see whether there is a sex-specific differential regulation of gene expressions by LC-PUFAs, we examined the expression levels of the same set of genes using eye samples from the male C57BL/6 mice, which can be enriched-LC-PUFA diet-resistant controls (Figs. S5 and S6). The only difference was the downregulation of *Pitx3* at E12.5 in only the female mice fed the ARA(+)/DHA(−) diet. This result implies that the ARA(+)/DHA(−)-diet-induced microphthalmia and cataract may be related to the diminished expression level of *Pitx3* at a critical point (e.g., around E12.5).

4. Discussion

C57BL/6 mice have eye abnormalities more frequently than C3H/He mice (information from Oriental Yeast Co., Ltd., and also see [23]). In the current study, we further revealed that the administration of enriched ARA- or ARA and DHA-containing diet during the developmental stage increases the incidence of eye abnormalities in only the C57BL/6 female pups, not in the male C57BL/6 or C3H/He mice of both sexes. These results add a palpable example of the gene-by-environment (GxE) (in this case dietary LC-PUFAs) interactions. For a GxE interaction on tumor growth and immune functions, it was reported in 1980 that a PUFA-rich diet enhances proliferative response to mitogens and the rate of growth of a spontaneous transplantable adenocarcinoma as compared with a PUFA-poor diet result in females, not males, of BALB/c mice, and that in female DBA/2 mice, low dietary PUFA determines a reduced tumor growth only, but it does not affect proliferative response to mitogens [24].

Because both the ARA(+)/DHA(−) and ARA(+)/DHA(+) diets increased the incidence of microphthalmia, it is likely that ARA has a more significant effect on the eruption of abnormalities than DHA. Regarding cataract and/or corneal opacity, since only the ARA(+)/DHA(+) diet significantly raised its incidence in female C56BL/6 mice, the excess of both ARA and DHA, or ARA alone in the diet is deemed responsible for this abnormality. It is suggested that females possess a greater capacity for α-linolenic acid conversion to eicosapentaenoic acid, docosapentaenoic acid and DHA than males. Such a metabolic capacity is thought to be important

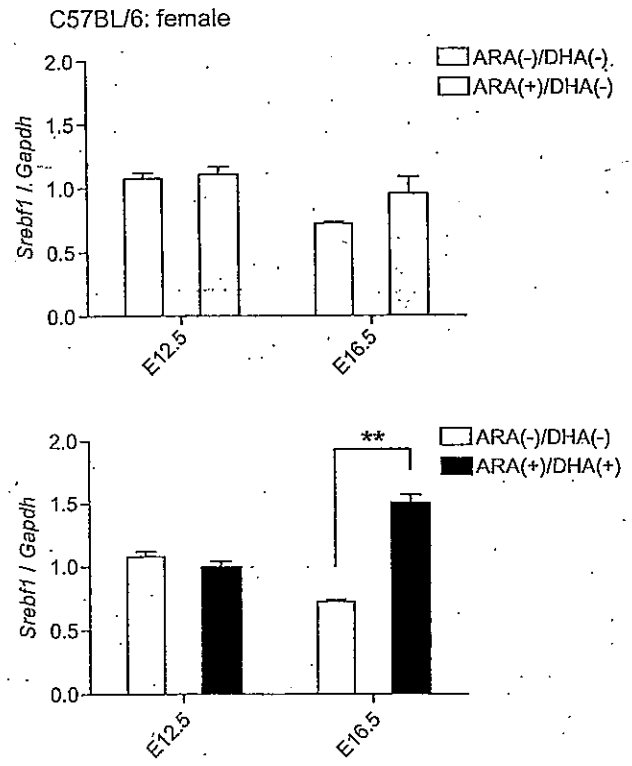

Fig. 4. qRT-PCR analysis of *Srebf1* transcript in ARA(−)/DHA(−), ARA(+)/DHA(−) and ARA(+)/DHA(+) mice at E12.5 and E16.5. Each error bar shows a mean ± SEM. $n = 6$ per group. $^{**}P < 0.01$.

for meeting the demands of fetuses and neonates for DHA during pregnancy and lactation [25]. The differential eye abnormality occurrences between the male and female C57/BL6 mice detected in this study may partly reflect a sex-dependent distinct capacity of lipid metabolism.

Conforming to the observed abnormal eye phenotypes, the expression levels of genes associated with the development of anterior ocular parts and causative for their abnormalities were diversely affected, particularly at E16.5 in the ARA(+)/DHA(+) group. These expressional changes were similar between the male and female C57BL/6 mice (Fig. 3B, right panel and Fig. S5B, right panel). Therefore, the perturbation of the gene expressions at E16.5 stated above, albeit remarkable, does not seem to be directly linked to the formation of eye abnormalities specific to female C57BL/6. The expression level of *Pax6* was reduced to about half in both males and females (Fig. 3B and Fig. S5B). Rats with a heterozygous *Pax6* gene defect show multipronged eye abnormalities, suggesting that *Pax6* has a mater-gene-like role in the eye formation [10]. Therefore, those diverse expressional changes, including those of *Pax6* may underlie the observation that this inbred strain is prone to be affected by LC-PUFA-rich diets in terms of eye abnormalities (particularly, cataract and/or corneal opacity: see Table 1). To address this speculation, it would be necessary to examine gene expressions in C3H/He mice in the future. Although we could not identify the genes truly associated with the observed eye abnormalities at E16.5, a notable gene is *Pitx3*, which showed downregulation at E12.5 in only the C57BL/6 female mice fed the ARA(+)/DHA(−) diet, not in C57BL/6 male mice. *Pitx3* is expressed during early vertebrate lens development. The deletion of this gene causes abnormal lens phenotypes in the mouse line *aphakia* (*ak*) [5,6]. In *ak* mice, the lens can start to form, but its development is disturbed. Eventually, lens development is arrested and the lens disappears [26–29]. The abnormal lens phenotypes observed in the female mice fed the ARA(+)/DHA(−) diet are similar to some aspects of lens abnormalities reported in the *ak* mutants [5,6] (Fig. 2B and Fig. S4B).

Other than eye abnormalities, small brains and hypolasia of lower jaws at E16.5 were observed in the female C57BL/6 mice fed the ARA(+)/DHA(+) diet (Figs. S3B, e, and f). These issues should also be pursued in future studies.

As stated in Introduction, we previously reported in animal experiments that dietary LC-PUFAs may have potential to improve the phenotypes relevant to human psychiatric illnesses [18]. Indeed, recently, Amminger et al. reported that long-chain ω-3 (n−3) PUFAs reduce the risk of progression to psychotic disorders and may offer a safe and efficacious strategy for indicated prevention in young people with subthreshold psychotic states [30]. Although an LC-PUFA-enriched diet was reported to be nontoxic in rats [31], considering our current results using mice, we need to be cautious about the intake of a high dose of LC-PUFAs from supplements by pregnant mothers.

In summary, this study provides pivotal evidence that rich dietary LC-PUFAs (particularly ARA) are associated with ocular abnormalities in a strain- and sex-dependent manner in mice. Further studies on the biological mechanisms linking LC-PUFA metabolism and eye development are warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.10.051.

References

[1] J.R. Marszalek, H.F. Lodish, Docosahexaenoic acid fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you, Annu. Rev. Cell Dev. Biol. 21 (2005) 633–657.
[2] A.S. Ryan, J.D. Astwood, S. Gautier, C.N. Kuratko, E.B. Nelson, N. Salem Jr., Effects of long-chain polyunsaturated fatty acid supplementation on neurodevelopment in childhood: a review of human studies, Prostaglandins, Leukotrienes Essent. Fatty Acids 82 (2010) 305–314.
[3] J. Graw, The genetic and molecular basis of congenital eye defects, Nat. Rev. Genet. 4 (2003) 876–888.
[4] D. Morrison, D. FitzPatrick, I. Hanson, K. Williamson, V. van Heyningen, B. Fleck, I. Jones, J. Chalmers, H. Campbell, National study of microphthalmia, anophthalmia, and coloboma (MAC) in Scotland: investigation of genetic aetiology, J. Med. Genet. 39 (2002) 16–22.
[5] E.V. Semina, J.C. Murray, R. Reiter, R.F. Hrstka, J. Graw, Deletion in the promoter region and altered expression of Pitx3 homeobox gene in aphakia mice, Hum. Mol. Genet. 9 (2000) 1575–1585.
[6] D.K. Rieger, E. Reichenberger, W. McLean, A. Sidow, B.R. Olsen, A double-deletion mutation in the Pitx3 gene causes arrested lens development in aphakia mice, Genomics 72 (2001) 61–72.
[7] O. Medina-Martinez, I. Brownell, F. Amaya-Manzanares, Q. Hu, R.R. Behringer, M. Jamrich, Severe defects in proliferation and differentiation of lens cells in Foxe3 null mice, Mol. Cell Biol. 25 (2005) 8854–8863.
[8] J. Fantes, N.K. Ragge, S.A. Lynch, N.I. McGill, J.R. Collin, P.N. Howard-Peebles, C. Hayward, A.J. Vivian, K. Williamson, V. van Heyningen, D.R. FitzPatrick, Mutations in SOX2 cause anophthalmia, Nat. Genet. 33 (2003) 461–463.
[9] T. Glaser, L. Jepeal, J.G. Edwards, S.R. Young, J. Favor, R.L. Maas, PAX6 gene dosage effect in a family with congenital cataracts aniridia, anophthalmia and central nervous system defects, Nat. Genet. 7 (1994) 463–471.
[10] S. Kanakubo, T. Nomura, K. Yamamura, J. Miyazaki, M. Tamai, N. Osumi, Abnormal migration and distribution of neural crest cells in Pax6 heterozygous mutant eye a model for human eye diseases, Genes Cells 11 (2006) 919–933.

[11] N.K. Ragge, A.G. Brown, C.M. Poloschek, B. Lorenz, R.A. Henderson, M.P. Clarke, I. Russell-Eggitt, A. Fielder, D. Gerrelli, J.P. Martinez-Barbera, P. Ruddle, J. Hurst, J.R. Collin, A. Salt, S.T. Cooper, P.J. Thompson, S.M. Sisodiya, K.A. Williamson, D.R. Fitzpatrick, V. van Heyningen, I.M. Hanson, Heterozygous mutations of OTX2 cause severe ocular malformations, Am. J. Hum. Genet. 76 (2005) 1008–1022.
[12] V.A. Voronina, E.A. Kozhemyakina, C.M. O'Kernick, N.D. Kahn, S.L. Wenger, J.V. Linberg, A.S. Schneider, P.H. Mathers, Mutations in the human RAX homeobox gene in a patient with anophthalmia and sclerocornea, Hum. Mol. Genet. 13 (2004) 315–322.
[13] J.P. Whitcher, M. Srinivasan, M.P. Upadhyay, Corneal blindness: a global perspective, Bull. World Health Organ 79 (2001) 214–221.
[14] A. Blixt, M. Mahlapuu, M. Aitola, M. Pelto-Huikko, S. Enerback, P. Carlsson, A forkhead gene, FoxE3 is essential for lens epithelial proliferation and closure of the lens vesicle, Genes Dev. 14 (2000) 245–254.
[15] M.F. Lyon, R.V. Jamieson, R. Perveen, P.H. Glenister, R. Griffiths, Y. Boyd, L.H. Glimcher, J. Favor, F.L. Munier, G.C. Black, A dominant mutation within the DNA-binding domain of the bZIP transcription factor Maf causes murine cataract and results in selective alteration in DNA binding, Hum. Mol. Genet. 12 (2003) 585–594.
[16] S. Nishiguchi, H. Wood, H. Kondoh, R. Lovell-Badge, V. Episkopou, Sox1 directly regulates the gamma-crystallin genes and is essential for lens development in mice, Genes Dev. 12 (1998) 776–781.
[17] N. Rajaram, T.K. Kerppola, Synergistic transcription activation by Maf and Sox and their subnuclear localization are disrupted by a mutation in Maf that causes cataract, Mol. Cell Biol. 24 (2004) 5694–5709.
[18] M. Maekawa, N. Takashima, M. Matsumata, S. Ikegami, M. Kontani, Y. Hara, H. Kawashima, Y. Owada, Y. Kiso, T. Yoshikawa, K. Inokuchi, N. Osumi, Arachidonic acid drives postnatal neurogenesis and elicits a beneficial effect on prepulse inhibition a biological trait of psychiatric illnesses, PLoS One 4 (2009) e5085.
[19] E.L. Berson, B. Rosner, M.A. Sandberg, C. Weigel-DiFranco, A. Moser, R.J. Brockhurst, K.C. Hayes, C.A. Johnson, E.J. Anderson, A.R. Gaudio, W.C. Willett, E.J. Schaefer, Further evaluation of docosahexaenoic acid in patients with retinitis pigmentosa receiving vitamin A treatment: subgroup analyses, Arch. Ophthalmol. 122 (2004) 1306–1314.
[20] D.R. Hoffman, K.G. Locke, D.H. Wheaton, G.E. Fish, R. Spencer, D.G. Birch, A randomized placebo-controlled clinical trial of docosahexaenoic acid supplementation for X-linked retinitis pigmentosa, Am. J. Ophthalmol. 137 (2004) 704–718.
[21] Z. Sheng, H. Otani, M.S. Brown, J.L. Goldstein, Independent regulation of sterol regulatory element-binding proteins 1 and 2 in hamster liver, Proc. Natl. Acad. Sci. USA 92 (1995) 935–938.
[22] J. Xu, H. Cho, S. O'Malley, J.H. Park, S.D. Clarke, Dietary polyunsaturated fats regulate rat liver sterol regulatory element binding proteins-1 and -2 in three distinct stages and by different mechanisms, J. Nutr. 132 (2002) 3333–3339.
[23] M.L. Robinson, A. Holmgren, M.J. Dewey, Genetic control of ocular morphogenesis: defective lens development associated with ocular anomalies in C57BL/6 mice, Exp. Eye Res. 56 (1993) 7–16.
[24] M. Giovarelli, E. Padula, G. Ugazio, G. Forni, G. Cavallo, Strain- and sex-linked effects of dietary polyunsaturated fatty acids on tumor growth and immune functions in mice, Cancer Res. 40 (1980) 3745–3749.
[25] G.C. Burdge, S.A. Wootton, Conversion of alpha-linolenic acid to eicosapentaenoic docosapentaenoic and docosahexaenoic acids in young women, Br. J. Nutr. 88 (2002) 411–420.
[26] D.S. Varnum, L.C. Stevens, Aphakia, a new mutation in the mouse, J. Hered. 59 (1968) 147–150.
[27] J. Zwaan, B.M. Kirkland, Malorientation of mitotic figures in the early lens rudiment of aphakia mouse embryos, Anat. Rec. 182 (1975) 345–354.
[28] J. Zwaan, Immunofluorescent studies on aphakia a mutation of a gene involved in the control of lens differentiation in the mouse embryo, Dev. Biol. 44 (1975) 306–312.
[29] C. Grimm, B. Chatterjee, J. Favor, T. Immervoll, J. Loster, N. Klopp, R. Sandulache, J. Graw, Aphakia (ak), a mouse mutation affecting early eye development: fine mapping, consideration of candidate genes and altered Pax6 and Six3 gene expression pattern, Dev. Genet. 23 (1998) 299–316.
[30] G.P. Amminger, M.R. Schafer, K. Papageorgiou, C.M. Klier, S.M. Cotton, S.M. Harrigan, A. Mackinnon, P.D. McGorry, G.E. Berger, Long-chain omega-3 fatty acids for indicated prevention of psychotic disorders: a randomized placebo-controlled trial, Arch. Gen. Psychiatry 67 (2010) 146–154.
[31] B.A. Lina, A.P. Wolterbeek, Y. Suwa, S. Fujikawa, Y. Ishikura, S. Tsuda, M. Dohnalek, Subchronic (13-week) oral toxicity study, preceded by an in utero exposure phase, with arachidonate-enriched triglyceride oil (SUNTGA40S) in rats, Food Chem. Toxicol. 44 (2006) 326–335.

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Research Section

A developmental safety study in rats using DHA- and ARA-rich single-cell oils

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Abstract—The long-chain omega-3 and omega-6 fatty acids, docosahexaenoic and arachidonic acids, are important in fetal development, but may be depleted from the mother during pregnancy as she transfers reserves to the developing fetus *in utero* and later to the infant through her breast milk. Pregnant women can increase their dietary intake of these nutrients to maintain adequate maternal reserves and ensure an optimal infant supply. DHASCO® and ARASCO® oils, concentrated sources of docosahexaenoic and arachidonic acids, respectively, have been tested in acute and subchronic studies without toxic effects. The present developmental toxicity study was undertaken to test for potential teratogenic activity of these oils to ensure their safe use during pregnancy. DHASCO and ARASCO oils were administered by oral gavage to pregnant rats at doses up to 1250 and 2500 mg/kg body weight/day, respectively, during the period of organogenesis. Caesarean sections and necropsies were performed on day 20 of gestation. Maternal reproductive outcomes were analyzed, and fetal external, soft and skeletal tissue were examined. Treatment with these oils did not produce overt maternal toxicity, nor did either oil result in changes in pre- or postimplantation losses, resorptions, live births or sex ratios. Neither oil caused fetal malformations. Increased frequencies of renal variations in development occurred in a non-dose-dependent manner and were not toxicologically significant. We conclude that these oils are not teratogenic at doses that represent a 100-fold safety factor over expected use levels.

Keywords: docosahexaenoic acid (DHA); arachidonic acid; fetal organogenesis; fetal development; teratogenicity and toxicity study.

Abbreviations: ARA = arachidonic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; LC-PUFAs = long-chain polyunsaturated fatty acids; NOAEL = no-observable-adverse-effect level.

INTRODUCTION

The long-chain omega-3 and omega-6 fatty acids docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (ARA; 20:4n-6) are important to the developing fetus, particularly for neural and vascular tissue development (Crawford, 1993). These fatty acids are incorporated into the membranes of fetal tissues at a particularly high rate during the third trimester of pregnancy, when the brain and vascular system are growing at a very rapid pace (Crawford *et al.*, 1997). Both DHA and ARA are efficiently transferred from maternal to fetal circulation through the placenta, which is adept at concentrating these long-chain polyunsaturated fatty acids (LC-PUFAs) in a process known as biomagnification (Ruyle *et al.*, 1990). An infant's DHA and ARA status at birth is correlated to the mother's levels of these fatty acids during pregnancy, especially during the last trimester (Connor *et al.*, 1996; Otto *et al.*, 1997a). The mother continues to supply the infant with DHA and ARA postnatally through her breast milk. Breast milk levels of DHA are linearly related to maternal DHA intake (Makrides *et al.*, 1996).

As the mother utilizes her stores of DHA to support the developing fetus, her own DHA status is compromised, as evidenced by a relative increase in mead acid (20:3n-9), a marker of essential fatty acid deficiency, in umbilical cord arteries, and more specifically by an

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increase in the maternal DHA deficiency index (the 22:5n-6 docosapentaenoic acid to DHA ratio in blood) as the pregnancy progresses (Al *et al.*, 1995; Hornstra *et al.*, 1989; Otto *et al.*, 1997a). The DHA status of the mother declines further in the postpartum period, especially if she is breastfeeding (Otto *et al.*, 1998). Multiple or multigravidae pregnancies place greater stress on the mother's LC-PUFA levels, with subsequently lower DHA status of the infant(s) at birth (Al *et al.*, 1997; Zeijdner *et al.*, 1997). Low DHA levels may be associated with an increased risk of pre-eclampsia and postpartum depression (Hibbeln *et al.*, 1995; Velzing-Aarts *et al.*, 1999; Williams *et al.*, 1995), and have been linked to shorter gestation times and poorer fetal growth (Hornstra *et al.*, 1989, 1995; Leaf *et al.*, 1992; Olsen *et al.*, 1992). Maternal relative ARA levels also decrease during pregnancy (Al *et al.*, 1995). The infant's ARA status at birth has also been positively correlated with fetal growth (Foreman-van Drongelen *et al.*, 1995; Koletzko and Braun, 1991; Leaf *et al.*, 1992).

Maternal LC-PUFA reserves depend on the mother's diet. Western diets, however, typically have low amounts of DHA. Based on the most recent USDA Continuing Survey of Food Intakes by Individuals (USDA, 1999), pregnant or nursing women on average consume 50 mg DHA per day (D. Benisek, personal communication), compared to the 300 mg per day recommended for pregnant or lactating mothers by a recent NIH workshop (Simopoulos *et al.*, 1999). Maternal LC-PUFA reserves, however, can be improved by supplementation with DHA and ARA during pregnancy (Connor *et al.*, 1996; Hamosh and Salem, 1998; van Houwelingen *et al.*, 1995). DHA can be obtained in the diet primarily from fatty fish or organ meats or alternatively by supplementation with DHA-rich oils. Fish oils have been used for this purpose; however, low eicosapentaenoic acid (EPA) fish oils are advised to minimize the risk of bleeding (Olsen *et al.*, 1992) and cod liver oil should be avoided because of potential vitamin A teratogenicity (Rice, 1996). Some scientists have suggested increasing both DHA and ARA intake to maintain a proper long-chain omega-3 to omega-6 ratio during pregnancy (Felton *et al.*, 1994; Hornstra *et al.*, 1996; Otto *et al.*, 1997b). Dietary ARA sources include meats and eggs.

With the recent development of single-cell sources of both DHA and ARA, alternative sources of concentrated, well-defined and specific DHA- and ARA-rich oils are now available to augment dietary intakes of these fatty acids. DHASCO® oil is derived from microalgae and contains 40–50% DHA, but no EPA or other LC-PUFAs (Kyle, 1996). ARASCO® oil is derived from a microfungus and contains 40–50% ARA and only minimal amounts of other LC-PUFAs (Kyle, 1997). These oils are produced by a fermentation process followed by oil extraction and purification using current Good Manufacturing Practices for foods. Both DHASCO and ARASCO oils have already been used for fortification of infant formulas in many parts of the world. Previous studies have shown that neither oil is toxic in acute or 28- or 90-day subchronic safety studies in rats, including one study with an *in utero* phase (Arterburn *et al.*, 2000; Boswell *et al.*, 1996; Burns *et al.*, 1999; Koskelo *et al.*, 1997; Wibert *et al.*, 1997). The focus of the present study is to specifically investigate teratogenic potential of these oils in a rat development model to ensure that they are safe to use during pregnancy, particularly during the period of fetal organogenesis.

MATERIALS AND METHODS

This study was conducted in the laboratories of Covance Laboratories Inc. (Madison, WI, USA) in accordance with the Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations for Nonclinical Laboratory Studies, 21 CFR 58. The FDA's *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food,* commonly referred to as the *Redbook I* and the draft *Redbook II* were used as guidelines for study design.

Test materials

DHASCO single-cell oil (lot no. D015-DS) and ARASCO single-cell oil (lot no. A013-DS), both produced by Martek Biosciences Corporation (Columbia, MD, USA), were used in this study. The final product was assayed for elemental composition, chemical characteristics and fatty acid composition and met previously set specifications (Table 1). Ascorbyl palmitate (0.025%) and tocopherol (0.025%) were added to the final product to protect the oils from oxidation.

Dosing

Test material doses of either 500 or 1250 mg/kg body weight/day of DHASCO (low and high DHASCO groups, respectively) or 1000 or 2500 mg/kg body weight/day ARASCO (low and high ARASCO groups, respectively) were prepared by diluting the test oils with high oleic sunflower oil (lot no. SC 4080, SVO Specialty Products, Eastlake, OH, USA) so as to provide a constant total oil dose of 2.5 g (2.78 ml) per kg body weight per day to each animal based on the most recently recorded body weights (Table 2). Dose preparations were mixed twice during the study. Controls were dosed with the high oleic sunflower oil vehicle. The preparations were delivered by gavage (to model oral intake) once daily at approximately the same time of day on days 6 through 15 of gestation.

Animals

Female Crl:CD® Sprague–Dawley BR VAF/Plus® rats approximately 10 wk of age were obtained from Charles River Laboratories, Inc. (Portage, MI,

Table 1. Fatty acid composition (% of total fatty acids) of DHASCO and ARASCO test oils and vehicle oil (high oleic sunflower oil)

Fatty acid	Common name	DHASCO oil	ARASCO oil	Vehicle oil
10:0	Capric acid	0.6	0	0
12:0	Lauric acid	4.5	0	0
14:0	Myristic acid	15.4	0.4	0
14:1n-5	Myristioleic	0.2	0	0
16:0	Palmitic acid	11.6	9.4	2.7
16:1n-7	Palmitoleic acid	2.3	0.1	0
18:0	Stearic acid	0.3	10.3	3.4
18:1n-9	Oleic acid	11.3	8.3	87.3
18:2n-6	Linoleic acid	0.8	6.3	4.6
18:3n-6	Gamma-linolenic acid	0	3.6	0
18:3n-3	Linolenic acid	0.2	0	0
20:0	Arachidic acid (ARA)	0.1	1.0	0.3
20:1	Eicosencoic acid	0.1	0.5	0.3
20:3n-6	Dihomo-gamma-linolenic acid	0	2.8	0
20:4n-6	Arachidonic acid (ARA)	0	51.4	0
20:5n-3	Eicosapentaenoic acid (EPA)	0	0	0
22:0	Behenic acid	0.1	1.8	1.0
22:5n-3	Docosapentaenoic acid (DPAn-3)	0	0	0
22:5n-6	Docosapentaenoic acid (DPAn-6)	0	0	0
22:6n-3	Docosahexaenoic acid (DHA)	51.7	0	0
24:0	Lignoceric acid	0	1.5	0.3

Table 2. Dosing scheme

Group	ARASCO (mg/kg bw/day)	DHASCO (mg/kg bw/day)	High oleic sunflower oil (mg/kg bw/day)	Number of mated females
Control	–	–	2500	25
Low ARASCO	1000	–	1500	25
High ARASCO	2500	–	–	25
Low DHASCO	–	500	2000	25
High DHASCO	–	1250	1250	25

USA). The animals were housed in individual cages in a temperature and humidity-controlled room with a 12-hr light/dark cycle. Water and certified rodent diet 5002 meal (PMI Feeds, Inc., Richmond, IN, USA) was provided *ad lib*. Females were acclimated for approximately 1 wk prior to starting the study and were examined by a veterinarian prior to release from acclimation. The females were approximately 11 wk old when paired for mating and weighed 214.2–263.0 g on gestation day 0. Males from the same strain and source as the females were used for mating.

Mating

Each female was paired with one male. Vaginal smears were taken daily during cohabitation, and the presence of a copulatory plug or sperm in the vaginal smear was considered positive evidence of mating. The date this evidence was found was designated as day 0 of gestation, and the female was then removed from the male's cage and housed individually as described above. 125 mated females were randomly assigned by consecutive block design to one of the four treatment groups or the control group (25 animals per group).

Observation, body weights and food consumption

The animals were observed twice daily for mortality, morbidity and signs of toxicity. A detailed examination was performed on each animal each time body weights were recorded. Individual weight data were recorded on day 0 and days 6 through 20 of gestation. Individual food consumption data were recorded during days 0–6, 6–9, 9–12, 12–16 and 16–20 of gestation.

Postmortem examinations

On day 20 of gestation, females were euthanized with Metofane®. The ovaries were examined for the number of corpora lutea. Uteri with visible implantations were excised and weighed, and the number and placement of implantation sites, live and dead fetuses, early and late resorptions and any abnormalities were recorded. The uterus was opened along the entire length, placental membranes were excised, and the conceptuses were removed.

The maternal necropsy included an examination of the external surface of the body, all orifices, the cranial cavity, external surfaces of the brain and spinal cord, nasal cavity and paranasal sinuses, and the thoracic, abdominal and pelvic cavities and viscera. Uteri with no visible implantations were excised and stained with a 10% ammonium sulfide solution for detection of implantations and confirmation of pregnancy status.

Fetal evaluations

Each live fetus was sexed, weighed and examined for external abnormalities. Viable fetuses were euthanized with an intrathoracic injection of Beuthanasia®-D

solution. Approximately one-half of all live fetuses from each litter (an average of seven animals per litter) were processed and examined for soft tissue development using a modification of the Wilson Technique (Wilson, 1965). The remaining fetuses were eviscerated, processed and examined for skeletal abnormalities. Soft tissue was examined for all litters in the control group and each treatment group, and skeletal specimens were examined for all litters in the control and high-dose groups. Specimens in the low-dose groups were held for possible examination.

Statistical analysis

The litter was the experimental unit for evaluation where appropriate. Treatment groups were compared with the control group. Levene's test was used to test for variance homogeneity. In the case of heterogeneity of variance at $P \leqslant 0.05$, rank transformation was used to stabilize the variance. Analysis of variance (ANOVA) was done on the homogeneous or transformed data. If the ANOVA was significant, Dunnett's multiple comparison t-test was used for pairwise comparisons between the control and test material-treated groups. When no transformation established variance homogeneity at $P \leqslant 0.05$, the data were also examined by non-parametric techniques. These statistics included the Kruskal–Wallis H-Test ANOVA and, if this test was significant, by the Nemenyi–Kruskal–Wallis test for multiple comparisons/Wilcoxon–Mann–Whitney two-sample rank-sum. One-way ANOVA was used to analyze continuous data such as body weights, body weight changes, food consumption, gravid uterine weights and caesarean section data. One-way analysis of covariance (ANCOVA) was used to analyze fetal body weights with the number of fetuses in the litter as the covariate. When appropriate, the proportion of litters and fetuses with fetal abnormalities in the treated groups was compared with that of the control group by the Fisher–Irwin exact test. A chi-square test was used to examine potential relationships between renal variants and fetal weights or maternal weight gain.

RESULTS

Clinical observations, body weights and food consumption

All 125 dams included in the study survived through the end of the study period (day 20 of gestation), and none of the animals had any significant clinical signs or symptoms, except two animals, one in the control and the other in the low DHASCO group, that presented with mouth malocclusions. This finding was not related to dose or treatment. Food consumption was similar between control and treatment groups (Fig. 1). The mean food consumption by the low-dose DHASCO group was higher than the control group ($P < 0.05$) during the first 6 days of gestation, but was not statistically significant thereafter. These animals had not received any test material during this interval, so the difference in food consumption was not related to the treatment. Dams in the control and treatment groups had similar weight gains (not shown) as well as actual weights (Fig. 2) throughout the gestation period. There were no indications that either DHASCO or ARASCO oils resulted in any overt maternal toxicity during the gestation period based on these in-life observations.

Caesarean section data

The results of the caesarean section data are summarized in Table 3. Between 23 and 25 of the mated females (92–100%) in each group became pregnant. None of the animals in any of the groups aborted, died or delivered early. All pregnant dams had viable fetuses at caesarean section, and the mean numbers of corpora lutea, implantation sites, preimplantation losses, resorptions or postimplantation losses per pregnant female were not significantly different between the treatment and control groups (Table 3). There were no dead fetuses in any of the pregnant females at the time of caesarean section, and the

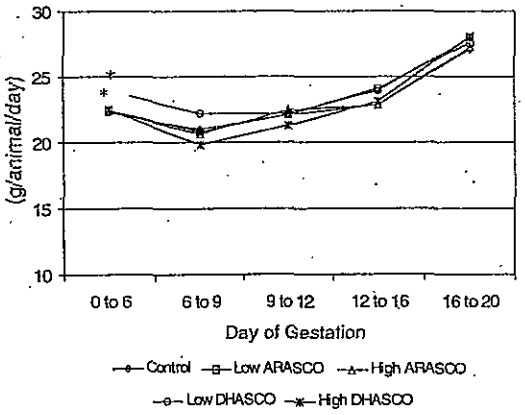

Fig 1. Mean food consumption. *Significant at the 5% level.

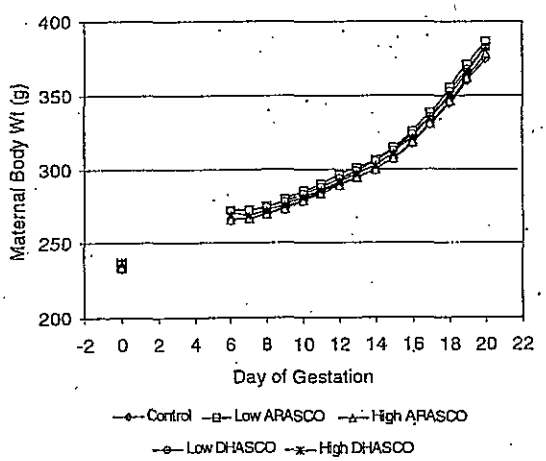

Fig. 2. Mean maternal body weights.

mean number of live fetuses (total, male or female) per dam, as well as the sex ratios, were similar in the treatment and the control groups. There were no significant differences noted in any of the reproductive outcomes between the treatment and control groups.

Postmortem observations

The mean weights of the gravid uterus as well as the mean corrected body weights (terminal body weight minus gravid uterine weight) of the dams did not differ between the treatment and control groups (Table 4). The net weight gain; that is, the corrected weight at study termination minus the weight on day 0 of gestation, varied considerably between 34 and 102 g for individual animals (data not shown). However, there were no significant differences in the mean net weight changes between treatment and control groups (Table 4). Maternal necropsy findings occurred sporadically in control and treatment groups at low frequencies (≤ three animals per group) and were not considered treatment related (data not shown).

Fetal observations

The mean weights for male and female fetuses were not different in treatment groups compared to controls (Table 4). There were also no differences in fetal weights of treatment groups after adjusting for litter size (not shown). External examination of the fetuses revealed several malformations that occurred at a low rate (Table 5). These findings occurred sporadically in either the control or treatment groups and were not related to dose or treatment. Several different fetal soft tissue malformations were found at a low frequency (no more than one animal per group) during fetal necropsy (Table 6). They were distributed between control and treatment groups in a non-dose- and non-treatment-related manner.

Several soft tissue variations in development were also observed (Table 6). The frequency of underdeveloped renal papilla was elevated in several of the

Table 3. Summary of caesarean section data[a]

Parameter	Control	Low ARASCO	High ARASCO	Low DHASCO	High DHASCO
Females mated	25	25	25	25	25
Pregnancies (n)	23	24	24	24	25
Aborted	0	0	0	0	0
Died	0	0	0	0	0
Delivered early	0	0	0	0	0
Animals pregnant at caesarean section (n)	23	24	24	24	25
Dams with viable fetuses (n)	23	24	24	25	25
Dams with no viable fetuses (n)	0	0	0	0	0
Corpora lutea (n per pregnant dam)	15.7±2.5	16.3±2.1	15.9±1.8	16.4±2.7	15.8±2.5
Implantation sites (n per pregnant dam)	14.6±2.2	15.4±1.9	15.2±1.5	14.8±3.5	14.7±2.5
Preimplantation loss (% per pregnant dam)	5.8±13.8	5.3±6.1	4.0±5.0	8.5±19.4	6.5±10.5
Resorptions (n per pregnant dam)- total	0.9±1.1	0.8±1.0	1.0±1.3	0.7±0.9	0.9±1.1
- early	0.7±1.0	0.8±1.0	1.0±1.2	0.7±0.9	0.6±0.9
- late	0.2±0.5	0.0±0.2	0.0±0.2	0.0±0.0	0.2±0.4
Dead fetuses- total	0	0	0	0	0
Postimplantation loss (% per pregnant dam)	6.0±7.1	4.9±6.1	6.3±7.9	4.7±5.5	6.1±6.9
Live fetuses (% per pregnant female)- total	13.7±2.1	14.6±1.7	14.2±1.6	14.1±3.4	13.8±2.6
- females	7.3±1.9	7.1±1.7	7.4±2.3	7.0±2.9	6.8±2.1
- males	6.3±1.5	7.5±2.2	6.8±2.0	7.2±2.2	7.0±2.5
Sex ratio M:F (% each sex)	46:54	52:48	48:52	51:49	51:49

[a]Values represent means±SD.

Table 4. Summary of maternal and fetal weight data[a]

	Group				
Parameter	Control	Low ARASCO	High ARASCO	Low DHASCO	High DHASCO
Dams or litters evaluated (n)	23	24	24	24	25
Maternal weight evaluations					
Gravid uterus weight (g)	73.1±10.4	78.8±9.0	76.5±7.2	75.9±16.0	74.9±13.4
Corrected maternal body weight[b] (g)	301.8±18.0	3.7.5±18.9	301.8±17.4	3.6.6±20.9	3.8.0±17.8
Net maternal wt change from day 0[c] (g)	68.9±12.6	70.7±15.0	67.8±11.1	70.3±15.7	70.7±10.0
Fetal weight evaluations					
Male fetal weight (g)	3.70±0.32	3.62±0.25	3.69±0.33	3.70±0.47	3.68±0.34
Female fetal weight (g)	3.47±0.34	3.42±0.20	3.55±0.33	3.51±0.52	3.50±0.28
All viable fetuses (male and female) (g)	3.57±0.33	3.53±0.20	3.61±0.32	3.61±0.49	3.59±0.32

[a]Data represent means±SD.
[b]Corrected weight is the terminal body weight minus gravid uterine weight.
[c]Net weight change from day 0 is the corrected weight minus day 0 body weight.

treatment groups and was significantly higher in the low DHASCO group ($P<0.05$) compared to the control group. The frequencies of this variation in the treatment groups were not related to dose, that is, the high DHASCO and ARASCO groups had fewer occurrences of this variation than the low-dose groups. Several fetal and litter incidence rates, mostly in the DHASCO groups, were outside the laboratory's historical control ranges for this condition, but all values fell within regional historical control ranges (Table 7). In addition, the fetal and litter frequencies of dilated renal pelvis were also statistically higher ($P<0.01$) in the low ARASCO and low DHASCO groups compared to the control group (Table 6). As with the underdeveloped renal papilla, the occurrences of dilated renal pelvis were not dose related. The fetal and litter frequencies, particularly in the low-dose groups, were outside the laboratory historical control ranges for this condition but were within the regional ranges (Table 7). These renal variants tended to be clustered within specific litters, and the differences in frequencies in the low-dose groups could be

Table 5. Summary of fetal external malformations

Malformation	Control	Low ARASCO	High ARASCO	Low DHASCO	High DHASCO
Fetuses (litters) evaluated	315 (23)	351 (24)	341 (24)	339 (24)	345 (25)
Cleft palate	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Cleft lip	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Ablepharia	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)
Tail absent	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
Anal atresia	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
Total fetal (litter) incidence	1 (1)	1 (1)	0 (0)	0 (0)	1 (1)

Table 6. Summary of fetal soft tissue observations

Observation	Control		Low ARASCO		High ARASCO		Low ARASCO		High ARASCO	
Number fetuses (litters) evaluated	157	(23)	174	(24)	174	(24)	169	(24)	172	(25)
Malformations—fetuses (litters) affected										
Cleft lip	1	(1)	0	(0)	0	(0)	0	(0)	0	(0)
Cleft palate	1	(1)	0	(0)	0	(0)	0	(0)	0	(0)
Cerebrum-dilated ventricles	0	(0)	1	(1)	0	(0)	0	(0)	1	(1)
Right sided aortic arch	1	(1)	0	(0)	0	(0)	0	(0)	0	(0)
Bulbous aortic arch	1	(1)	0	(0)	0	(0)	0	(0)	0	(0)
Pulmonary trunk stenosis	1	(1)	0	(0)	0	(0)	0	(0)	0	(0)
Intraventricular septal defect	1	(1)	0	(0)	0	(0)	0	(0)	0	(0)
Diaphragmatic hernia	0	(0)	0	(0)	0	(0)	1	(1)	0	(0)
Variations—fetuses (litters) affected										
Retroesophageal subclavian	2	(2)	0	(0)	0	(0)	0	(0)	0	(0)
Underdeveloped renal papilla	8	(4)	14	(8)	8	(7)	22[b]	(10)	17	(10)
Dilated renal pelvis	0	(0)	15[b]	(7)[b]	1	(1)	19[b]	(9)[b]	4	(4)
Distended ureter(s)	0	(0)	0	(0)	0	(0)	6	(4)	1	(1)
Total fetal (litter) incidence	10	(5)	25[a]	(12)	10	(9)	33[b]	(12)	19	(11)

[a]$P<0.05$; [b]$P<0.01$.

Table 7. Comparison of fetal variant rates to laboratory and regional historical control rates[a]

Aberration		Percent incidence: Control	Low ARASCO	High ARASCO	Low DHASCO	High DHASCO	Historical control maximums: Lab	Regional
Underdeveloped Renal papilla	Fetal	5.1	8.0	4.7	13	9.9	5.3	14
	Litter	17	33	29	42	40	35	57
Dilated renal Pelvis	Fetal	0	8.6	0.6	11	2.3	4.0	20
	Litter	0	29	42	38	16	22	60
Distended Ureter	Fetal	0	0	0	3.6	0.6	6.2	31
	Litter	0	0	0	17	4	22	90
Unossified Stenebra(e)	Fetal	41	NE	38	NE	33	41	72
	Litter	83		79		80	100	80

[a]Data represent mean percents for each variant. The maximum historical incidence (as a percent) of these variations in control animals was compiled by Covance Laboratories, Madison, Wisconsin (10 studies performed between 1992 and 1996) and the regional maximums were compiled and published by the Mid-Atlantic Regional Teratology Association and the Midwest Teratology Association (MARTA, 1995). NE = not examined.

largely attributed to two litters in each group where 60–100% of the examined fetuses were affected. A *post hoc* analysis revealed, however, that the variants were not related to maternal weight gain in the latter part of gestation or to fetal weight. Dilated renal pelvis and underdeveloped renal papilla represent variations in development, usually caused by slight delays, and because they have no persistent effects, these findings were not considered toxicologically significant. The incidence of distended ureter, another renal variant often related to hydronephrosis, was not significantly higher in treatment groups than the control group (Table 6) and generally did not accompany either of the other two renal variants. The fetal and litter incidence of distended ureter fell within both laboratory and regional historical control ranges for this condition (Table 7).

Several different fetal skeletal abnormalities occurred with a fetal incidence of 5% or less and were randomly distributed among control and treatment groups (Table 8). The only fetal skeletal abnormality occurring in more than 5% of the fetuses was unossified sternebrae, which was found with a 30–40% fetal incidence and approximately 80% litter incidence in both control and treatment groups (Table 7). These rates were similar among the groups, and all fell within the historical control ranges for this condition. None of the rates of observed skeletal abnormalities were statistically different in treatment than in control groups.

DISCUSSION

This developmental study was designed to assess potential adverse effects of DHASCO and ARASCO oils on fetal development, with special emphasis on developmental abnormalities and delays. The design is particularly effective for detecting teratogenic effects since the interval of exposure to the test materials (days 6–15 of gestation) coincides with the period of fetal organogenesis in rats (MacKenzie and Hoar, 1995). Organogenesis in human development, a particularly vulnerable time, occurs primarily during the first 2 months of pregnancy, when all major body organs and systems are formed. Organ maturation and growth occur throughout the rest of the pregnancy.

Table 8. Fetal skeletal observations

Observation	Control	High ARASCO	High DHASCO
Fetuses (litters) evaluated	158 (23)	171 (24)	173 (25)
Malformations			
Thoracic arches absent	0 (0)	1 (1)	0 (0)
Thoracic centra absent	0 (0)	1 (1)	0 (0)
Malformed lumbar centrum	0 (0)	0 (0)	1 (1)
Lumbar centrum absent	0 (0)	0 (0)	1 (1)
Sacral centrum absent	0 (0)	0 (0)	1 (1)
Intraventricular septal defect	1 (1)	0 (0)	0 (0)
Caudal vertebra absent	0 (0)	0 (0)	1 (1)
Absent rib(s)	2 (2)	1 (1)	0 (0)
Variations			
Skull bones reduced ossification	4 (3)	3 (3)	1 (1)
Skull bone unossified	5 (3)	2 (2)	6 (4)
Less than 26 presacral vertebra	1 (1)	4 (1)	1 (1)
Sternebra unossified	64 (19)	65 (19)	57 (20)
Short rib(s)	8 (4)	4 (1)	3 (2)
Less than 13 full pairs of ribs	8 (4)	0 (0)	4 (3)
Seventh cervical rib(s)	0 (0)	3 (1)	0 (0)
Rudimentary rib(s)	0 (0)	2 (2)	2 (2)
Ribs reduced ossifiaction	0 (0)	0 (0)	1 (1)
Pelvic bone reduced ossification	1 (1)	1 (1)	2 (2)
Total fetal (litter) incidence	72 (20)	71 (19)	68 (23)

Under the conditions of this study, administration of ARASCO and DHASCO oils at up to 2.5 and 1.25 g/kg body weight/day, respectively, to pregnant rats did not produce significant adverse developmental effects, and more specifically, no teratogenic effects. Despite the high doses, there were no overt signs of maternal toxicity or test material-related necropsy findings in the dams. This was not surprising, since similar doses had not produced adverse effects in rats in previous subchronic studies. Reproductive outcomes were not modified by these oils; there were no significant differences in the mean preimplantation loss, postimplantation loss, percent live fetuses (male, female and total), resorptions (early, late and total) or sex ratio for any test material-treated groups. The treatments with these oils did not lead to any fetal malformations. Fetal external, soft tissue and skeletal variations were present in control and treated groups, but occurred in a non-dose-related pattern and were not considered toxicologically significant.

A high incidence of unossified sternebrae was noted in both control and treatment groups. This is a common skeletal aberration in rodent development (MARTA, 1995) and is caused by a slight delay (less than 1 day) in ossification that normally occurs between days 19 and 20 of gestation (Khera, 1981). This aberration was not related to the treatment in this study, but rather suggests that both the control and the treatment groups were slightly delayed.

There were also statistically higher instances of renal variants, specifically underdeveloped renal papilla and dilated renal pelvis, with some of the low but none of the high doses of ARASCO and DHASCO oils. In only a few instances were either of these findings accompanied by distended ureter(s), and no renal malformations were found at any doses. The occurrence of these variations was clearly not dose related, and the incidence rates were always within the regional but sometimes exceeded laboratory historical control ranges. The laboratory database, however, was rather small (10 studies). The incidence of these aberrations tend to vary widely within a rodent species (Khera, 1981), and enlarged renal pelvis is a relatively common finding in rodent teratology bioassays, especially since the peak renal development occurs near the end of gestation. Therefore, even slight delays are apparent in fetal examinations performed on day 20 of gestation

(Woo and Hoar, 1972). Both underdeveloped renal papilla and dilated renal pelvis are considered variations or aberrations, not malformations. If not accompanied by other renal malformations, as is the case in this study, they are generally reversible with normal kidney function by the time of weaning (Woo and Hoar, 1972). The renal variants in this study were not related to fetal weights, suggesting that the variants were not diagnostic of a more severe retardation in growth or development. Rather, they more likely reflect a very slight delay, similar to the delay in stenebral ossification, possibly caused by fetal or maternal stress. We cannot completely rule out the possibility that renal variants are related to the treatment, as they can be acquired or genetic, and maternal stress is a known contributory factor. However, the fact that the frequencies are not dose related, a general criteria for establishing a causal relationship, and that dilated renal pelvis and undeveloped renal papilla are common findings with variable prevalence within rodent species, these aberrations could also be the result of normal variation. In either case, the findings were not considered toxicologically significant since the conditions represent only slight delays that are a minor divergence from normal, and there are no persistent effects on the pups.

The doses used in this developmental study are the same doses used in previous 90-day subchronic studies performed with these materials (Arterburn *et al.*, 2000; Koskelo *et al.*, 1997). The no-observable-adverse-effect level (NOAEL) for DHASCO and ARASCO oils under the conditions of this study, based on the lack of maternal or fetal toxicity, correspond to the highest doses used, 1250 and 2500 mg/kg body weight/day. Assuming an average woman weighs 55 kg and a pregnant woman should consume up to 300 mg DHA per day (approx. 600 mg DHASCO oil), a daily intake suggested at a recent NIH Workshop on the Essentiality of Recommended Daily Intakes of Omega-6 and Omega-3 Fatty Acids (Simopoulos *et al.*, 1999), the NOAEL represents over a 100-fold safety factor for the mother and developing fetus. Currently, there are no recommendations for arachidonic acid consumption for pregnant and lactating women, but assuming similar amounts of ARASCO as DHASCO oil might be consumed, the NOAEL for ARASCO represents over a 200-fold safety factor.

This is the first report of a developmental study on these single-cell oils. Earlier acute and subchronic studies did not find any adverse treatment-related effects of these oils (Arterburn *et al.*, 2000; Boswell *et al.*, 1996; Burns *et al.*, 1999; Koskelo *et al.*, 1997; Wibert *et al.*, 1997). Of particular relevance was the subchronic study by Burns *et al.* (1999) which included an *in utero* phase. In that study, male and female rats were treated with a combination of ARASCO and DHASCO oils at doses up to 120 g/kg diet (approx. 8–9 g/kg body weight/day of a 2:1 mixture of ARASCO plus DHASCO oils) during the premating and mating intervals. Female rats also received the same oil doses during gestation and lactation, and the F_1 pups were fed the oils subchronically during the post-weaning period for 90 days. No adverse treatment-related toxicological effects on reproductive performance or fertility, on fetal development or pup survival, or on clinical observations or necropsy findings during the subchronic phase were noted in that study, despite the very high doses (up to 2.5-fold higher than this developmental study). Of note, there were no treatment-related renal abnormalities observed. These two studies together cover the use of these oils during preconceptual, gestational and lactation and post-weaning periods, and neither found evidence of treatment-related adverse toxicological effects on the dams or their offspring.

A number of recent studies have suggested that a woman's DHA status decreases as the pregnancy progresses, and that the infant's DHA level at birth is related to maternal DHA status (Al *et al.*, 1995; Connor *et al.*, 1996; Otto *et al.*, 1997a). Several studies suggest that low DHA levels may be associated with decreased gestation length, postpartum depression and pre-eclampsia as well as lower birth size (Hibbeln and Salem, 1995; Hornstra *et al.*, 1989, 1995; Leaf *et al.*, 1992; Olsen *et al.*, 1992; Velzing-Aarts *et al.*, 1999; Williams *et al.*, 1995). Consequently, several investigators have suggested that pregnant women increase their intake of DHA along with ARA, which is also associated with improved fetal growth (Foreman-van Drongelen *et al.*, 1995; Koletzko and Braun, 1991; Leaf *et al.*, 1992), in order to maintain the proper omega-3 to omega-6 ratio during pregnancy. DHASCO and ARASCO oils offer women and their physicians alternative, non-teratogenic dietary sources of DHA and ARA, respectively, when their usual dietary intake may not be adequate to meet the needs of pregnancy.

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REFERENCES

Al M. D., van Houwelingen A. C. and Hornstra G. (1997) Relation between birth order and the maternal and neonatal docosahexaenoic acid status. *European Journal of Clinical Nutrition* 51, 548–553.

Al M. D., van Houwelingen A. C., Kester A. D., Hasaart T. H., de Jong A. E. and Hornstra G. (1995) Maternal essential fatty acid patterns during normal pregnancy and their relationship to the neonatal essential fatty acid status. *British Journal of Nutrition* 74, 55–68.

Arterburn L. M., Boswell K. D., Koskelo E.-K., Kassner S. L., Kelly C. M. and Kyle D. J. (2000) A combined subchronic (90-day) toxicity and neurotoxicity study of a single-cell source of docosahexaenoic acid triglyceride (DHASCO® oil). *Food and Chemical Toxicology* 38, 35–49.

Boswell K., Koskelo E. K., Carl L., Glaza S., Hensen D. J., Williams K. D. and Kyle D. J. (1996) Preclinical evalua-

tion of single-cell oils that are highly enriched with arachidonic acid and docosahexaenoic acid. *Food and Chemical Toxicology* 34, 585–593.

Burns R. A., Wibert G. J., Diersen-Schade D. A. and Kelly C. M. (1999) Evaluation of single-cell sources of docosahexaenoic acid and arachidonic acid: 3-month rat oral safety study with an in utero phase. *Food and Chemical Toxicology* 37, 23–36.

Connor W. E., Lowensohn R. and Hatcher L. (1996) Increased docosahexaenoic acid levels in human newborn infants by administration of sardines and fish oil during pregnancy. *Lipids* 31, S183–3187.

Crawford M. A. (1993) The role of essential fatty acids in neural development: implications for perinatal nutrition. *American Journal of Clinical Nutrition* 57, 703S–709S.

Crawford M. A., Costeloe K., Ghebremeskel K., Phylactos A., Skirvin L. and Stacey F. (1997) Are deficits of arachidonic and docosahexaenoic acids responsible for the neural and vascular complications of preterm babies? *American Journal of Clinical Nutrition* 66, 1032S–1041S.

Felton C. V., Chang T. C., Crook D., Marsh M., Robson S. C. and Spencer J. A. (1994) Umbilical vessel wall fatty acids after normal and retarded fetal growth. *Archives of Disease in Childhood* 70, F36–39.

Foreman-van Drongelen M. M., van Houwelingen A. C., Kester A. D., Hasaart T. H., Blanco C. E. and Hornstra G. (1995) Long-chain polyunsaturated fatty acids in preterm infants: status at birth and its influence on postnatal levels. *Journal of Pediatrics* 126, 611–618.

Hamosh M. and Salem N. Jr (1998) Long-chain polyunsaturated fatty acids. *Biology of Neonate* 74, 106–120.

Hibbeln J. R. and Salem N. Jr (1995) Dietary polyunsaturated fatty acids and depression: when cholesterol does not satisfy. *American Journal of Clinical Nutrition* 62, 1–9.

Hornstra G., Al M. D., van Houwelingen A. C. and Foreman-van Drongelen M. M. (1995) Essential fatty acids in pregnancy and early human development. *European Journal of Obstetrics, Gynecology and Reproductive Biology* 61, 57–62.

Hornstra G., Al M. D., van Houwelingen A. C. and Foreman-van Drongelen M. M. H. P. (1996) Essential fatty acids, pregnancy and pregnancy outcomes. In *Recent Developments in Infant Nutrition*, ed. J. G. Bindels, A. C. Hoedhart and H. K. A. Visser, pp. 51–63. Kluwer Academic Publishers, Great Britain.

Hornstra G., van Houwelingen A. C., Simonis M. and Gerrard J. M. (1989) Fatty acid composition of umbilical arteries and veins: possible implications for the fetal EFA-status. *Lipids* 24, 511–517.

Khera K. S. (1981) Common fetal aberrations and their teratologic significance: a review. *Fundamental and Applied Toxicology* 1, 13–18.

Koletzko B. and Braun M. (1991) Arachidonic acid and early human growth: is there a relation? *Annals of Nutrition and Metabolism* 35, 128–131.

Koskelo E. K., Boswell K., Carl L., Lanoue S., Kelly C. and Kyle D. (1997) High level of dietary arachidonic acid triglyceride exhibit no subchronic toxicity in rats. *Lipids* 32, 397–405.

Kyle D. J. (1996) Production and use of a single cell oil which is highly enriched in docosahexaenoic acid. *Lipid Technology* 2, 109–112.

Kyle D. J. (1997) Production and use of a single cell oil highly enriched in arachidonic acid. *Lipid Technology* 9, 116–121.

Leaf A. A., Leighfield M. J., Costeloe K. L. and Crawford M. A. (1992) Long chain polyunsaturated fatty acids and fetal growth. *Early Human Development* 30, 183–191.

MacKenzie K. M. and Hoar R. M. (1995) Developmental toxicology. In *C.R.C. Handbook of Toxicology*, ed. M. J. Derelanko and M. A. Hollinger, pp. 379–402. CRC Press, Boca Raton, FL.

Makrides M., Neumann M. A. and Gibson R. A. (1996) Effect of maternal docosahexaenoic acid (DHA) supplementation on breast milk composition. *European Journal of Clinical Nutrition* 50, 352–357.

Middle Atlantic Reproduction and Teratology Association (MARTA) and Midwest Teratology Association (December, 1995) *Historical Control Data* (1992-1994) *for Development and Reproductive Toxicity Studies Using the Crl:CD BR Rat.* Published and distributed by Charles River Laboratories.

Olsen S., Sorensen J., Secher N., Hedegaard M., Henriksen T., Hansen H. and Grant A. (1992) Randomised controlled trial of effect of fish-oil supplementation on pregnancy duration. *Lancet* 339, 1003–1007.

Otto S. J., van Houwelingen A. C., Antal M., Manninen A., Godfrey K., Lopez-Jaramillo P. and Hornstra G. (1997) Maternal and neonatal essential fatty acid status in phospholipids: an international comparative study. *European Journal of Clinical Nutrition* 51, 232–242.

Otto S. J., van Houwelingen A. C., Badart-Smook A. and Hornstra G. (1998) The post-partum docosahexaenoic acid status of lactating and non-lactating mothers (abstract). *Abstract Book, 3rd ISSFAL Congress, Lyon, France*, pp. 111.

Otto S. J., van Houwelingen A. C. and Hornstra G. (1997) Search for an LCP supplement for pregnant women (abstract). *Prostaglandins, Leukotrienes and Essential Fatty Acids* 57, 190.

Rice R. (1996) Fish and healthy pregnancy: more than just a red herring!. *Professional Care of Mother and Child* 6, 171–173.

Ruyle M., Connor W. E., Anderson G. J. and Lowensohn R. I. (1990) Placental transfer of essential fatty acids in humans: venous-arterial difference for docosahexaenoic acid in fetal umbilical erythrocytes. *Proceedings of the National Academy of Sciences of the U.S.A* 87, 7902–7906.

Simopoulos A. P., Leaf A. and Salem N. (1999) Workshop on the essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. *Journal of the American College of Nutrition* 18, 487–489.

USDA Website (1999) 1994–96 Continuing Survey of Food Intakes by Individuals and Diet and Health Knowledge Survey. *http://www.barc.usda.gov/foodsurvey/home.htm.*

van Houwelingen A. C., Sorensen J. D., Hornstra G., Simonis M. M., Boris J., Olsen S. F. and Secher N. J. (1995) Essential fatty acid status in neonates after fish-oil supplementation during late pregnancy. *British Journal of Nutrition* 74, 723–731.

Velzing-Aarts F. V., van der Klis R. F. M., van der Dijs F. P. L. and Muskiet F. A. J. (1999) Umbilical vessels of preeclamptic women have low contents of both n-3 and n-6 long-chain polyunsaturated fatty acids. *American Journal of Clinical Nutrition* 69, 293–298.

Wibert G. J., Burns R. A., Diersen-Schade D. A. and Kelly C. M. (1997) Evaluation of single cell sources of docosahexaenoic acid and arachidonic acid: a 4-week oral safety study in rats. *Food and Chemical Toxicology* 35, 967–974.

Williams M. A., Zingheim R. W., King I. B. and Zebelman A. M. (1995) Omega-3 fatty acids in maternal erythrocytes and risk of preeclampsia. *Epidemiology* 6, 232–237.

Wilson J. G. (1965) Methods for administering agents and detecting malformation in experimental animals. In *Teratology: Principles and Techniques*, ed. J. G. Wilson and J. Warkany, pp. 262–277. University of Chicago, Chicago.

Woo D. C. and Hoar R. M. (1972) "Apparent hydronephrosis" as a normal aspect of renal development in late gestation of rats: the effect of methyl salicylate. *Teratology* 6, 191–196.

Zeijdner E. E., van Houwelingen A. C., Kester A. D. and Hornstra G. (1997) Essential fatty acid status in plasma phospholipids of mother and neonate after multiple pregnancy. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 56, 395–401.

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Arachidonate-enriched triglyceride oil does not promote tumor development in a rat medium-term multi-organ carcinogenesis model

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ABSTRACT

The modifying potential on tumor development of arachidonate-enriched triglyceride oil (ARA-oil) containing approximately 40% arachidonic acid was investigated in a medium-term multi-organ carcinogenesis bioassay using male and female F344 rats. The animals were sequentially given five carcinogens with different target sites in the first 4 weeks, and then administered ARA-oil for 24 weeks at dietary levels of 0% (control), 1.25%, 2.5% or 5.0%. No statistically significant differences in incidences and multiplicities of hyperplastic and neoplastic lesions were showed in the large intestine in either sex. In the liver, kidney, and lung in both sexes, and the mammary gland and uterus in females, tumor promoting potential was not evident with ARA-oil treatment. ARA-oil did not affect the quantitative data for glutathione S-transferase placental form positive foci of the liver. Increased induction of hyperplastic or neoplastic lesions in the urinary bladder and thyroid in ARA-oil-treated groups was without dose dependence. In addition, a second experiment with ARA-oil only administration for 8-week revealed no effects on cellular proliferation in the urinary bladder or thyroid in either sex. These results indicate that ARA-oil has no tumor promoting potential in any organs or tissues initiated with the five carcinogens applied in the present study.

Introduction

Polyunsaturated fatty acids (PUFAs), such as arachidonic acid (ARA) and docosahexaenoic acid (DHA), are natural nutrients found in common foodstuffs (e.g., egg yolk, meat and fish oil). ARA and DHA are also contained in human and animal breast milk and play pivotal roles in brain growth and neurodevelopment in late pregnancy and in neonatal infants (Birch et al., 2000; Bouwstra et al., 2003; Clandinin et al., 2005; Woods et al., 1996). A broad range of ARA and DHA concentrations occur in human breast milk depending on the maternal nutritional status (Brenna et al., 2007). Many advisory boards (British Nutrition Foundation's Task Force, 1992; ESPGAN, 1991; SCF, 1993) and scientists have recommended use of an infant formula providing DHA with the minimum amount of ARA equivalent to the contents of DHA, when breastfeeding is not possible (Hoffman et al., 2009; Koletzko et al., 2008). Supply of ARA with DHA has also been approved by the CODEX Alimentarius Commission for infant formulas (Joint FAO/WHO Food Standards Programme, 2007).

Arachidonate-enriched triglyceride oil (ARA-oil) can be industrially manufactured by fermentation using a fungus *Mortierella alpine*, and has been widely used as a food additive for infant formulas all over the world. Many safety studies have examined this ARA-oil. Based on the Ames test and a mouse lymphoma TK(+/−) forward mutation assay, ARA-oil is not mutagenic, and it was not clastogenic in chromosomal aberration assays performed with Chinese hamster ovary cells (Arterburn et al., 2000b). Furthermore neither maternal toxicity nor teratogenic potential were demonstrated in rats administered up to 2500 mg/kg body weight/day of this oil (Arterburn et al., 2000a), and no adverse effects were observed on repeated dose 28-day and 90-day oral toxicity studies in rats (Hempenius et al., 1997, 2000). Moreover, when ARA levels in the brain, heart, and liver were elevated by 8%, 59% and 76%, respectively, as a result of ARA-oil gavage (2.5 g/kg/day), no developmental, histopathological or neuropathological differences were seen between rats fed ARA versus vehicle controls in a subchronic toxicity study (Koskelo et al., 1997).

Prostaglandins (PGs), major lipid mediators, are involved in regulation of many physiological functions, such as inflammatory responses, control of blood pressure, and blood coagulation. Prostaglandin E_2 (PGE_2) is detected at high levels in human cancer

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tissues, indicating that it may play a role in the promotion stage of carcinogenesis, affecting cell proliferation, immunosuppression, angiogenesis and/or apoptosis (Wang and DuBois, 2006). ARA is contained in membrane phospholipids of various tissues, and free ARA is released from phospholipids by phospholipase A_2 and then converted to a variety of lipid mediators, such as PGE_2. However, whether dietary ARA-oil modifies tumor-promoting process is still unknown.

Our rat medium-term multi-organ carcinogenesis bioassay is based on the two-stage theory of chemical carcinogenesis (Hagiwara et al., 1993; Ito et al., 2000; Shirai et al., 1999; Yoshida et al., 1993) and was accepted as one alternative *in vivo* approach for elucidating the carcinogenic potential of pharmaceuticals at the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH4) (Ito et al., 1998; Usui, 1998). This bioassay is advantageous for elucidating the carcinogenic promoting or protective effects of chemical substances because it includes a whole-body survey (Hasegawa et al., 1994; Hirose et al., 1993; Ito et al., 1996; Shibata et al., 1995; Takahashi et al., 1992; Toriyama-Baba et al., 2001; Yamamoto et al., 1995).

In the present study, we focused on tumor promoting potential of ARA-oil in this rat medium-term multi-organ carcinogenesis model (Experiment I). Stimulation of cellular proliferation activity was also evaluated (Experiment II), since tumor promoters generally lead to elevation in cellular proliferation in target organs (Fukushima et al., 1991; Hasumura et al., 2005; Hood et al., 1999; Shibata et al., 1989, 1992).

2. Materials and methods

All experimental procedures were performed in accordance with the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain, Notification No. 88, from the Japanese Ministry of Environment dated April 28th, 2006 and with the in-house guidelines for the Care and Use of Laboratory Animals at DIMS Institute of Medical Science, Inc. (April 1st, 1991). The current study was also conducted in compliance with the Good Laboratory Practice (GLP) Standards of the Japanese Ministry of Health and Welfare Ordinance No. 21 dated March 26th, 1997, Amendment, Notice No. 114 dated June 13th, 2008.

2.1. Materials

2.1.1. Carcinogens (initiators of medium-term multi-organ carcinogenesis)

Diethylnitrosamine (DEN) and *N*-*n*-butyl-*N*-butan-4-ol-nitrosamine (BBN) were purchased from Tokyo Chemical Industry Co. Ltd., (Tokyo, Japan), 1,2-dimethylhydrazine dihydrochloride (DMH) and *N*-methyl-*N*-nitrosourea (MNU) from Sigma-Aldrich Japan Inc., (Tokyo, Japan); and dihydroxy-di-*N*-propylnitrosamine (DHPN) from Nacalai Tesque, Inc., (Kyoto, Japan). The purities of DEN, BBN, DMH, MNU and DHPN were 99% or more, 90% or more, 99% or more, 50% (containing 12% acetic acid and 38% water), and 97.2%, respectively. These carcinogens were all used to initiate tumor formation.

2.1.2. Test substance

ARA-oil, rich in *n*–6 fatty acids (mainly arachidonic acid; about 40%) was supplied by Suntory Wellness Ltd. (SUNTGA40S; lot No. 08042351 for Experiment I, and lot No. 100621A1 for Experiment II; Osaka, Japan) for use as the test substance. Soybean oil was purchased from Showa Sangyo Co. Ltd. (Tokyo, Japan) and was employed as the reference substance. Fatty acid compositions of the ARA-oil and soybean oil used in this study are summarized in Table 1.

A semi-synthetic AIN-93M powder diet (Oriental Yeast Co. Ltd., Tokyo, Japan), from which 4% soybean oil and 3% corn starch were removed, was used in Experiments I and II. The complete ingredients for semi-synthetic AIN-93M are listed on Table 2. ARA-oil was mixed with 4% soybean oil- and 3% corn starch-depleted semi-synthetic AIN-93M powder diet at final concentrations of 0% (control groups 2 and 6), 1.25% (group 3), 2.5% (group 4) or 5.0% (groups 5 and 7) ARA-oil; soybean oil was added back to each diet at final concentrations of 7.0%, 5.75%, 4.5% or 2.0%, respectively, to ensure that each diet had the same total lipid level and total caloric value. The highest dose of ARA-oil used was 5.0%, because that amount was authorized in the Japanese Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives, and 5.0% ARA-oil was used in the 13-week subchronic oral toxicity study (Lina et al., 2006). The lower used, 2.5% and 1.25% ARA-oil, simply represented a 2-fold dilution series. MF powder diet (Oriental Yeast Co. Ltd., Tokyo, Japan), a standard rodent diet, was fed to group 1 in both experiments.

Table 1
Fatty acid compositions of the ARA-oil and soybean oil.

Fatty acid	Fatty acid composition (%)	
	ARA-oil	Soybean oil
Palmitic acid (16:0)	10.3	10.4
Stearic acid (18:0)	7.1	3.9
Oleic acid (18:1)	7.2	23.8
Linoleic acid (18:2 *n*–6)	9.9	52.8
α-Linolenic acid (18:3 *n*–3)	0.5	7.3
γ-Linolenic acid (18:3 *n*–6)	2.6	0.0
Dihomo-γ-linolenic acid (20:3 *n*–6)	3.5	0.0
Arachidonic acid (20:4 *n*–6)	42.0	0.0
Behenic acid (22:0)	3.3	0.4
Lignoceric acid (24:0)	8.5	0.0
Others	5.1	1.4

Table 2
Ingredients of the semi-synthetic AIN-93M powder diet.

Ingredient	Semi-synthetic AIN-93M (%)
Casein	14.0
L-Cysteine	0.18
Corn starch[a]	46.569
α-Corn starch	15.5
Sucrose	10.0
Soybean oil[a]	4.0
Cellulose powder	5.0
Mineral mix	3.5
Vitamin mix	1.0
Choline bitartrate	0.25
3-Butylhydroquinone	0.001

[a] A modified semi-synthetic AIN-93M powder diet, from which 3% corn starch and 4% soybean oil were eliminated beforehand, was mixed with test substance and soybean oil.

The diets were stored in a refrigerator (2–10 °C) until fed to rats. This storage regime was based on results of an earlier study on the stability of this oil in the diet that was performed by Suntory Wellness Ltd. The oil was confirmed to be stable in a prepared diet for about 9 weeks under the following conditions: refrigerated, protected from light for 6 weeks; protected from light for 2 weeks at room temperature; kept in a lighted area for 10 days. The concentration of arachidonic acid in the diet was measured six or seven times in the course of Experiment I and two times in the course of Experiment II, and each time dietary arachidonic acid was measured it was found to be within an acceptable range and homogeneity was consistently satisfactory (Table 3 for Experiment I and data not shown for Experiment II).

2.2. Experimental animals

Male and female F344/DuCrlCrlj (F344) rats were purchased from Charles River Japan, Inc. (Atsugi, Japan) and allowed free access to semi-synthetic AIN-93M powder diet for a quarantine/acclimation period of 7–8 days, during which the body weight and health conditions were monitored. After confirmation of normal health status, they were entered into the experiment at the age of 6 weeks. Healthy animals were housed two to a polycarbonate cage (257 × 426 × 200 mm, W × D × H, Tokiwa Kagaku Kikai Co. Ltd., Tokyo, Japan) on hardwood chip bedding (Beta chip, Northeastern Products Co., Warrensburg, NY, USA) in an environment-controlled room. Constant conditions of temperature 20–25 °C, humidity 45–68%, and ventilation (more than 10 times/h) were maintained, and the room was artificially illuminated to provide a 12-h light/dark cycle (7:00/19:00) each day.

2.3. Experimental procedures

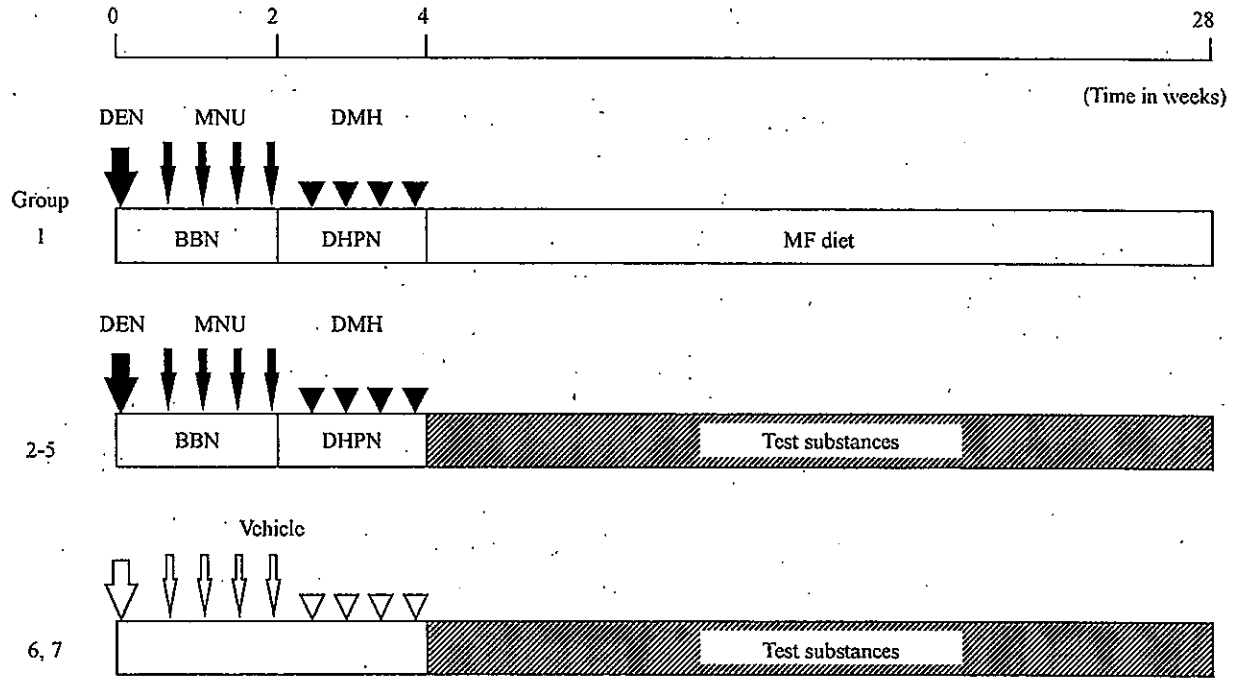
2.3.1. Experiment I: examination of tumor promoting potential

A schematic representation of the experimental protocol used in Experiment I is shown in Fig. 1. The tumor-promoting potential of ARA-oil was assessed using an established medium-term multi-organ carcinogenesis model (Doi et al., 2010; Hasegawa et al., 1994; Hirose et al., 1998; Ichihara et al., 2008; Shibata et al., 1995; Takahashi et al., 1992; Yada et al., 2002). A total of 120 rats in each sex were

Table 3
Compositions of total fat and fatty acids in each diet.

Groups	DMBDD treatment	Basal diet	Oil (%)		Crude fat (%)	Linoleic acid (%)	Arachidonic acid (%)
			ARA-oil	Soybean oil			
1	+	MF	0	0	4.9[a]	47.1[a]	Not detected[a]
2	+	AIN-93M[b]	0	7.0	7.14 ± 0.05[c]	3.44 ± 0.02	<0.01
3	+	AIN-93M	1.25	5.75	7.09 ± 0.04	2.95 ± 0.02	0.47 ± 0.00
4	+	AIN-93M	2.5	4.5	7.10 ± 0.00	2.47 ± 0.03	0.95 ± 0.01
5	+	AIN-93M	5.0	2.0	7.10 ± 0.06	1.53 ± 0.01	1.92 ± 0.02
6	–	AIN-93M	0	7.0	7.14 ± 0.05	3.44 ± 0.02	<0.01
7	–	AIN-93M	5.0	2.0	7.10 ± 0.06	1.53 ± 0.01	1.92 ± 0.02

[a] Data certificated from January to December 2009 by the supplier were cited from the catalogue.
[b] Modified semi-synthetic AIN-93M powder diet.
[c] Values indicate mean ± SD.



1. Design of Experiment I. DMBDD treatment (see detail in Section 2) and animals in group 1 (naïve control) were fed MF powder diet. Groups 2, 3, 4 and 5 were fed a modified semi-synthetic AIN-93M powder diet supplemented with 7.0% soybean oil, 1.25% ARA-oil and 5.75% soybean oil, 2.5% ARA-oil and 4.5% soybean oil, and 5.0% ARA-oil and 2.0% soybean oil, respectively. Animals in groups 6 and 7 (non-DMBDD treatment) were fed a modified semi-synthetic AIN-93M powder diet supplemented with 7.0% soybean oil and 5.0% ARA-oil with 2.0% soybean oil, respectively. All surviving animals were euthanized at week 29.

randomly allocated to seven groups (20 rats each in groups 1–5, 10 rats each in groups 6 and 7) using a computerized body-weight stratification technique. The animals in groups 1–5 were treated first with DEN (100 mg/kg body weight, in saline, i.p. single injection) at the commencement, then with MNU (20 mg/kg body weight, in saline, i.p., 4 times in weeks 1 and 2) and BBN (0.05% in drinking water, during weeks 1 and 2), and finally with DMH (40 mg/kg body weight, in saline, s.c., four times in weeks 3 and 4) and DHPN (0.1% in drinking water, during weeks 3 and 4) for multi-organ initiation. This regime is referred to as DMBDD treatment. The animals in groups 6 and 7 each received just the vehicle used for each carcinogen; vehicle was administered at the same time points and via the same route as the corresponding carcinogen. The regime used for groups 6 and 7 is referred to as the non-DMBDD treatment. All animals were fed semi-synthetic AIN-93M powder diet during the 4-week DMBDD treatment or during the 4-week non-DMBDD treatment. After the 4-week DMBDD and non-DMBDD treatments, animals were given free access to MF powder diet or one of the modified semi-synthetic AIN-93M powder diets supplemented with ARA-oil and soybean oil for an additional 24 weeks as detailed in Table 3. The MF powder diet group (group 1) served as a naïve control group. All animals were observed daily to inspect general health, and were weighed weekly for the first 14 weeks, and then biweekly until the end of the experiment. Almost every rat that was euthanized under a moribund condition, or that was found dead during experiment could be subjected to histopathological examination. Feed and water consumption per cage was also measured over a 2-day period before each weighing. At the end of experimental week 28, all surviving rats were deprived of feed (but not water) overnight and then weighed; blood samples were then collected by exsanguination via the abdominal aorta while the rats were under deep ether anesthesia.

During the autopsies, the gross anatomy of each animal was examined. The brain, heart, thymus, liver, kidneys, spleen, adrenals and testes/ovaries were immediately excised and weighed, and organ-to-body weight ratios were calculated using the body weights before euthanasia. The pituitary and thyroid (including parathyroid) were also weighed after fixation, and organ-to-body weight ratios were calculated. Buffered formalin solution (10%) was used to fix the organs and tissues for further analysis. Organs and tissues were processed, embedded in paraffin wax, and sectioned using a standard protocol. The paraffin sections were stained with hematoxylin and eosin (H&E) solution, and then examined histopathologically. Lymph nodes (mandibular, mesenteric), spleen, bone marrow, thymus, pituitary, thyroid (including parathyroid), adrenals, nasal cavity, trachea, lungs (including bronchus), tongue, esophagus, stomach (forestomach and glandular stomach), small intestine (duodenum, jejunum and ileum), cecum, large intestine (colon and rectum), pancreas, liver, kidneys, urinary bladder, testes, prostate, seminal vesicles, mammary gland, ovaries, uterus, vagina, bone (femur, sternum), Zymbal's glands, brain (cerebrum, cerebellum), spinal cord (thoracic portion), and any other tissues with abnormal findings were examined. Histopathological evaluations were performed with reference to incidences and multiplicities of preneoplastic and neoplastic lesions. However, it should be pointed out that our multiplicity calculation in fact reflects both multiplicity and incidence.

Glutathione S-transferase placental form (GST-P) positive foci, considered as preneoplastic lesions in the liver (Ito et al., 1988), were measured as follows. Two slices (3 mm thick) were cut from fresh left and median lobes with a razor blade and fixed in ice-chilled 10% buffered formalin solution; these slices were then embedded in paraffin wax and sectioned and immunohistochemically stained by the avidin–biotin–peroxidase complex (ABC) method, using a rabbit Vectastain Elite ABC kit (Vector Lab. Inc., Burlingame, CA, USA). Rabbit anti-rat liver GST-P polyclonal antiserum (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) was used at a 1:2000 dilution as the primary antibody. The numbers and areas of GST-P positive foci larger than 0.2 mm in diameter were counted and measured, respectively, using a color video image processor (IPAP-WIN; Sumika Technos, Co., Osaka, Japan) as described previously (Shirai et al., 1999) and evaluated quantitatively.

2.3.2. Experiment II: an 8-week feeding study

Experiment II was performed to confirm whether ARA-oil stimulates cell proliferation in two representative organs, the urinary bladder and thyroid. The following indicators were inspected: 5-bromo-2′-deoxyuridine (BrdU) labeling, urine parameters and hormone activity related to thyroid follicular cell function. A total of 100 rats in each sex at 6 weeks of age were randomly allocated to five groups (20 rats per group) using a computerized body-weight stratification technique. Beginning the day after allocation, rats were fed MF powder diet (group 1) or the modified semi-synthetic AIN-93M powder diet mixed with 0%, 1.25%, 2.5% or 5.0% ARA-oil (groups 2, 3, 4 and 5, respectively) for 8 weeks. The animals were observed daily to inspect general health, and weighed weekly. Feed and water consumption per cage was also measured over a 2-day period before each weighing.

Urinalysis samples were collected over 4 h (from 9:00 AM to 13:00 PM) from 20 males and 20 females per group at week 8. Semi quantitative estimations (Multistix, Bayer Medical Co. Ltd., Tokyo, Japan) of protein, glucose, ketones, bilirubin, occult blood and urobilinogen were performed. The color of the urine was inspected macroscopically. Specific gravity values were measured using a refractometer (Atago Co. Ltd., Tokyo, Japan). The levels of electrolytes (sodium, potassium, and chlorine) were determined using a Hitachi Biochemical-Automatic Analyzer 7070 (Hitachi Co. Ltd., Tokyo, Japan). Urine samples were weighed. Urine was inspected macroscopically, and sediments were assessed by microscopic analysis of Sternheimer and Malbin stained specimens. In addition, pH values of fresh urine samples, obtained by forced micturition, were measured using a pH meter, F-51 (Horiba Ltd., Kyoto, Japan).

After 8-week dietary treatment, 5 mL/kg of BrdU (Sigma-Aldrich Japan Inc., Tokyo, Japan) dissolved in saline was injected intraperitoneally one hour before euthanasia; animals were fully fed and had not fasted overnight. Triiodothyronine (T3), thyroxine (T4) and thyroid-stimulating hormone (TSH) levels in serum samples were measured; the serum samples were derived from blood of the abdominal aorta collected during the autopsy. Serum T3, T4 and TSH were measured using the chemiluminescent enzyme immunoassay (CLEIA) method and IMMULYZE (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). Urinary bladders were inflated with 10% buffered formalin solution, excised, and then weighed after fixation. The thyroid glands were weighed after fixation. Serial paraffin sections of these organs were prepared for H&E staining and histopathological examination using standard protocol, and immunohistochemical staining of incorporated BrdU was performed using an ABC technique and a mouse Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). A mouse-bromodeoxyuridine monoclonal antiserum (Dako-Cytomation, Glostrup, Denmark) was used at a 1:1500 dilution as the primary anti-BrdU antibody. The numbers of cells with nuclei that had incorporated BrdU per 1000 or more epithelial cells were counted, and the labeling indices were expressed as percentage values.

3. Statistical analysis

The significance of differences in each parameter (excluding general conditions) measured in Experiments I and II were analyzed and evaluated at $P < 0.05$ or $P < 0.01$. Differences in variability of parameters between the 7.0% soybean oil (group 2) and 1.25%, 2.5% and 5.0% ARA-oil (groups 3–5) were assessed using the Bartlett's test. Differences in numerical data on body weights, feed consumption, water intake, organ weight, macroscopic lesions in the large intestine (colon and rectum), microscopic lesions in the thyroid, urinary bladder, kidneys and large intestine, number and area of GST-P positive foci of the liver, BrdU labeling indices, concentrations of T3, T4 and TSH, and quantitative urinalysis data were considered significant when $P < 0.05$. If homogeneity of variance was assured, the data were analyzed with the parametric Dunnett's multiple comparison test (two sided), and if not, they were analyzed with the non-parametric Steel's test (two sided) (Steel, 1959). Statistical pairwise comparisons between MF diet (group 1) versus 7.0% soybean oil (group 2), and 7.0% soybean oil (group 6) versus 5.0% ARA-oil (group 7) (for Experiment I only) of the numerical data mentioned above were assessed using the F test and considered significant when $P < 0.05$. If homogeneity of variance was assured, the data were analyzed by the Student's t-test (two sided), and if not by the Welch test (two sided).

The significance of differences between group 2 and all groups 3–5 and statistical pairwise comparisons of group 1 versus group 2 and group 6 versus group 7 in incidences of gross pathology and histopathology were analyzed using the Fisher's exact probability test (one sided). Comparison of graded data from different groups and quantitative urinalysis data from different groups was performed using the Wilcoxon test (two-sided).

4. Results

4.1. Experiment I: examination of tumor promoting potential

4.1.1. Survival rates and general conditions

The survival rates of male DMBDD-treated rats receiving 1.25%, 2.5% or 5.0% ARA-oil (groups 2, 3, 4 or 5, respectively) at 28 weeks (experiment termination) were 60%, 70%, 70% and 65%, respectively; those of female rats receiving 0%, 1.25%, 2.5% or 5.0% ARA-oil (groups 2, 3, 4 or 5, respectively) were 95%, 85%, 80% and 85%, respectively. Survival rates of male and female rats fed MF diet (group1) were 80% and 95%, respectively. Those for male groups 2–5 were slightly lower than for males fed MF diet (group 1), but there were no statistically significant differences. No mortality in any of the groups was related to the treatment of test substance. No ARA-oil treatment-related impairment of general health was observed in any groups from week 5 to the end of the experiment (week 28). Emaciation, irregular respiration, anemia, abdominal distention and/or other ill signs, which were elicited by treatment with the five potent carcinogens, were observed in some males and females in groups 1–5, but without influence of the ARA-oil. No deaths or abnormal conditions were observed in any male or female rats not subjected to DMBDD treatment and fed 7.0% soybean oil (group 6) or 5.0% ARA-oil (group 7).

4.1.2. Body weights, feed and water consumption, intake of test substance, and intakes of BBN and DHPN

Initial and terminal body weights, mean feed consumption (experimental weeks 5–28) (g/animal/day) and intake of ARA-oil (g/kg/day) are summarized in Table 4. During the DMBDD treatment period (weeks 1–4), average body weights in groups of male and female with DMBDD were clearly lower than those in groups without DMBDD treatment through weeks 1–4 and then continued to the termination. Throughout the experiment, there were no statistically significant differences in mean body weights of male or female rats in 1.25%, 2.5% or 5.0% ARA-oil groups (groups 3, 4 and 5) as compared with control values (group 2); similarly, there were no differences in mean body weights of male or female fed 7.0% soybean oil (group 6) versus 5.0% ARA-oil (group 7) with non-DMBDD treatment groups. Body weights of males fed 7.0% soybean oil (group 2) from weeks 6 to 11 were significantly smaller than for males fed MF diet (group 1) (data not shown). However, these differences in change in body weight were slight and transient.

Occasionally, during ARA-oil treatment, average feed consumption was significantly lowered in males fed 1.25%, 2.5% and 5.0% ARA-oil (groups 3, 4 and 5) as compared to 7.0% soybean oil (group 2). This variation was considered incidental and not related to the test substance treatment, because it was not continuous. As shown in Table 4, a slightly but significantly lower value for mean feed consumption was noted in only male rats fed 2.5% ARA-oil (group

Table 4
Body weights, mean feed consumption and ARA-oil intake for F344 rats treated with ARA-oil with or without prior DMBDD treatment.

Groups	Treatment		Body weight (g)		Mean feed consumption (g/rat/day)	Mean ARA-oil intake (g/kg/day)
	DMBDD	ARA-oil	Initial[a]	Terminal[b]	Weeks 5–28	Weeks 5–28
Males						
1	+	0% (MF diet)	114.0 ± 5.1[c]	302.3 ± 17.1[c]	13.93 ± 1.34[c]	0
2	+	0%	113.5 ± 5.3	301.2 ± 40.8	13.19 ± 1.80[##]	0
3	+	1.25%	113.8 ± 5.5	294.4 ± 25.6	12.69 ± 1.56	0.613
4	+	2.5%	113.4 ± 5.3	303.3 ± 17.0	12.59 ± 1.52[**]	1.212
5	+	5.0%	113.6 ± 5.1	298.7 ± 22.0	12.79 ± 1.94	2.469
6	–	0%	113.7 ± 4.9	420.9 ± 14.9	15.46 ± 1.21	0
7	–	5.0%	113.2 ± 4.9	422.8 ± 18.1	15.69 ± 1.19	2.198
Females						
1	+	0% (MF diet)	102.0 ± 4.5	179.0 ± 7.8	9.69 ± 1.24	0
2	+	0%	100.7 ± 4.4	175.1 ± 14.0	9.43 ± 1.58	0
3	+	1.25%	101.8 ± 5.1	177.0 ± 13.4	9.47 ± 1.30	0.727
4	+	2.5%	101.0 ± 4.8	181.7 ± 8.3	9.52 ± 1.50	1.433
5	+	5.0%	101.2 ± 5.0	177.2 ± 11.1	9.58 ± 1.44	2.913
6	–	0%	100.9 ± 4.3	215.0 ± 11.6	9.93 ± 1.21	0
7	–	5.0%	100.9 ± 5.4	214.3 ± 10.1	10.37 ± 1.22	2.626

[a] Values were obtained when the DMBDD treatment started.
[b] Values were obtained at the day before euthanasia.
[c] Values are mean ± SD.
[**] Significantly different from the group 2 at $P < 0.01$.
[##] Significantly different from the group 1 at $P < 0.01$.

4) as compared with 7.0% soybean oil group (group 2) without dose dependence. Similarly, mean feed consumption was significantly lower in male rats fed 7.0% soybean oil (group 2) than in males fed the MF diet (group 1). The mean ARA-oil intakes by males and females, respectively, were 0.613 and 0.727 g/kg/day in the 1.25% group, 1.212 and 1.433 g/kg/day in the 2.5% group, and 2.469 and 2.913 g/kg/day in the 5.0% group, showing a dose-dependent proportional increase.

Average water intake during ARA-oil treatment was significantly higher in male rats fed 2.5% and 5.0% ARA-oil (groups 4 and 5) and in female rats fed 1.25%, 2.5% and 5.0% ARA-oil (groups 3, 4 and 5) than in males and females fed 7.0% soybean oil (group 2). The increased water intake of males in groups 4 and 5 and females in group 5 was apparently related to ARA-oil intake, but the effect was slight. In contrast, water intake was considerably and significantly higher in males and females fed the MF diet (group 1) than in male and female rats fed 7.0% soybean oil (group 2); this difference could be ascribed to differences in the palatability of the MF and semi-synthetic AIN-93M powder diet. In the non-DMBDD-treated rats, water intake was slightly higher in male and female rats fed 5.0% ARA-oil (group 7) than in the 7.0% soybean oil group (group 6) (data not shown). During the DMBDD treatment, water intakes were similar in all DMBDD treatment groups and average intakes of BBN and DHPN were 28.3–31.5 mg/kg/day and 61.1–70.4 mg/kg/day for males, and 26.7–31.5 mg/kg/day and 53.7–65.1 mg/kg/day for females, respectively. There were no remarkable differences among the groups.

4.1.3. Organ weights.

Organ weight data in the DMBDD treatment groups were excluded from the evaluation, because of the large variation due to nodule development. Therefore, organ weights were assessed only in the non-DMBDD treatment groups. Relative weights of the adrenal glands were significantly higher in both sexes receiving 5.0% ARA-oil (group 7) as compared with control values (group 6). Relative spleen and liver weights were significantly higher in females receiving 5.0% ARA-oil (group 7) than the control values (group 6) (data not shown).

4.1.4. Gross pathology

Based on macroscopic observation of multiple organs, organ enlargement, discolored spots/areas, and nodules were observed in various organs (e.g., lymph nodes, spleen, thymus, thyroid, lungs, digestive tract, liver, kidneys, urinary bladder, skin/subcutis and abdominal cavity) of DMBDD-treated male and female rats (groups 1–5). In contrast, no macroscopic lesions were observed in male and female rats that were not subjected to DMBDD treatment (groups 6 and 7) (data not shown). Taken together, these findings demonstrated that treatment with the five carcinogens induced tumor formation in multiple organs.

4.1.5. Histopathology

In the large intestine (colon and rectum), there was no significant elevation in either incidence or multiplicity of hyperplastic and neoplastic lesions in either sex in groups 3, 4, and 5 (ARA-oil) as compared with those of males and females in group 2 (Table 5).

In the urinary bladder, the multiplicity values for transitional cell papillary or nodular (PN) hyperplasias were significantly higher in male rats fed 2.5% ARA-oil (without dose–response manner) than in males fed 7.0% soybean oil (group 2), but this was not true for female rats from groups 2 and 4. The incidence of transitional cell papillomas was significantly higher in female rats fed 1.25% ARA-oil (without dose-dependence) than in females fed 7.0% soybean oil (group 2), but this was not true for males in groups 2 and 3 (Table 6).

In the thyroid, the incidence of follicular cell hyperplasias was significantly higher in male rats fed 5.0% ARA-oil (group 5) than in males fed 7.0% soybean oil (group 2) and the incidences of follicular cell adenomas were significantly higher in male rats fed 1.25% and 5.0% ARA-oil (groups 3 and 5) than in males fed 7.0% soybean oil (group 2). In addition, the incidence of follicular cell carcinomas was significantly higher in male rats fed 1.25% ARA-oil (group 3) than in males fed 7.0% soybean oil (group 2). However, multiplicities of follicular cell lesions did not significantly differ among males from groups 3, 4 and 5 (ARA-oil) and group 2 (7.0% soybean oil). Furthermore, in females, no significant differences in the incidences or multiplicities of the thyroid lesions were found between ARA-oil-treated groups and group 2 (7.0% soybean oil) (Table 7).

The incidences of neoplastic lesions in organs other than the large intestine, urinary bladder or thyroid in ARA-oil-treated groups were given in Tables 8 (males) and 9 (females). A significantly higher incidence of bronchiolo-alveolar adenomas in the lungs was observed in male rats fed 1.25% or 2.5% ARA oil (groups

Table 5
Incidences and multiplicities of hyperplastic and neoplastic lesions of the large intestine in F344 rats treated with ARA-oil with or without prior DMBDD treatment.

Groups	Treatment		No. of rats examined	Hyperplasia	Atypical hyperplasia	Adenoma	Adenocarcinoma	Adenocarcinoma, mucinous
	DMBDD	ARA-oil						
Males								
1	+	0% (MF diet)	20	8 (0.5 ± 0.6)[a]	8 (0.5 ± 0.7)	13 (1.4 ± 1.4)	8 (0.4 ± 0.5)	2 (0.1 ± 0.3)
2	+	0%	20	6 (0.5 ± 0.9)	2[#] (0.1 ± 0.3[#])	18 (2.0 ± 1.6)	9 (0.5 ± 0.6)	1 (0.1 ± 0.2)
3	+	1.25%	20	9 (0.7 ± 0.9)	6 (0.4 ± 0.6)	17 (2.4 ± 2.0)	11 (0.7 ± 0.7)	0
4	+	2.5%	20	12 (0.9 ± 0.9)	5 (0.3 ± 0.4)	15 (1.9 ± 1.8)	13 (0.8 ± 0.7)	0
5	+	5.0%	20	3 (0.2 ± 0.5)	5 (0.3 ± 0.4)	18 (2.1 ± 1.2)	6 (0.6 ± 1.2)	0
6	–	0%	10	0	0	0	0	0
7	–	5.0%	10	0	0	0	0	0
Females								
1	+	0% (MF diet)	20	4 (0.2 ± 0.4)	3 (0.2 ± 0.4)	4 (0.3 ± 0.6)	2 (0.2 ± 0.5)	1 (0.1 ± 0.2)
2	+	0%	20	1 (0.1 ± 0.2)	2 (0.1 ± 0.3)	5 (0.3 ± 0.6)	3 (0.2 ± 0.4)	0
3	+	1.25%	19	3 (0.2 ± 0.4)	2 (0.1 ± 0.3)	4 (0.3 ± 0.6)	5 (0.4 ± 0.7)	0
4	+	2.5%	20	4 (0.2 ± 0.4)	6 (0.3 ± 0.5)	7 (0.4 ± 0.5)	4 (0.2 ± 0.4)	0
5	+	5.0%	20	2 (0.1 ± 0.3)	4 (0.3 ± 0.6)	7 (0.4 ± 0.5)	6 (0.4 ± 0.7)	1 (0.1 ± 0.2)
6	–	0%	10	0	0	0	0	0
7	–	5.0%	10	0	0	0	0	0

A hemangioma, sarcoma, NOS and Schwannoma were found in male groups 3 and 2 or female group 3, respectively.
[a] Values are incidences (average number of lesions per rat, mean ± SD).
[#] Significantly different from the group 1 at $P < 0.05$.

Table 6
Incidences and multiplicities of hyperplastic and neoplastic lesions of the urinary bladder in F344 rats treated with ARA-oil with or without prior DMBDD treatment.

Groups	Treatment		No. of rats examined	Hyperplasia, Transitional cell, papillary/nodular	Hyperplasia, squamous cell	Papilloma, transitional cell	Papilloma, squamous cell	Leiomyoma
	DMBDD	ARA-oil						
Males								
1	+	0% (MF diet)	20	12 (1.5 ± 2.0)[a]	1 (0.1 ± 0.2)	13 (1.2 ± 1.1)	0	0
2	+	0%	20	12 (0.9 ± 1.0)	0	11 (1.2 ± 1.5)	0	0
3	+	1.25%	20	12 (1.4 ± 1.5)	0	10 (1.1 ± 1.5)	0	1 (0.1 ± 0.2)
4	+	2.5%	20	16 (2.5 ± 2.4[*])	0	14 (1.4 ± 1.3)	1 (0.1 ± 0.4)	1 (0.1 ± 0.2)
5	+	5.0%	20	13 (1.4 ± 1.3)	0	16 (1.8 ± 1.5)	3 (0.2 ± 0.4)	0
6	–	0%	10	0	0	0	0	0
7	–	5.0%	10	0	0	0	0	0
Females								
1	+	0% (MF diet)	20	8 (0.6 ± 0.8)	0	4 (0.3 ± 0.6)	0	0
2	+	0%	20	10 (0.5 ± 0.5)	0	2 (0.1 ± 0.3)	0	0
3	+	1.25%	19	9 (0.9 ± 1.4)	0	8[*] (0.4 ± 0.5)	1 (0.1 ± 0.2)	0
4	+	2.5%	20	12 (0.9 ± 1.1)	0	7 (0.5 ± 0.8)	0	0
5	+	5.0%	20	12 (1.4 ± 1.5)	0	6 (0.3 ± 0.5)	0	0
6	–	0%	10	0	0	0	0	0
7	–	5.0%	10	0	0	0	0	0

[a] Values are incidences (average number of lesions per rat, mean ± SD).
[*] Significantly different from the group 2 at $P < 0.05$.

3 and 4) as compared with control values (group 2), but this was without dose dependence and equivalent to the value for the MF diet group (group 1). The significantly higher incidence of endometrial stromal polyps in the uterus observed in female rats fed 1.25% ARA-oil (group 3) than control values (group 2), was again not dose-dependent. Under the present conditions, the statistical maximum detection power ($1 - \beta$) was 84.9% for bronchiolo-alveolar adenomas in the male lung between group 2 (control group) and group 3 (1.25% group), with a probability value (α) of 0.05 (Table 8), when analyzed with public software "R-2.14.2" (http://cran.md.tsukuba.ac.jp/).

In non-DMBDD initiated groups (groups 6 and 7), no neoplastic lesions were found in any organs/tissues of either male or female rats; except for a few spontaneous hyperplastic lesions in some organs observed in rats from groups 6 and 7 (data not shown).

4.1.6. Quantitative evaluation of GST-P positive foci of the liver

There were no statistically significant differences in either the number or area of GST-P positive foci between groups 3, 4 or 5 (ARA-oil) and group 2 (7.0% soybean oil control) (Table 10).

4.2. Experiment II: an 8-week feeding study

4.2.1. Cellular proliferation potential in the urinary bladder and thyroid

In both organs, there were no statistically significant differences in BrdU labeling indices between groups 3, 4 or 5 (ARA-oil) and group 2 (7.0% soybean oil) (Table 11). No statistically significant increase in the urinary bladder and thyroid weights was exhibited in any ARA-oil-treated groups as compared with control values (group 2). No histopathological alterations suggestive of increase in cellular proliferation, were observed in these organs. Moreover, no epithelial cells were found in urine sediment specimens from any groups (data not shown).

4.2.2. Serum concentrations of T3, T4 and TSH

As shown in Table 12, serum T3 levels in the all male rats fed ARA-oil (groups 3, 4 and 5) were slightly but significantly higher than those for males fed 7.0% soybean oil control (group 2), but not in the all female rats fed ARA-oil. The serum TSH level was significantly higher in females fed 5.0% ARA-oil (group 5) than in

Table 7
Incidences and multiplicities of hyperplastic and neoplastic lesions of the thyroid in F344 rats treated with ARA-oil with or without prior DMBDD treatment.

Groups	Treatment		No. of rats examined	Follicular cell			C-cell		
	DMBDD	ARA-oil		Hyperplasia	Adenoma	Carcinoma	Hyperplasia	Adenoma	Carcinoma
Males									
1	+	0% (MF diet)	20	16 (1.8 ± 1.3)[a]	14 (1.5 ± 1.4)	5 (0.3 ± 0.6)	0	2 (0.1 ± 0.3)	0
2	+	0%	20	10[*] (0.9 ± 1.0[*])	4[**] (0.3 ± 0.6[**])	1 (0.1 ± 0.2)	1 (0.1 ± 0.2)	0	0
3	+	1.25%	20	8 (0.5 ± 0.7)	10[*] (0.7 ± 0.8)	6[*] (0.3 ± 0.5)	1 (0.1 ± 0.2)	1 (0.1 ± 0.2)	0
4	+	2.5%	20	9 (0.6 ± 0.7)	7 (0.6 ± 0.9)	2 (0.1 ± 0.3)	2 (0.2 ± 0.5)	0	0
5	+	5.0%	20	16[*] (1.1 ± 0.9)	10[*] (0.8 ± 1.0)	2 (0.1 ± 0.3)	1 (0.1 ± 0.2)	0	1 (0.1 ± 0.2)
6	−	0%	10	0	0	0	0	0	0
7	−	5.0%	10	0	0	0	1 (0.1 ± 0.3)	0	0
Females									
1	+	0% (MF diet)	20	2 (0.2 ± 0.5)	4 (0.2 ± 0.4)	1 (0.1 ± 0.2)	0	0	0
2	+	0%	20	2 (0.1 ± 0.3)	3 (0.2 ± 0.4)	2 (0.1 ± 0.3)	0	0	0
3	+	1.25%	19	1 (0.1 ± 0.2)	4 (0.3 ± 0.6)	1 (0.1 ± 0.2)	1 (0.1 ± 0.2)	1 (0.1 ± 0.2)	0
4	+	2.5%	20	4 (0.2 ± 0.4)	4 (0.2 ± 0.4)	1 (0.1 ± 0.2)	0	0	0
5	+	5.0%	20	2 (0.1 ± 0.3)	1 (0.1 ± 0.2)	0	0	0	0
6	−	0%	10	0	0	0	0	0	0
7	−	5.0%	10	0	0	0	0	0	0

[a] Values are incidences (average number of lesions per rat, mean ± SD).
[*] Significantly different from the group 2 at $P < 0.05$.
[*] Significantly different from the group 1 at $P < 0.05$.
[**] Significantly different from the group 1 at $P < 0.01$.

females fed 7.0% soybean oil (group 2). However, there were no statistically significant differences in T4 levels in either sex or TSH levels in males between any groups fed ARA-oil (groups 3, 4 and 5) and the group fed 7.0% soybean oil (groups 2).

4.2.3. Urinalysis

As shown in Table 13, the urine pH levels were significantly lower in males fed 2.5% or 5.0% ARA-oil (groups 4 and 5) than in males fed 7.0% soybean oil (group 2). Sodium ion concentrations in urine were significantly lower in all males fed ARA-oil (groups 3, 4 and 5) and in females fed 2.5% or 5.0% ARA-oil (groups 4 and 5) than in males and females fed 7.0% soybean oil (group 2). Potassium ion concentrations in urine were significantly lower in males fed 5.0% ARA-oil (group 5) and in females fed 2.5% or 5.0% ARA-oil (groups 4 and 5) than in males and females fed 7.0% soybean oil (group 2). Chlorine ion concentrations in urine were significantly lower in males fed 1.25% or 5.0% ARA-oil (groups 3 and 5), and in females received ARA-oil (groups 3, 4, and 5) than in males and females fed 7.0% soybean oil (group 2). Urine volume was significantly higher in male and female rats fed 2.5% or 5.0% ARA-oil (groups 4 and 5) than in male and female rats fed 7.0% soybean oil (group 2). The specific gravity of urine samples was significantly lower in male and female rats fed ARA-oil (groups 3, 4 and 5) than in male and female rats fed 7.0% soybean oil (group 2). Variation (increases or decreases) in urinalysis parameters was generally dependent on the dose of ARA-oil. However, no urinary sediment epithelial cells, erythrocytes and leukocytes were found in any groups receiving ARA-oil, MF diet or 7.0% soybean oil in both sexes. Moreover, no urinary calculi were observed in any group (data not shown).

5. Discussion

In the present study, we investigated the potential of ARA-oil to modify tumor promotion using a medium-term multi-organ carcinogenesis bioassay in male and female 6-week-old F344/DuCrlCrlj rats. In this bioassay, when the incidences of hyperplastic (preneoplastic) or neoplastic lesions are increased significantly and dose-dependently in comparison to control values, test substances are generally judged as positive for tumor promoting activity at the whole-body level. The results of the present study indicated that ARA-oil supplementation did not demonstrate any consistent carcinogenic or promoting influence in any organs or tissues of either sex (Experiment I). Next, the effect of ARA-oil on cellular proliferation in the urinary bladder and thyroid was examined in an 8-week continuous-feeding study (Experiment II), considering the generally accepted role of cellular proliferation in tumor-promotion processes (Fukushima et al., 1991; Hasumura et al., 2005; Hood et al., 1999; Shibata et al., 1989, 1992). No cellular proliferation was found in either the urinary bladder or the thyroid in the current study. This result strongly supported the conclusion that ARA-oil did not show tumor promotion potential in Experiment I.

In this study, we chose 5.0% ARA-oil as the highest treatment level, based on a 13-week subchronic oral toxicity study because 5.0% ARA-oil was the highest dose tested that did not result in any signs of toxicity (Lina et al., 2006). Taking into account (1) the amount of ARA-oil (which contains 42.0% ARA) in test diet, (2) feed consumption, and (3) body weight in rats, dietary ARA intake of rat fed 5.0% ARA-oil was estimated to be about 1037 mg ARA/kg/day in male rats and 1223 mg ARA/kg/day in female rats in Experiment I. The concentration of fat in human breast milk is 3.5%, and average ARA contents in total fatty acids of breast milk in Japanese woman is 0.35% (Tanaka et al., 1994). The daily average intake of breast milk by infants is about 117 mL/kg/day (Ministry of Health, Labour and Welfare, 1999). Based on these data, daily ARA intake by infants via breast milk is approximately 14.3 mg ARA/kg/day. Compared with the amounts of ARA tested in this animal study, this represents about 73- to 86-fold less.

In the large intestine, the test substance did not promote any hyperplastic and neoplastic lesions developed by DMBDD treatment in any of the test groups. An important theme has been discussed in the field of chemo-prevention; specifically, a decline in $n-6/n-3$ polyunsaturated fatty acid ratio plays a role in cancer prevention when food is supplemented with various fatty acids. Many experimental and epidemiological studies have identified a positive relationship between excessive fat intake and cancer development (McCormick et al., 1989; Miller et al., 1983; Noguchi et al., 1997; Rose, 1997). It is now certain that the development of colorectal cancer is inhibited by diets rich in $n-3$ fatty acid (Bartram et al., 1993; Minoura et al., 1988; Narisawa et al., 1991; Rao et al., 1996, 2001; Reddy and Sugie, 1988; Toriyama-Baba et al., 2001). These reports suggest that western-style meals, in which $n-6$ fatty acid is dominant, may cause the development of human

Table 8
Incidences of neoplastic lesions in male F344 rats treated with ARA-oil with or without prior DMBDD treatment.

Organ and Findings	Groups	1[a]	2	3	4	5	6	7
	DMBDD	+	+	+	+	+	–	–
	ARA-oil	0%	0%	1.25%	2.5%	5.0%	0%	5.0%
No. of rats examined		20	20	20	20	20	10	10
Mandibular lymph node								
Hemangioma		0	0	0	1	0	0	0
Hemangiosarcoma		0	0	0	1	0	0	0
Mesenteric lymph node								
Hemangioma		1	1	0	0	0	0	0
Spleen								
Hemangioma		2	0	0	1	0	0	0
Hemangiosarcoma		2	0	0	0	1	0	0
Bone marrow								
Hemangioma		0	0	0	0	1	0	0
Hemangiosarcoma		0	0	0	0	1	0	0
Thymus								
Malignant lymphoma		0	0	0	1	0	0	0
Pituitary								
Adenoma, pars distalis		0	0	1	0	0	0	0
Adrenals								
Hemangioma		1	0	1	2	1	0	0
Nasal cavity								
Adenoma		4	1	3	1	2	0	0
Papilloma, squamous cell		1	0	0	0	1	0	0
Lungs/bronchial								
Adenoma, bronchiolo-alveolar		10	4[#]	13[**]	11[*]	9	0	0
Hemangioma		0	0	0	1	0	0	0
Adenocarcinoma, bronchiolo-alveolar		2	4	2	2	2	0	0
Esophagus								
Papilloma, squamous cell		0	2	1	1	1	0	0
Stomach								
Leiomyoma		0	0	0	1	0	0	0
Papilloma, squamous cell		4	0	1	2	1	0	0
Schwannoma		0	0	0	0	1	0	0
Adenocarcinoma		0	0	0	1	0	0	0
Duodenum								
Adenoma		0	0	0	1	0	0	0
Adenocarcinoma		4	2	1	3	5	0	0
Adenocarcinoma, mucinous		0	0	1	0	0	0	0
Jejunum								
Leiomyoma		1	0	0	0	0	0	0
Adenocarcinoma		2	2	3	0	2	0	0
Cecum								
Adenoma		0	1	0	1	3	0	0
Leiomyoma		0	0	0	0	1	0	0
Adenocarcinoma		0	0	4	3	0	0	0
Pancreas								
Schwannoma		0	0	1	0	0	0	0
Liver								
Cholangiocarcinoma		0	0	0	0	1	0	0
Kidneys								
Adenoma, tubular cell		16	14	10	10	8	0	0
Hemangioma		0	0	1	0	1	0	0
Carcinoma, tubular cell		0	0	0	1	1	0	0
Carcinoma, transitional cell		1	4	6	3	6	0	0
Hemangiosarcoma		1	0	1	0	1	0	0
Nephroblastoma		20	17	16	13	18	0	0
Prostate								
Schwannoma		1	1	0	0	0	0	0
Malignant schwannoma		0	0	1	0	0	0	0
Seminal vesicles								
Leiomyoma		0	0	0	1	0	0	0
Zymbal's glands								
Adenoma		1	0	1	0	1	0	0
Carcinoma		0	1	1	0	3	0	0
Brain								
Astrocytoma		0	0	0	0	1	0	0
Malignant reticulosis		0	0	1	0	0	0	0
Meningeal sarcoma		1	0	0	0	0	0	0
Spinal cord								
Schwannoma		1	0	0	2	0	0	0
All sites								
Malignant lymphoma		10	10	10	11	11	0	0
Malignant lymphoma/leukemia		0	1	0	0	1	0	0

[a] MF diet group.
[*] Significantly different from the group 2 at $P < 0.05$.
[**] Significantly different from the group 2 at $P < 0.01$.
[#] Significantly different from the group 1 at $P < 0.05$.

Table 9
Incidences of neoplastic lesions in female F344 rats treated with ARA-oil with or without prior DMBDD treatment.

Organ and findings	Groups	1[a]	2	3	4	5	6	7
	DMBDD	+	+	+	+	+	–	–
	ARA-oil	0%	0%	1.25%	2.5%	5.0%	0%	5.0%
No. of rats examined		20	20	19	20	20	10	10
Spleen								
Hemangiosarcoma		1	0	0	0	0	0	0
Pituitary								
Adenoma, pars distalis		0	0	0	1	0	0	0
Craniopharyngioma		1	0	0	0	0	0	0
Adrenals								
Adenoma, cortical		0	1	0	0	0	0	0
Hemangioma		2	0	0	0	0	0	0
Nasal cavity								
Adenoma		0	1	0	2	1	0	0
Fibrosarcoma		0	1	0	0	0	0	0
Lungs/bronchial								
Adenoma, bronchiolo-alveolar		10	9	8	4	5	0	0
Adenocarcinoma, bronchiolo-alveolar		0	2	4	0	1	0	0
Carcinoma, squamous cell		0	0	0	1	1	0	0
Tongue								
Papilloma, squamous cell		0	1	0	0	0	0	0
Esophagus								
Papilloma, squamous cell		1	1	2	1	0	0	0
Stomach								
Papilloma, squamous cell		2	0	1	0	1	0	0
Duodenum								
Adenoma		0	0	1	0	0	0	0
Adenocarcinoma		0	1	0	2	1	0	0
Adenocarcinoma, mucinous		0	0	0	0	1	0	0
Jejunum								
Adenocarcinoma		0	0	0	0	3	0	0
Cecum								
Adenocarcinoma		0	0	0	0	1	0	0
Adenocarcinoma, mucinous		0	0	1	0	0	0	0
Liver								
Adenoma, hepatocellular		1	0	0	1	0	0	0
Cholangioma		0	0	0	0	1	0	0
Hemangioma		0	0	0	1	0	0	0
Carcinoma, hepatocellular		1	0	0	0	1	0	0
Cholangiocarcinoma		1	0	0	0	0	0	0
Kidneys								
Adenoma, tubular cell		4	5	8	9	7	0	0
Hemangioma		0	2	1	0	1	0	0
Carcinoma, tubular cell		0	0	1	0	0	0	0
Carcinoma, transitional cell		0	0	1	1	2	0	0
Nephroblastoma		15	15	16	12	13	0	0
Mammary gland								
Adenoma		7	4	5	2	1	0	0
Fibroadenoma		1	0	0	0	0	0	0
Adenocarcinoma		3	4	1	4	6	0	0
Uterus								
Polyp, endometrial stromal		2	0	4*	1	1	0	0
Sarcoma, endometrial stromal		0	0	1	0	0	0	0
Zymbal's glands								
Adenoma		1	0	0	1	1	0	0
All sites								
Malignant lymphoma		5	6	3	3	8	0	0

[a] MF diet group.
* Significantly different from the group 2 at $P < 0.05$.

colorectal, breast, and prostate cancers. Therefore, we focused to investigate whether ARA-oil rich polyunsaturated fatty acid had a tumor-promoting potential. Our findings indicated that ARA-oil, containing about 40% ARA, did not exert tumor promoting potential in any organs or tissues of rats, including the large intestine, mammary gland and prostate.

The incidence of transitional cell papillomas in the urinary bladder developing in female rats fed 1.25% ARA-oil and the multiplicity of transitional cell PN hyperplasias in male rats fed 2.5% ARA-oil were higher than those of females and males, respectively, fed 7.0% soybean oil group. However, these increases were not ascribable to the ARA-oil supplements because of the lack of dose-dependence. Some tumor promoters in the urinary bladder are known to elevate urine pH and sodium or potassium ion excretion in the urine, and are associated with an increase in cell proliferation in urinary bladder epithelium (Fukushima et al., 1991; Shibata et al., 1989, 1992). However, none of these alterations was observed in the 8-week feeding study (Experiment II). Therefore, we concluded that the higher incidence of transitional cell papillomas in female rats fed 1.25% ARA-oil and the increased in multiplicity of transitional cell PN hyperplasias in male rats fed 2.5% ARA-oil that we observed in Experiment I were incidental.

Table 10
Quantitative data for GST-P positive foci in the liver of F344 rats treated with ARA-oil with or without prior DMBDD treatment.

Groups	Treatment		No. of rats examined	GST-P positive foci	
	DMBDD	ARA-oil		Number (No./cm^2)	Area (mm^2/cm^2)
Males					
1	+	0% (MF diet)	16	3.18 ± 1.63[a]	0.18 ± 0.10
2	+	0%	12	4.47 ± 2.28	0.27 ± 0.19
3	+	1.25%	14	4.27 ± 3.29	0.26 ± 0.22
4	+	2.5%	14	4.07 ± 3.02	0.22 ± 0.18
5	+	5.0%	13	4.21 ± 2.15	0.22 ± 0.13
6	–	0%	10	0.00 ± 0.00	0.00 ± 0.00
7	–	5.0%	10	0.00 ± 0.00	0.00 ± 0.00
Females					
1	+	0% (MF diet)	19	11.23 ± 5.17	1.38 ± 0.74
2	+	0%	19	16.64 ± 4.14[##]	1.67 ± 0.61
3	+	1.25%	17	16.49 ± 4.16	1.87 ± 0.57
4	+	2.5%	16	16.21 ± 5.48	2.02 ± 1.02
5	+	5.0%	17	16.77 ± 5.76	1.76 ± 0.78
6	–	0%	10	0.00 ± 0.00	0.00 ± 0.00
7	–	5.0%	10	0.00 ± 0.00	0.00 ± 0.00

[a] Values are mean ± SD.
[##] Significantly different from the group 1 at $P < 0.01$.

Table 11
BrdU labeling indices in the epithelial cells of the urinary bladder and thyroid of F344 rats fed on AIN-93M diet containing ARA-oil in the 8-week feeding study.

Groups	ARA-oil	No. of rats examined	BrdU labeling index (%)	
			Urinary bladder	Thyroid
Males				
1	0% (MF diet)	20	0.13 ± 0.05[a]	0.33 ± 0.21
2	0%	20	0.09 ± 0.09	0.39 ± 0.34
3	1.25%	20	0.09 ± 0.11	0.42 ± 0.38
4	2.5%	20	0.09 ± 0.09	0.38 ± 0.30
5	5.0%	20	0.10 ± 0.08	0.44 ± 0.32
Females				
1	0% (MF diet)	20	0.14 ± 0.11	0.21 ± 0.14
2	0%	20	0.05 ± 0.09[##]	0.18 ± 0.13
3	1.25%	20	0.05 ± 0.06	0.26 ± 0.23
4	2.5%	20	0.07 ± 0.09	0.25 ± 0.21
5	5.0%	20	0.11 ± 0.11	0.25 ± 0.26

[a] Values are mean ± SD.
[##] Significantly different from the group 1 at $P < 0.01$.

There were statistically significant increases in the incidences of follicular cell adenomas or carcinomas in the thyroid of male rats fed 1.25% ARA-oil. The incidences of follicular cell hyperplasias or adenomas were significantly higher in male rats fed 5.0% ARA-oil than in males fed 7.0% soybean oil (group 2). These differences were thought to be independent of the administration of test substance because the increases were not dose-dependent and/or the incidence levels were equivalent to those in rats fed the MF diet. Moreover, there was no evidence of an increase in follicular cell lesions in any group of female rats fed ARA-oil. In the 8-week feeding study (Experiment II), no increase in the thyroid weights was showed, and there were no statistically significant differences in BrdU indices in the thyroid epithelium in any groups fed ARA-oil in either sex. In addition, ARA-oil treatment was associated with a very slight but statistically significant increase in serum T3 levels, whereas T4 and TSH levels were unchanged in all male ARA-oil-treated groups. On the other hand, slightly but significantly higher TSH levels were only shown in the female 5.0% ARA-oil-treated group (group 5) as compared with control values (group 2), whereas T3 and T4 levels were not affected in any of the fem

Table 12
Serum concentrations of triiodothyronin (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) in F344 rats fed on AIN-93M diet containing ARA-oil in the 8-week feeding study.

Groups	ARA-oil	No. of rats examined	T3 (ng/dL)	T4 (μg/dL)	TSH (ng/mL)
Males					
1	0% (MF diet)	20	120.4 ± 16.8[a]	6.96 ± 0.98	1.48 ± 1.22
2	0%	20	122.1 ± 10.5	6.80 ± 0.66	1.21 ± 1.08
3	1.25%	20	133.5 ± 11.9[*]	6.68 ± 0.92	1.38 ± 0.98
4	2.5%	20	135.4 ± 14.5[**]	6.55 ± 0.76	1.60 ± 1.17
5	5.0%	19	134.3 ± 12.5[**]	6.69 ± 0.79	0.99 ± 0.84
Females					
1	0% (MF diet)	19	136.8 ± 15.0	5.84 ± 1.17	1.65 ± 0.83
2	0%	20	149.2 ± 20.0[#]	5.55 ± 1.00	2.92 ± 0.87[##]
3	1.25%	20	151.7 ± 22.6	5.73 ± 1.72	2.93 ± 1.36
4	2.5%	20	156.8 ± 30.6	5.43 ± 1.53	3.48 ± 1.48
5	5.0%	20	167.0 ± 20.0	6.18 ± 1.18	4.90 ± 1.76[**]

[a] Values are mean ± SD.
[*] Significantly different from the group 2 at $P < 0.05$.
[**] Significantly different from the group 2 at $P < 0.01$.
[#] Significantly different from the group 1 at $P < 0.05$.
[##] Significantly different from the group 1 at $P < 0.01$.

Table 13
Urinalysis data for F344 rats fed on AIN-93M diet containing ARA-oil in the 8-week feeding study.

Groups	ARA-oil	No. of rats examined	pH	Na (mEq/L)	K (mEq/L)	CL (mEq/L)	Urine volume (in g)	Specific gravity
Males								
1	0% (MF diet)	20	6.92 ± 0.26[a]	127.0 ± 36.5	242.2 ± 36.0	128.4 ± 31.6	1.46 ± 0.53	1.058 ± 0.009
2	0%	19	6.26 ± 0.37[##] (n = 20)	113.9 ± 54.3	148.3 ± 67.3[##]	90.9 ± 41.1[##]	0.53 ± 0.34[##]	1.056 ± 0.021
3	1.25%	20	6.05 ± 0.25	62.6 ± 25.7[**]	118.1 ± 44.8	57.4 ± 28.6[**]	0.73 ± 0.28	1.039 ± 0.012[*]
4	2.5%	20	5.98 ± 0.15[*]	66.9 ± 33.4[*]	110.2 ± 29.4	67.9 ± 30.4	0.92 ± 0.32[**]	1.037 ± 0.009[**]
5	5.0%	20	6.00 ± 0.25[*]	40.3 ± 22.6[**]	82.2 ± 20.7[**]	46.8 ± 26.6[**]	1.09 ± 0.35[**]	1.031 ± 0.009[**]
Females								
1	0% (MF diet)	20	7.43 ± 0.22	75.8 ± 43.7	121.6 ± 79.7	57.6 ± 43.9	1.20 ± 0.42	1.029 ± 0.016
2	0%	20	6.11 ± 0.39[##]	55.7 ± 35.8	178.5 ± 70.2[#]	102.0 ± 41.3[##]	0.41 ± 0.13[##]	1.054 ± 0.015[#]
3	1.25%	20	5.88 ± 0.19	43.5 ± 25.6	125.8 ± 57.0	59.8 ± 28.5[**]	0.53 ± 0.25	1.040 ± 0.014[**]
4	2.5%	20	6.03 ± 0.21	21.6 ± 11.7[**]	122.2 ± 48.3[*]	54.4 ± 31.6[**]	0.66 ± 0.30[*]	1.035 ± 0.011[**]
5	5.0%	20	5.98 ± 0.25	16.1 ± 8.4[**]	93.2 ± 33.0[**]	45.6 ± 12.0[**]	0.61 ± 0.31[*]	1.029 ± 0.008[**]

[a] Values are mean ± SD.
[*] Significantly different from the group 2 at $P < 0.05$.
[**] Significantly different from the group 2 at $P < 0.01$.
[#] Significantly different from the group 1 at $P < 0.05$.
[##] Significantly different from the group 1 at $P < 0.01$.

ARA-oil-treated groups. In the present study, because those hormonal alterations were varied and inconsistent, they may be considered unrelated to follicular cell proliferation due to thyroid tumor promoter increase of the TSH level by negative-feedback via the thyroid-pituitary axis (Alison et al., 1994; Hasumura et al., 2005; Hood et al., 1999). Based on our findings, we concluded that ARA-oil did not exert tumor-promoting activity in the thyroid.

In conclusion, the results indicated that arachidonate-enriched triglyceride oil did not have tumor-promoting activity in any organs or tissues in a medium-term multi-organ carcinogenesis bioassay in rats.

Conflict of Interest

Six of the authors (YK, NT, WF, HK, HS and YS) are employees of Suntory Business Expert Ltd. or Suntory Wellness Ltd., which is a manufacturer of foods including ARA-oil. The remaining authors had no conflicts of interest.

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References

Alison, R.H., Capen, C.C., Prentice, D.E., 1994. Neoplastic lesions of questionable significance to humans. Toxicol. Pathol. 22, 179–186.
Arterburn, L.M., Boswell, K.D., Henwood, S.M., Kyle, D.J., 2000a. A developmental safety study in rats using DHA- and ARA-rich single-cell oils. Food Chem. Toxicol. 38, 763–771.
Arterburn, L.M., Boswell, K.D., Lawlor, T., Cifone, M.A., Murli, H., Kyle, D.J., 2000b. *In vitro* genotoxicity testing of ARASCO® and DHASCO® oils. Food Chem. Toxicol. 38, 971–976.
Bartram, H.P., Gostner, A., Scheppach, W., Reddy, B.S., Rao, C.V., Dusel, G., Richter, F., Richter, A., Kasper, H., 1993. Effects of fish oil on rectal cell proliferation, mucosal fatty acids, and prostaglandin E_2 release in healthy subjects. Gastroenterology 105, 1317–1322.
Birch, E.E., Garfield, S., Hoffman, D.R., Uauy, R., Birch, D.G., 2000. A randomized controlled trial of early dietary supply of long-chain polyunsaturated fatty acids and mental development in term infants. Dev. Med. Child Neurol. 42, 174–181.
Bouwstra, H., Dijck-Brouwer, D.A., Wildeman, J.A., Tjoonk, H.M., van der Heide, J.C., Boersma, E.R., Muskiet, F.A., Hadders-Algra, M., 2003. Long-chain polyunsaturated acids have a positive effect on the quality of general movements of health term infants. Am. J. Clin. Nutr. 78, 313–318.
Brenna, J.T., Varamini, B., Jensen, R.G., Diersen-Schade, D.A., Boettcher, J.A., Arterburn, L.M., 2007. Dochosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. Am. J. Clin. Nutr. 85, 1457–1464.
British Nutrition Foundation's Task Force, 1992. Recommendations for intakes of unsaturated fatty acids. In: Unsaturated Fatty Acids: Nutritional and Physiological Significance. Chapman and Hall, London, UK, pp. 152–163.
Clandinin, M.T., Van Aerde, J.E., Merkel, K.L., Harris, C.L., Springer, M.A., Hansen, J.W., Diersen-Schade, D.A., 2005. Growth and development of preterm infants fed infant formulas containing docosahexaenoic acid and arachidonic acid. J. Pediatr. 146, 461–468.
Doi, Y., Furukawa, F., Suguro, M., Ito, H., Imai, N., Nabae, K., Toda, Y., Inatomi, S., Kinugasa, S., Kobayashi, H., 2010. Rat medium-term multi-organ carcinogenesis bioassay of *Agaricus blazei* Murrill fruit-body extract. Food Chem. Toxicol. 48, 402–408.
ESPGAN (European Society of Pediatric Gastroenterology and Nutrition) committee on nutrition, 1991. Comment on the content and composition of lipids in infant formulas. Acta Paediatr. Scand. 80, 887–896.
Fukushima, S., Shibata, M.-A., Kurata, Y., Hasegawa, R., 1991. The roles of L-ascorbic acid, urinary pH, and Na or K ion concentration in rat bladder epithelial cell proliferation with special reference to tumor promotion. Prog. Clin. Biol. Res. 369, 195–208.
Hagiwara, A., Tanaka, H., Imaida, K., Tamano, S., Fukushima, S., Ito, N., 1993. Correlation between medium-term multi-organ carcinogenesis bioassay data and long-term observation results in rats. Jpn. J. Cancer Res. 84, 237–245.
Hasegawa, R., Tanaka, H., Tamano, S., Shirai, T., Nagao, M., Sugimura, T., Ito, N., 1994. Synergistic enhancement of small and large intestinal carcinogenesis by combined treatment of rats with five heterocyclic amines in a medium-term multi-organ bioassay. Carcinogenesis 15, 2567–2573.
Hasumura, M., Imai, T., Takizawa, T., Ueda, M., Onose, J., Hirose, M., 2005. Promotion of thyroid carcinogenesis by *para*-aminobenzoic acid in rats initiated with *N*-bis(2-hydroxypropyl)nitrosamine. Toxicol. Sci. 86, 61–67.
Hempenius, R.A., Lina, B.A., Haggitt, R.C., 2000. 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. Food Chem. Toxicol. 38, 127–139.
Hempenius, R.A., Van Delft, J.M., Prinsen, M., Lina, B.A., 1997. Preliminary safety assessment of an arachidonic acid-enriched oil derived from *Mortierella alpina*: summary of toxicological data. Food Chem. Toxicol. 35, 573–581.
Hirose, M., Takesada, Y., Tanaka, H., Tamano, S., Kato, T., Shirai, T., 1998. Carcinogenicity of antioxidants BHA, caffeic acid sesamol, 4-methoxyphenol and catechol at low doses, either alone or in combination, and modulation of their effects in a rat medium-term multi-organ carcinogenesis model. Carcinogenesis 19, 207–212.
Hirose, M., Tanaka, H., Takahashi, S., Futakuchi, M., Fukushima, S., Ito, N., 1993. Effects of sodium nitrite, and catechol, 3-methoxycatechol, or butylated hydroxyanisole in combination in a rat multiorgan carcinogenesis model. Cancer Res. 53, 32–37.
Hoffman, D.R., Boettcher, J.A., Diersen-Schade, D.A., 2009. Toward optimizing vision and cognition in term infants by dietary docosahexaenoic and arachidonic acid supplementation: A review of randomized controlled trials. Prostaglandins Leukot. Essent. Fatty Acids 81, 151–158.
Hood, A., Liu, J., Klaassen, C.D., 1999. Effects of phenobarbital, pregnenolone-16α-carbonitrile, and propylthiouracil on thyroid follicular cell proliferation. Toxicol. Sci. 50, 45–53.
Ichihara, T., Yoshino, H., Doi, Y., Nabae, K., Imai, N., Hagiwara, A., Tamano, S., Morita, O., Tamaki, Y., Suzuki, H., 2008. No enhancing effects of diacylglycerol oil on tumor development in a medium-term multi-organ carcinogenesis bioassay using male F344 rats. Food Chem. Toxicol. 46, 157–167.

Ito, N., Hasegawa, R., Imaida, K., Hirose, M., Shirai, T., 1996. Medium-term liver and multi-organ carcinogenesis bioassays for carcinogens and chemopreventive agents. Exp. Toxic Pathol. 48, 113–119.
Ito, N., Imaida, K., Tamano, S., Hagiwara, A., Shirai, T., 1998. Medium-term bioassays as alternative carcinogenicity test. J. Toxicol. Sci. 23 (Suppl. II), 103–106.
Ito, N., Imaida, K., Asamoto, M., Shirai, T., 2000. Early detection of carcinogenic substances and modifiers in rats. Mutat. Res. 462, 209–217.
Ito, N., Tsuda, H., Tatematsu, M., Inoue, T., Tagawa, Y., Aoki, T., Uwagawa, S., Kagawa, M., Ogiso, T., Masui, T., Imaida, K., Fukushima, S., Asamoto, M., 1988. Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase placental form positive foci in rats–an approach for a new medium-term bioassay system. Carcinogenesis 9, 387–394.
Joint FAO/WHO Food Standards Programme, CODEX Alimentarius Commission, 30th Section, Rome, Italy, 2–7 July 2007.
Koletzko, B., Lien, E., Agostoni, C., Böhles, H., Campoy, C., Cetin, I., Decsi, T., Dudenhausen, J.W., Dupont, C., Forsyth, S., Hoesli, I., Holzgreve, W., Lapillonne, A., Putet, G., Secher, N.J., Symonds, M., Szajewska, H., Willatts, P., Uauy, R., ; World Association of Perinatal Medicine Dietary Guidelines Working Group, 2008. The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. J. Perinat. Med. 36, 5–14.
Koskelo, E.-K., Boswell, K., Carl, L., Lanoue, S., Kelly, C., Kyle, D., 1997. High level of dietary arachidonic acid triglyceride exhibit no subchronic toxicity in rats. Lipids 32, 397–405.
Lina, B.A.R., Wolterbeek, A.P.M., Suwa, Y., Fujikawa, S., Ishikura, Y., Tsuda, S., Dohnalek, M., 2006. Subchronic (13-week) oral toxicity study, preceded by an in utero exposure phase, with arachidonate-enriched triglyceraide oil (SUNTGA40S) in rats. Food Chem. Toxicol. 44, 326–335.
McCormick, D.L., Spicer, A.M., Hollister, J.L., 1989. Differential effects of tranylcypromine and imidazole on mammary carcinogenesis in rats fed low and high fat diets. Cancer Res. 49, 3168–3172.
Miller, A.B., Howe, G.R., Jain, M., Craib, K.J.P., Harrison, L., 1983. Food items and food groups as risk factors in a case-control study of diet and colo-rectal cancer. Int. J. Cancer 32, 155–161.
Ministry of Health, Labour and Welfare, 6th version of the Japanese Standard Food Composition Table, 1999.
Minoura, T., Takata, T., Sakaguchi, M., Takada, H., Yamamura, M., Hioki, K., Yamamoto, M., 1988. Effect of dietary eicosapentaenoic acid on azoxymethane-induced colon carcinogenesis in rats. Cancer Res. 48, 4790–4794.
Narisawa, T., Takahashi, M., Kotanagi, H., Kusaka, H., Yamazaki, Y., Koyama, H., Fukaura, Y., Nishizawa, Y., Kotsugai, M., Isoda, Y., Hirano, J., Tanida, N., 1991. Inhibitory effect of dietary perilla oil rich in the n–3 polyunsaturated fatty acid α-linolenic acid on colon carcinogenesis in rats. Jpn. J. Cancer Res. 82, 1089–1096.
Noguchi, M., Minami, M., Yagasaki, R., Kinoshita, K., Earashi, M., Kitagawa, H., Taniya, T., Miyazaki, I., 1997. Chemoprevention of DMBA-induced mammary carcinogenesis in rats by low-dose EPA and DHA. Br. J. Cancer 75, 348–353.
Rao, C.V., Hirose, Y., Indranie, C., Reddy, B.S., 2001. Modulation of experimental colon tumorigenesis by types and amounts of dietary fatty acids. Cancer Res. 61, 1927–1933.
Rao, C.V., Simi, B., Wynn, T.-T., Garr, K., Reddy, B.S., 1996. Modulating effect of amount and types of dietary fat on colonic mucosal phospholipase A_2, phosphatidylinositol-specific phospholipase C activities, and cyclooxygenase metabolite formation during different stages of colon tumor promotion in male F344 rats. Cancer Res. 56, 532–537.
Reddy, B.S., Sugie, S., 1988. Effect of different levels of omega-3 and omega-6 fatty acids on azoxymethane-induced colon carcinogenesis in F344 rats. Cancer Res. 48, 6642–6647.
Rose, D.P., 1997. Dietary fatty acids and cancer. Am. J. Clin. Nutr. 66 (suppl), 998S–1003S.
Report of the SCF (Scientific Committee for Food) on the Essential Requirements for Infant Formulae and Follow-on Formulae. Opinion expressed on 17 September 1993.
Shibata, M.-A., Fukushima, S., Asakawa, E., Hirose, M., Ito, N., 1992. The modifying effects of indomethacin or ascorbic acid on cell proliferation induced by different types of bladder tumor promoters in rat urinary bladder and forestomach mucosal epithelium. Jpn. J. Cancer Res. 83, 31–39.
Shibata, M.-A., Hasegawa, R., Imaida, K., Hagiwara, A., Ogawa, K., Hirose, M., Ito, N., Shirai, T., 1995. Chemoprevention by dehydroepiandrosterone and indomethacin in a rat multiorgan carcinogenesis model. Cancer Res. 55, 4870–4874.
Shibata, M.-A., Yamada, M., Asakawa, E., Hagiwara, A., Fukushima, S., 1989. Responses of rat urine and urothelium to bladder tumor promoters: possible roles of prostaglandin E_2 and ascorbic acid synthesis in bladder carcinogenesis. Carcinogenesis 10, 1651–1656.
Shirai, T., Hirose, M., Ito, N., 1999. Medium-term bioassays in rats for rapid detection of the carcinogenic potential of chemicals. In: McGregor, D.B., Rice, J.M., Venitt, S. (Eds.), The Use of Short- and Medium-Term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard evaluation. International Agency for Research on Cancer, Scientific Publications No. 146, Lyon, pp. 251–272.
Steel, R.G.D., 1959. A multiple comparison rank sum test: treatments versus con Biometrics 15, 560–572.
Takahashi, S., Hakoi, K., Yada, H., Hirose, M., Ito, N., Fukushima, S., 1992. Enhancing effects of diallyl sulfide on hepatocarcinogenesis and inhibitory actions of the related diallyl disulfide on colon and renal carcinogenesis in rats. Carcinogenesis 13, 1513–1518.
Tanaka, Y., Konishi, Y., Nishimune, T., 1994. Fatty acid composition of mature human milk in Osaka prefecture – relationship between the content of mother's diet and fatty acid composition of human milk – Osakafuritsu Koshueisei Kenkyusho Kenkyuhokoku Shokuhin Eiseihen 25, 21–31.
Toriyama-Baba, H., Iigo, M., Asamoto, M., Iwahori, Y., Park, C.B., Han, B.S., Takasuka, N., Kakizoe, T., Ishikawa, C., Yazawa, K., Araki, E., Tsuda, H., 2001. Organotropic chemopreventive effects of n–3 unsaturated fatty acids in a rat multi-organ carcinogenesis model. Jpn. J. Cancer Res. 92, 1175–1183.
Usui, T., 1998. Testing human pharmaceuticals for carcinogenic potential: new approach in ICH process. J. Toxicol. Sci. 23 (Suppl. II), 258–261.
Wang, D., DuBois, R.N., 2006. Prostaglandins and cancer. Gut 55, 115–122.
Woods, J., Ward, G., Salem Jr., N., 1996. Is docosahexaenoic acid necessary in infant formula? Evaluation of high linolenate diets in the neonatal rat. Pediatr. Res. 40, 687–694.
Yada, H., Hirose, M., Tamano, S., Kawabe, M., Sano, M., Takahashi, S., Futakuchi, M., Miki, T., Shirai, T., 2002. Effects of antioxidant 1-O-Hexyl-2,3,5-trimethylhydroquinone or ascorbic acid on carcinogenesis induced by administration of aminopyrine and sodium nitrite in a rat multi-organ carcinogenesis model. Jpn. J. Cancer Res. 93, 1299–1307.
Yamamoto, S., Konishi, Y., Matsuda, T., Murai, T., Shibata, M.-A., Matsui-Yuasa, I., Otani, S., Kuroda, K., Endo, G., Fukushima, S., 1995. Cancer induction by an organic arsenic compound, dimethylarsinic acid (cacodylic acid), in F344/DuCri rats after pretreatment with five carcinogens. Cancer Res. 55, 1271–1276.
Yoshida, Y., Tatematsu, M., Takaba, K., Iwasaki, S., Ito, N., 1993. Target org specificity of cell proliferation induced by various carcinogens. Toxicol. Pathol. 21, 436–442.