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Effects of arachidonate-enriched triacylglycerol supplementation on serum fatty acids and platelet aggregation in healthy male subjects with a fish diet

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The changes in fatty acid composition of serum and in platelet aggregation induced by supplementation of arachidonate-enriched TAG were investigated in twenty-four healthy Japanese men in a double-blind, placebo-controlled study. The arachidonate-enriched TAG ingested was an edible oil, extracted and purified from a biomass of submerged fermented *Mortierella alpina*. Mean daily intake of fish and shellfish by subjects was 87·2 (SE5·3) g/d, while dietary intakes of arachidonic acid (ARA) by the ARA group and placebo group were 175 (SE12) and 179 (SE13) mg/d, respectively. In the ARA group, after 2-week supplementation of 838 mg ARA/d, ARA concentration in serum phospholipids was increased from 9·6 (SE0·4) to 13·7 (SE0·4) g/100 g total fatty acids, and was significantly different from that in the placebo group ($P<0·001$). This level was maintained for 4 weeks but returned to baseline level after a 4-week washout period. Linoleic acid concentration in serum phospholipids decreased from 19·2 (SE0·8) to 16·3 (SE0·6) g/100 g total fatty acids in the ARA group. Similarly, ARA content of serum TAG increased after ARA supplementation. Neither the EPA nor DHA content of serum phospholipids or TAG was altered by ARA supplementation. The platelet aggregation induced in platelet-rich plasma by adding adenosine diphosphate, collagen and ARA, physical characteristics of subjects, and biochemical parameters were unchanged throughout the test period. These findings suggest that ARA concentration in serum phospholipids and TAG can be safely increased by supplementation of arachidonate-enriched TAG oil.

Arachidonic acid: Serum: Platelet aggregation: Man

Arachidonic acid (ARA; 20 : 4*n*-6) is a PUFA and major constituent of the cell membrane, and plays important roles in the preservation of physiological function. For example, yolk, lean meat and fish contain comparatively large amounts of ARA. In the last several decades, many studies have focused on the usefulness of *n*-6 PUFA[1–3] and *n*-3 PUFA[2–6] in atherosclerosis, and of the latter in dementia[7–10]. There are many official recommendations of the ratio of *n*-6 to *n*-3 fatty acids. For example, the WHO/FAO suggests a ratio of 5 : 1–10 : 1[11], the National Institutes of Health recommends a ratio of 2 : 1–3 : 1[12] and Canada recommends 4 : 1–10 : 1[13]. Also, there are recommended dietary intakes for specific fatty acids, such as linoleic acid (18 : 2*n*-6), α-linolenic acid (18 : 3*n*-3) and EPA (20 : 5*n*-3) + DHA (20 : 6*n*-3), proposed by the UK[14], National Institutes of Health[3] and American Heart Association. Whereas there are no official recommendations for ARA.

In the human body, ARA is synthesized through desaturation and elongation of linoleic acid. That process is similar to the synthesis of EPA and not as complex as that of DHA, from α-linolenic acid. However, human fetuses and neonates are unable to synthesize ARA and DHA sufficiently, and initially obtain ARA and DHA by placental transfer and from human milk[15–18]. It has been reported that premature babies given infant formulas containing ARA and DHA grew better than babies given formulas not containing them[19,20]. Even for term infants, use of infant formulas containing ARA and DHA yielded better developmental outcomes than unsupplemented formulas[21–23]. ARA and DHA are commonly found in infant formulas in Europe and the USA, because the usefulness of addition of them to the diet has been examined by many advisory bodies (e.g. ESPGAN[24], the British Nutrition Foundation Task Force[25] and SCF[26]). It has been found that the capacity to convert α-linolenic acid to EPA and DHA is low beyond infancy[27–29], and that the principal converting enzyme, Δ6-desaturase, becomes less efficient with ageing[30]. In healthy women, it has been reported that the conversion of [^{13}C]linoleic acid and [^{13}C]α-linolenic acid to longer-chain PUFA is a quantitatively minor route of utilization[31].

The content of ARA in membrane phospholipids has been found to be lower in aged than in young animals. Several studies have found that the ability of aged rats to sustain long-term potentiation, which is thought to be a strong model for neuronal plasticity such as learning and memory in the hippocampus[32], is impaired. Also it has been revealed

Abbreviations: ARA, arachidonic acid.

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that the impairment of sustaining long-term potentiation is coupled with a decrease in membrane ARA concentration in the dentate gyrus[33,34]. In addition, results of spatial task performance and ARA studies as well as studies of ARA and long-term potentiation have suggested the possibility that spatial learning deficits in aged rats may be improved by administration of ARA[33–38].

It has been expected that highly purified oils rich in ARA will come into use not only in infant formulas but also nutritional products and food supplements. In the last few decades, advances in microbiological technology have enabled supplementation of ARA in TAG oil[39].

Safety studies of ARA-enriched TAG oil obtained from *Mortierella alpina* have been performed both *in vitro* and *in vivo*[40–43]. There have been several human studies of ARA-enriched TAG, including one in healthy subjects using 1·5 g/d for 50 d[44–48] and one in patients with cirrhosis using 2 g/d for 8 weeks[47]. Both studies found that supplementation of ARA-enriched TAG safely increased ARA concentration in plasma and erythrocytes. Because the body composition of fatty acids is affected by diet, studies are needed in other types of individuals, e.g. those with moderate fish intake and who thus have comparatively high levels of EPA and DHA in the blood and cell membranes, such as the Japanese[48–52].

In the present study, we examined the effects of ARA-enriched TAG supplementation on the composition of serum fatty acids, platelet aggregation and biomedical parameters in healthy Japanese men, who have comparatively high levels of EPA and DHA in blood.

Experimental methods

Subjects

Healthy male subjects were recruited after assessment of medical and dietary histories. Exclusion criteria were the presence of overt vascular, haematological or respiratory disease, hyperlipidaemia, infection, BMI less than 19 or greater than 27 kg/m^2, smoking and consumption of drugs that affect lipid metabolism or haemostatic function. All subjects were asked not to change anything related to nutrition or lifestyle during the test period, in order to eliminate effects of such changes on certain haemostatic factors. The subjects were supplied with sufficient explanation concerning the study, they understood it well and they provided written informed consent.

Study design

This double-blind, placebo-controlled study included three periods, run-in (1 week), supplementation (4 weeks) and post-supplementation (4 weeks, washout), which together formed the test period. The study was approved by the Ethics Committee on Human Experimentation of Suntory and conformed to the Helsinki Declaration. Subjects were randomly divided into two groups. One group received capsules containing 200 mg/capsule TAG enriched with ARA (corresponding to 83·8 mg/capsule), which was extracted from a biomass of submerged fermented *M. alpina* and refined by high-purification processes (SUNTGA40S; Suntory Ltd, Osaka, Japan). Another group received capsules containing 200 mg/capsule commercially available olive oil as a placebo. The fatty acid compositions of the two oils are shown in Table 1. Subjects were instructed to take ten capsules every morning during the supplementation period. ARA content in the capsules was 83·8 mg/capsule and therefore the daily ARA dose was 838 mg/d in the ARA group.

Dietary design

The subjects were asked to continue their habitual diet and keep a diary regarding their health condition and daily intake of eggs, fish and meat, in order to roughly monitor consumption of ARA and other nutrients. Daily food intake by each subject was estimated by 7 d weighed intake. Nutrient intakes were calculated by the software Eiyoukun (version 4.0) for Microsoft Excel (Kenpakusha, Japan), which was based on the *Standard Tables of Food Composition in Japan*[53]. Because certain dietary components, such as garlic, onion, fermented soyabeans, some spices and alcohol, are known to modify platelet aggregation, the subjects were given written and verbal instructions not to exceed the usual intake of each during the test period. Each subject was instructed to have the same dinner each evening before blood sampling, and they did not allowed alcohol and fermented soyabeans the day before blood sampling.

Procedure for measurements and blood sampling

The physical characteristics of subjects were measured and blood and urine were collected for fatty acid analysis, determination of biochemical parameters and platelet aggregation, and urinalysis. Samples were obtained after a 13 h fast in the early morning before consumption of the capsules at 0, 2 and 4

Table 1. Fatty acid composition of the study oils (as % of total fatty acids)

Fatty acid	Placebo	ARA
8 : 0	0·7	0·4
10 : 0	0·2	0·2
14 : 0	0·0	0·5
15 : 0	0·0	0·2
16 : 0	11·9	10·0
16 : 1*n*-7	1·2	0·0
17 : 0	0·0	0·4
18 : 0	2·8	7·3
18 : 1*n*-9	70·8	6·9
18 : 2*n*-6	11·1	9·7
18 : 3*n*-6	0·0	2·4
18 : 3*n*-3	0·6	0·5
20 : 0	0·4	0·9
20 : 1*n*-9	0·3	0·4
20 : 2*n*-6	0·0	0·6
20 : 3*n*-6	0·0	3·5
20 : 4*n*-6 (ARA)	0·0	41·9
20 : 5*n*-3 (EPA)	0·0	0·1
22 : 0	0·0	3·5
22 : 4*n*-6	0·0	0·6
24 : 0	0·0	9·2
24 : 1*n*-9	0·0	0·3
Unknown	0·0	0·5

ARA, arachidonic acid.

weeks and after the 4-week washout period. Times of measurement and sampling were standardized across the test period to minimize effects of circadian variation. Samples were obtained and measurements performed after subjects had been sitting quietly for 15 min. Body weight and blood pressure were measured as physical characteristics. Blood pressure measurements were made using the HEM-1000 automatic digital manometer (Omron Healthcare Co., Kyoto, Japan). Recordings were made in duplicate, and consistent values or averages were taken. Venous blood samples were taken from the arm not used for measurement of blood pressure using a 21-gauge needle and syringe.

Fatty acid analysis

Serum was prepared from 3 ml blood using the method described earlier. Lipids in the serum were extracted and purified[54] and separated into neutral lipids and phospholipids by TLC with silica gel 60 (Merck, Darmstadt, Germany). The solvent system consisted of hexane–diethyl ether (7 : 3, v/v). Fatty acid residues in the phospholipid and TAG fractions were analysed[39]. In brief, each fraction with an additional internal standard (pentadecanoic acid) was incubated in methanolic HCl at 50°C for 3 h for transmethylation of fatty acid residues to fatty acid methyl esters. Fatty acid methyl esters were extracted with *n*-hexane and analysed by capillary GLC using pentadecanoic acid as an internal standard.

Analyses of haematological and biochemical parameters

Standard haematological tests were determined using a Sysmex SE-9000 (Sysmex Co., Kobe, Japan). Standard clinical chemistry tests, phospholipids (choline oxidase method[55]), HDL-cholesterol[56], LDL-cholesterol[57] and C-reactive protein (latex agglutination immunoassay[58,59]) were performed on a Model 7450 (7170) or 7070 Hitachi Automatic Clinical Analyzer (Hitachi High Technologies Co., Tokyo, Japan). Serum IgE was measured by fluorescence enzyme immunoassay, using a Uni CAP 1000 (Aloka Co., Tokyo, Japan). Plasma for blood glucose determination was prepared from blood (2 ml) collected into a tube containing heparin as an anticoagulant and Na-fluorine as a stop reagent, by immediate centrifugation at 2150 ***g*** for 10 min at 4°C, and was analysed by the hexokinase–glucose-6-phoshate dehydrogenase method in a JCA-BM12 (Jeol, Tokyo, Japan). Plasma for prothrombin time and antithrombin III determination was prepared from 1·8 ml blood collected into a tube containing 0·2 ml sodium citrate as an anticoagulant (final concentration 13 mM), and centrifuged at 2150 ***g*** for 10 min at 4°C. Prothrombin time was determined by the Quick one-step test and antithrombin III activity by the chromogenic method, using the Sysmex CA7000. Urinalysis was performed using the AUTION MAX AX-4280 (Eiken Chemical Co., Tokyo, Japan) to determine density and pH and for detection of glucose, protein, blood, urobilinogen, bilirubin and ketone bodies.

Platelet aggregation study

Venous blood (13·5 ml) was collected through siliconized needles into three plastic tubes, with 4·5 ml in each tube containing 0·5 ml 3·8 % sodium citrate (final concentration 13 mM). Platelet-rich plasma was obtained by centrifugation of blood at 190 ***g*** for 15 min at 22°C. Platelet-poor plasma was prepared by respinning the blood after removal of the platelet-rich plasma at 1700 ***g*** for 10 min. Platelet aggregation was measured in duplicate in a twelve-channel MCM Hematracer (MC Medical Inc., Tokyo, Japan). A 200 μl portion of the platelet-rich plasma was added to a silicon-coated tube containing a stirring bar at a speed of 1000 rpm at 37°C. The blank was 200 μl of the individual's platelet-poor plasma, unstirred. Aggregation of platelets in platelet-rich plasma was induced by adding specified concentrations of ADP (Sigma-Aldrich Co., St Louis, MO, USA), collagen (Hormon-Chemie Munchen GmbH[60,61]) and ARA (Nacalai Tesque, Inc., Kyoto, Japan), and measured as the increase in transmission of light through platelet-rich plasma for 15 min. Values are expressed as percentages of maximal aggregation induced by the aggregating agents, which were 1 and 2 μM-ADP, 0·125 and 0·25 μg collagen/ml, and 0·5 and 1 μM-ARA, and as concentration of aggregating agent required to elicit half-maximal response (ED_{50}) for ADP and collagen. Platelet aggregation was performed within 3 h of blood withdrawal.

Statistical analysis

All values are expressed as means with their standard errors of the means for the subjects in each experimental group. Findings were examined by two-way ANOVA without repeated measures, using time and group as sources of variation. Student's unpaired *t* test was used to determine differences between two treatment groups. Determination of the significance of differences between measurements within the same group before and after the intervention was performed with Bonferroni correction for multiple comparisons. Significance of findings was accepted at $P<0{\cdot}05$. The Statistical Package for the Social Sciences for Windows version 11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

Results

A total of twenty-eight subjects entered the study, and ranged in age from 26 to 60 years. Baseline characteristics were similar in the two groups. There was no statistically significant difference between the two groups with respect to age, body weight or BMI. Four subjects were withdrawn (one in the placebo group had tonsillitis and took anti-inflammatory drugs; one in the ARA group had an infectious disease and fever at the time of the 4-week blood draw; the other two subjects were withdrawn because of ingestion of anti-inflammatory drugs (one in the placebo group, one in the ARA group)). All of the twenty-eight subjects were included in the safety analysis. None of the subjects reported any detrimental side-effects. All adverse events observed during the test period were considered unrelated to supplementation. A total of twenty-four subjects (twelve in the placebo and twelve in the ARA group) completed the trial. The mean ages of these subjects in the placebo and ARA groups were 38·6 (SE 3·3) and 39·8 (SE 2·2) years, respectively.

Nutrient intakes

Table 2 shows the proportions of macronutrients and intakes of energy and some fatty acids associated with the background diet of subjects (not including supplements for the placebo and ARA groups). None of daily food intakes differed between the two groups (Table 2). The fish and shellfish intake of allsubjects in the present study was 87·2 (SE 5·3) g/d, while their meat intake was 115·5 (SE 8·1) g/d.

Serum fatty acid composition

The fatty acid compositions of serum phospholipids and TAG are shown in Tables 3 and 4. They did not differ between the two groups at baseline. Following ARA supplementation, ARA content of serum phospholipids increased significantly at 2 weeks ($P<0·001$) and 4 weeks ($P<0·001$), and returned to baseline level after the 4-week washout period. However, no other serum phospholipid fatty acid was significantly changed in the ARA supplementation group compared with the placebo group. In the ARA group alone, stearic acid (18 : 0) content in serum phospholipids was significantly increased and linoleic acid (18 : 2*n*-6) content decreased at 2 and 4 weeks compared to baseline. EPA content was 27·7 (SE 2·5) μg/ml and DHA was 92·3 (SE 2·9) μg/ml at 0 weeks, and neither EPA nor DHA content was changed after ARA supplementation.

The ARA content of serum TAG also increased significantly after ARA supplementation at 2 and 4 weeks ($P<0·01$), and returned after the washout period. As for serum phospholipids, EPA and DHA contents in serum TAG were not altered by ARA supplementation. In the ARA group, 16 : 1 and 18 : 1*n*-9 contents of serum TAG negligibly but significantly changed after washout, compared with baseline.

Physical characteristics, haematological and biochemical parameters

The physical characteristics and main blood biochemical parameters of the subjects of the two groups are shown in Table 5. In neither group did mean values of the parameters change during the test period, and no significant differences were observed between the two groups at pretreatment or at any time-point during the test period. No differences was observed in results of haematological tests, which were blood Hb, packed cell volume, counts of erythrocytes, total leucocytes, thrombocytes, mean of corpuscular volume and Hb concentration, and urinalysis between the two groups or during the test period.

Platelet aggregation

Table 6 shows platelet aggregation as percentages of maximal aggregation by ADP, collagen and ARA. No significant differences were observed in aggregation response between the two groups. In both groups, maximal aggregation by 0·25 μg collage/ml was significantly increased at 4 weeks compared with pretreatment (0 weeks), while in the placebo group alone it was still increased after the washout period. Table 7 shows the concentrations of aggregating agents, ADP and collagen, required to induce approximately half-maximal response (ED_{50}). No significant difference was observed in ED_{50} between the two groups, and in neither group was a significant difference observed between pretreatment (0 weeks) and other time-points.

Table 2. Subjects' daily food intake estimated by 7 d weighed intake for the arachidonic acid (ARA) treatment group (*n* 12) and placebo group (*n* 12)‡

(Mean values with their standard errors)

	Placebo				ARA			
Nutrient	Mean	SE	Minimum	Maximal	Mean	SE	Minimum	Maximal
Energy (MJ)	8·64	0·27			9·32	0·37		
Protein (E%)	13·9	0·4			13·8	0·4		
Carbohydrate (E%)	49·0	1·7			49·7	1·8		
Total fat (E%)	28·0	1·1			27·6	1·5		
SFA (E%)	7·83	0·51			7·61	0·54		
MUFA (E%)	10·79	0·46			10·37	0·66		
PUFA (E%)	5·98	0·30			6·13	0·34		
Protein (g)	71·5	2·5			76·1	1·9		
Carbohydrate (g)	252	10			277	16		
Total fat (g)	64·8	4·2			67·7	3·8		
SFA (g)	18·1	1·5			18·7	1·4		
MUFA (g)	25·0	1·7			25·5	1·7		
PUFA (g)	13·8	1·0			15·0	0·8		
n-3 Fatty acids (g)	2·66	0·24			2·74	0·19		
n-6 Fatty acids (g)	11·2	0·8			12·2	0·8		
18 : 3*n*-3 (g)	1·51	0·14	0·753	2·36	1·66	0·11	1·07	2·29
18 : 3*n*-6 (g)	10·8	0·8	6·76	16·1	11·9	0·7	8·58	16·7
20 : 4*n*-6 (ARA) (mg)	175	12	110	230	179	13	110	271
20 : 5*n*-3 (EPA) (mg)	318	46	42	634	311	56	59	691
22 : 6*n*-3 (DHA) (mg)	564	71	98	991	530	72	180	935

E%, percentage energy intake.

‡ No significant difference was observed between the two groups for any of the parameters.

Table 3. Fatty acid composition (g/100 g total fatty acids) of serum phospholipids during the test period for the arachidonic acid (ARA) treatment group (*n* 12) and placebo group (*n* 12)
(Mean values with their standard errors)

	Pretreatment (0 weeks)				Supplementation for 2 weeks				Supplementation for 4 weeks				Washout (4 weeks)			
	Placebo		ARA		Placebo		ARA		Placebo		ARA		Placebo		ARA	
Fatty acids	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
16 : 0	27·8	0·3	27·7	0·4	27·9	0·3	27·5	0·3	28·2	0·3	27·3	0·3	28·0	0·4	27·8	0·4
18 : 0	13·7	0·3	13·7	0·1	14·1	0·2	14·3††	0·2	14·0	0·3	14·3††	0·2	13·8	0·3	13·8	0·2
18 : 1*n*-9	8·05	0·32	8·35	0·22	8·14	0·25	7·57	0·16	8·16	0·29	8·17	0·41	8·53	0·42	9·28	0·37
18 : 2*n*-6	17·7	0·9	19·2	0·8	18·0	0·9	16·3††	0·2	18·0	0·7	16·2†††	0·6	17·6	0·7	17·4	0·7
20 : 4*n*-6 (ARA)	9·52	0·41	9·55	0·36	9·23	0·48	13·74***†††	0·44	9·25	0·40	13·89***†††	0·42	9·25	0·37	9·78	0·30
20 : 5*n*-3 (EPA)	3·07	0·35	2·35	0·22	2·87	0·39	2·29	0·28	2·67	0·21	2·22	0·15	2·95	0·27	3·12	0·26
22 : 5*n*-3	1·35	0·07	1·28	0·05	1·33	0·06	1·21	0·04	1·31	0·06	1·18	0·04	1·35	0·05	1·33	0·04
22 : 6*n*-3 (DHA)	8·63	0·36	7·70	0·33	8·48	0·35	7·67	0·32	8·38	0·37	7·38	0·34	8·84	0·36	8·08	0·35
24 : 1	2·03	0·15	1·74	0·12	1·82	0·08	1·49	0·17	1·85	0·10	1·66	0·10	1·69	0·10	1·54	0·11

Mean values were significantly different from those of the placebo (ANOVA and unpaired *t* test): ***$P<0·001$.
Mean values were significantly different from those of the pretreatment (ANOVA and Bonferroni's correction): ††$P<0·01$, †††$P<0·001$.

Table 4. Fatty acid composition (g/100 g total fatty acids) of serum TAG during the test period for the arachidonic acid (ARA) treatment group (*n* 12) and placebo group (*n* 12)
(Mean values with their standard errors)

	Pretreatment (0 weeks)				Supplementation for 2 weeks				Supplementation for 4 weeks				Washout (4 weeks)			
	Placebo		ARA		Placebo		ARA		Placebo		ARA		Placebo		ARA	
Fatty acids	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
16 : 0	22·1	0·6	22·1	0·8	21·9	0·5	21·8	0·7	22·4	1·0	21·3	0·9	22·7	0·8	22·8	0·9
16 : 1	4·51	0·34	3·98	0·25	4·39	0·26	3·92	0·23	4·59	0·35	4·15	0·17	4·89	0·35	4·86††	0·23
18 : 0	2·76	0·12	2·91	0·18	2·76	0·12	3·09	0·17	2·99	0·20	3·19	0·27	2·96	0·16	3·11	0·17
18 : 1*n*-9	38·0	0·5	39·7	0·9	37·4	0·8	37·9	0·9	37·8	0·5	38·1	0·7	37·7	0·7	37·5†	0·5
18 : 2*n*-6	20·3	0·9	21·1	1·0	21·1	1·0	20·7	0·6	20·4	1·2	20·7	0·6	19·4	1·1	19·6	0·9
20 : 4*n*-6 (ARA)	1·41	0·10	1·38	0·11	1·57	0·23	2·32†††	0·28	1·38	0·12	2·15**††	0·17	1·44	0·16	1·60	0·14
20 : 5*n*-3 (EPA)	1·24	0·17	0·87	0·10	1·21	0·19	1·13	0·18	1·09	0·16	1·11	0·12	1·19	0·18	1·28	0·12
22 : 5*n*-3	1·02	0·06	0·82	0·05	1·00	0·07	0·96	0·08	0·97	0·06	0·95	0·06	0·99	0·07	1·00	0·07
22 : 6*n*-3 (DHA)	4·94	0·52	3·48	0·36	5·00	0·52	4·21	0·48	4·87	0·57	4·24	0·47	5·15	0·69	4·63	0·50

Mean values were significantly different from those of the placebo (ANOVA and unpaired *t* test): **$P<0·01$.
Mean values were significantly different from those of the pretreatment (ANOVA and Bonferroni's correction): †$P<0·05$, ††$P<0·01$, †††$P<0·001$.

Table 5. Physical characteristics and blood biochemical parameters during the test period for the arachidonic acid (ARA) treatment group (*n* 12) and placebo group (*n* 12)‡
(Mean values with their standard errors)

	Pretreatment (0 weeks)				Supplementation for 2 weeks				Supplementation for 4 weeks				Washout (4 weeks)			
	Placebo		ARA		Placebo		ARA		Placebo		ARA		Placebo		ARA	
Variable	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Body weight (kg)	67·9	2·3	70·1	1·4	67·8	2·3	70·0	1·3	68·0	2·2	70·0	1·4	67·7	2·2	70·1	1·4
BMI	23·2	0·6	23·8	0·5	23·2	0·61	23·8	0·5	23·2	0·6	23·7	0·5	23·1	0·6	23·8	0·5
SBP (mmHg)	120	3	125	3	122	3	122	4	119	3	122	4	121	3	124	4
DBP (mmHg)	77	3	78	3	74	3	76	3	74	2	74	4	77	3	75	3
TAG (mg/dl)	102	19	105	15	106	17	100	14	121	22	110	20	120	24	121	17
Total-Chol (mg/dl)	194	12	201	11	195	11	204	9	196	12	198	9	192	11	206	13
HDL-Chol (mg/dl)	56	4	60	3	55	4	60	3	56	4	60	2	56	4	62	3
LDL-Chol (mg/dl)	119	11	122	10	120	9	120	8	117	11	113	7	114	8	120	10
NEFA (μEq/l)	455	38	553	70	479	38	500	76	433	39	438	40	514	52	514	82
PL (mg/dl)	199	11	208	13	206	14	221	12	207	12	207	10	202	10	219	11
AST (GOT) (U/l)	23	2	20	2	24	3	20	3	24	3	21	2	24	2	21	2
ALT (GPT) (U/l)	24	4	19	3	26	5	19	4	25	5	20	3	26	5	19	3
γ-GTP (U/l)	44	10	31	6	44	10	30	7	45	10	29	6	51	13	31	6
Glucose (mg/dl)	86	2	91	2	87	2	91	2	87	2	92	2	88	2	92	2
IgE (IU/ml)	238	95	124	46	247	101	127	47	253	111	133	46	264	120	113	37
PT (s)	10·4	0·2	10·6	0·1	10·4	0·2	10·6	0·1	10·3	0·2	10·6	0·1	10·3	0·1	10·4	0·1
ATIII (%)	109	2	108	3	109	3	108	2	106	3	104	3	108	3	108	2

ALT, alkaline phosphatase; AST, aspartate aminotransferase; ATIII, antithrombin III; Chol, cholesterol; DBP, diastolic blood pressure; γ-GTP, γ-glutamyl transpeptidase; PL, phospholipids; PT, prothrombin time; SBP, systolic blood pressure.
‡ No significant difference was observed between the two groups or between pretreatment and other periods for any of these parameters.

Table 6. Platelet aggregation as percentage of maximal aggregation for the arachidonic acid (ARA) treatment group (*n* 12) and placebo group (*n* 12)‡
(Mean values with their standard errors)

	Pretreatment (0 weeks)				Supplementation for 4 weeks				Washout (4 weeks)			
	Placebo		ARA		Placebo		ARA		Placebo		ARA	
Aggregating agent	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
ADP												
1 μM	29·8	5·8	27·3	4·9	35·7	7·1	30·9	7·5	33·3	6·9	30·7	7·0
2 μM	63·6	6·3	57·8	6·2	64·1	6·4	55·3	7·4	63·9	5·9	60·3	7·1
Collagen												
0·125 μg/ml	22·5	8·2	13·8	5·7	25·5	8·4	32·5	9·6	20·5	8·0	19·8	8·0
0·25 μg/ml	41·3	9·3	30·9	7·8	54·1††	8·9	48·4††	9·6	51·4†	8·4	36·6	8·3
ARA												
0·5 μM	20·1	8·7	13·3	6·1	32·8	11·0	26·3	9·8	23·4	9·2	19·6	8·7
1 μM	57·8	10·9	40·0	11·0	60·0	10·9	46·1	11·5	65·4	10·1	40·6	11·5

Mean values were significantly different from those of the pretreatment (ANOVA and Bonferroni's correction): †$P<0·05$, ††$P<0·01$.
‡ No significant difference was observed between the two groups in any of these parameters.

Table 7. Platelet aggregation ED_{50} (concentration of aggregating agent required to induce half-maximal response) for the arachidonic acid (ARA) treatment group (*n* 12) and placebo group (*n* 12)‡
(Mean values with their standard errors)

	Pretreatment (0 weeks)				Supplementation for 4 weeks				Washout (4 weeks)			
	Placebo		ARA		Placebo		ARA		Placebo		ARA	
Aggregating agent	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
ADP (μM)	1·84	0·26	2·04	0·26	1·84	0·37	2·71	0·63	1·85	0·31	2·67	0·79
Collagen (μg/ml)	0·396	0·113	0·601	0·168	0·245	0·036	0·396	0·104	0·285	0·050	0·444	0·097

‡ No significant difference was observed between the two groups or between pretreatment (0 weeks) and other periods for these parameters.

Discussion

This double-blind, placebo-controlled study is the first to have determined the changes in fatty acid composition of serum phospholipids and TAG and in platelet aggregation induced by supplementation of ARA-enriched TAG in healthy Japanese men with a fish diet. After 2-week supplementation of 838 mg ARA/d, ARA concentrations in serum phospholipids and TAG were significantly increased, without decrease in EPA or DHA concentrations (Tables 2 and 4) or effects on platelet aggregation (Tables 6 and 7).

All subjects of the present study kept relatively regular hours performing white-collar work and ate a set lunch or similar dishes in a cafeteria for weekday lunch. The subjects of both groups had a diet that was nearly normal in terms of energy and energy proportion (Table 2; estimated by 7 d weighed intake, the following similar), as did those in NILS-LSA[62,63] and the INTERLIPID study[64,65] for Japanese men. The fish and shellfish intake and meat intake of the subjects in the present study were nearly normal too.

Compared with Europeans[66,67] and Americans[44,68–70], the subjects in the present study consumed smaller amounts of total energy, fat and meat, and larger amounts of fish and shellfish and *n*-3 fatty acids such as EPA and DHA, but had a similar intake of ARA, in their daily diet. Although it is _generally assumed that meat provides *n*-6 fatty acids and fish and shellfish *n*-3 fatty acids, the subjects of the present study had almost the same intake of ARA as Europeans and Americans. One possible explanation for this discrepancy in findings concerning ARA intake is that fish and shellfish are comparatively common sources of ARA, next to yolk and meat, for Japanese.

Despite the variation among subjects in intake of fatty acids, especially EPA and DHA, ARA content in serum/plasma phospholipids at baseline in the present study was similar to pretreatment levels in healthy men in the USA[46,69], patients with cirrhosis in Italy[47] and healthy vegetarians in Germany[71]. Even at baseline in the present study, EPA and DHA contents of serum/plasma phospholipids were at the levels obtained several weeks after change to a fish diet[68,69] or supplementation of fish oil[72], DHA[71] or both of them[67,73] in previous studies in non-Japanese individuals.

The ARA concentration of serum phospholipids was increased by about 4 % 2 weeks after initiation of ARA supplementation, and this increase was still present 4 weeks later. It is possible that ARA concentration had already reached maximal level after 2 weeks of supplementation. No studies have demonstrated the existence of a maximal ARA concentration in plasma phospholipids, or that a duration of less than 2 weeks is required to reach the maximal level of ARA after initiation of supplementation of ARA using ARA-enriched TAG. In the ARA group, significant reductions (approximately 3 %) in 18 : 2*n*-6 were observed after 2 and 4 weeks of treatment as well as after a 4-week washout, compared with baseline. Most of the increase in ARA concentration (which was about 4 %) can be explained by the

decrease in 18 : 2*n*-6 (approximately 3 %). The proportions of increase in ARA and reduction of 18 : 2*n*-6 by ARA supplementation in the present study were consistent with the results obtained in previous ARA supplementation studies using ARA-enriched TAG in healthy subjects[46] and patients with liver cirrhosis[47]. Notably, EPA and DHA concentrations in plasma phospholipids did not change with ARA supplementation in the present study, although decreases in ARA concentration in plasma or serum have been reported in various subjects with a fish diet or undergoing EPA and DHA supplementation[68,71]. The present findings suggest the existence of multiple mechanisms for preservation of ARA, EPA and DHA in blood.

The relationship between fatty acid intake and platelet aggregation has been examined in interventional studies with healthy subjects. It has been reported that dietary fatty acid composition, i.e. of SFA, MUFA, *n*-6 and *n*-3, had a significant effect on ADP- and collagen-induced platelet aggregation[69]. Several studies have found that platelet aggregation decreased with consumption of large or normal amounts of salmon[68], fish oil[74] or *n*-3 fatty acid[75]. In a study of four subjects for whom ARA supplementation significantly decreased the threshold concentration of platelet aggregation by ADP, ethyl arachidonate was used in free and not TAG form, and an excessively large dose, 6 g/d, was used for supplementation[76]. In patients with cirrhosis who had reduced platelet aggregation, addition of 2 g/d of this agent improved platelet aggregation[47].

Addition of 1·5 g ARA-enriched TAG/d for 50 d induced no significant physiological changes in the blood coagulation and platelet aggregation systems compared with placebo treatment in the healthy American[45]. Although in that study they did not particularly mention intakes of EPA and DHA[44], the levels of their intakes were similar to those of general European and American people. In addition, the degree of increase in plasma ARA was approximately the same as in our present study. Therefore, it may be possible to say that the fact the serum/plasma ARA elevation by supplementation does not affect platelet aggregation can be observed in not only the Japanese but also in Westerners. Of course, further study in Western countries must be performed to make the hypothesis clear.

Consumption of an appropriate variety and quantity of nutrients in the diet is important for living a healthy, long life, and habitual fish intake has been considered one of the causes of Japanese longevity. Improvement in sanitary conditions and medical advances have extended average life expectancy, especially in metropolitan areas. As a result, the number of elderly individuals unable to eat well by themselves, e.g. due to light eating or illness, is increasing. It is important to determine not only which nutrients are essential for maintaining life but also those producing a healthy, long life. The importance of supplementation of ARA and DHA, both of which are essential fatty acids, must be determined, since it has been found that most of the ARA, EPA and DHA in blood is obtained from food and that only a part of it is synthesized from linoleic acid and α-linolenic acid in the human body[27–31].

Further studies are needed to determine the effects of supplementation of ARA in nutritional products beyond infant milk in healthy subjects with or without a fish diet. Changes in fatty acid composition, not only in serum and plasma but also in erythrocyte membranes and platelets, following ARA supplementation need to be determined. The 24 h excretion of 2,3-dinor-6-keto prostaglandin F1α and 11-dehydro-thromboxane B2, which are metabolites of ARA, should be measured. In addition, the changes that occur in fatty acid composition in serum and plasma within 2 weeks of initiation of ARA supplementation and whether platelet aggregation is altered after more than 4 weeks of supplementation should be determined.

In conclusion, no subjects discontinued participation in the study due to adverse events resulting from supplementation of ARA. No clinically significant changes in the results of physical examination or vital signs occurred in either group in the present study. It was shown that supplementation of ARA-enriched TAG oil obtained from *Mortierella alpina* safely increased ARA concentration in serum phospholipids without decreasing EPA or DHA concentrations in healthy Japanese men with daily, moderate intake of fish in their diet.

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