Lipid Peroxidation During n-3 Fatty Acid and Vitamin E Supplementation in Humans

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ABSTRACT: The purpose of this study was to investigate in healthy humans the effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake, alone or in combination with dL-α-tocopherol acetate (vitamin E) supplements on lipid peroxidation. Eighty men were randomly assigned in a double-blind fashion to take daily for 6 wk either menhaden oil (6.26 g, n-3 fatty acids) or olive oil supplements with either vitamin E (900 IU) or its placebo. Antioxidant vitamins, phospholipid composition, malondialdehyde (MDA), and lipid peroxides were measured in the plasma at baseline and week 6. At the same time, breath alkane output was measured. Plasma α-tocopherol concentration increased in those receiving vitamin E ($P < 0.0001$). In those supplemented with n-3 fatty acids, EPA and DHA increased in plasma phospholipids ($P < 0.0001$) and plasma MDA and lipid peroxides increased ($P < 0.001$ and $P < 0.05$, respectively). Breath alkane output did not change significantly and vitamin E intake did not prevent the increase in lipid peroxidation during menhaden oil supplementation. The results demonstrate that supplementing the diet with n-3 fatty acids resulted in an increase in lipid peroxidation, as measured by plasma MDA release and lipid peroxide products, which was not suppressed by vitamin E supplementation.

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Fish oils, the most common of which are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown in humans to lower plasma triglycerides (1), to be vasodilatory, to prolong bleeding time (2), and to decrease fibrinogen concentrations (3): all recognized as risk factors for the development of cardiovascular disease. Experimental studies have indicated that the most likely mechanism for the biochemical effects of fish oil relates to the competitive interaction between arachidonic acid (AA) and EPA for incorporation into cellular phospholipids and their further metabolism to eicosanoid products (4).

Fish oils are also highly unsaturated and readily susceptible to peroxidation (5). Peroxidation will give rise to lipid peroxides including malondialdehyde (MDA) and aldehyde breakdown products which have been implicated in the etiology of a number of diseases in humans including cardiovascular disease (6). Vitamin E, because of its antioxidant properties, may reduce the peroxidative damage caused by fish oils and thereby promote its beneficial effects. Fish oil supplements usually contain a small amount of vitamin E as an antioxidant (1 IU), but whether this is sufficient to achieve any beneficial effect is questionable (7).

The purpose of this study was to investigate in humans, in a randomized, double-blind, placebo-controlled fashion, the effect of n-3 fatty acid supplementation, alone or in combination with dL-α-tocopherol acetate (vitamin E), on different lipid peroxidation indices.

MATERIALS AND METHODS

Subjects and protocol. Eighty healthy men (20–60 yr) were recruited, primarily hospital staff and local area workers (social middle class). Informed consent was obtained, and the study protocol was approved by the Toronto Hospital Committee for Research on Human Subjects. Subjects were screened and excluded if they were smokers, had a medical condition (i.e., cancer, heart disease, hypertension, hyperlipidemia, diabetes, obesity, kidney/liver dysfunction, gastrointestinal abnormality, HIV+), had a high alcohol consumption (>30 g/d), or if any vitamin or medications had been taken in the past 4 wk.

The volunteers were also interviewed by a dietitian to ensure that they consumed a diet similar to a typical North American diet (30–40% of energy from fat and providing a polyunsaturated-to-saturated-fatty-acid (PUFA/SFA) ratio of 0.3:1. They were further instructed to avoid all types of fish (i.e., canned, frozen, fresh including shellfish) for the duration of the study and to avoid strenuous exercise.

The subjects returned 2 wk later and were randomized (using random number tables) in a double-blind fashion to supplement their usual diet for 6 wk with either menhaden oil

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Abbreviations: AA, Arachidonic acid; BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LA, linoleic acid; LDL, low density lipoprotein; MDA, malondialdehyde; OA, oleic acid; PUFA, polyunsaturated fatty acid; Se-GSHPx, selenium-dependent glutathione peroxidase; TBA, thiobarbituric acid; TBHQ, *tert*-butylhydroquinone.

ethyl esters with *tert*-butylhydroquinone (TBHQ: 0.017%) (8 caps/d = EPA 3.062 g, DHA 2.262 g, total n-3: 6.26 g) (National Marine Fisheries Service, Charleston, SC) or olive oil ethyl esters with TBHQ (0.01%) and fish flavoring (8 caps/d = 6.08 g monoenes) with dL-α-tocopheryl acetate (vitamin E; Hoffmann-La Roche Ltd., Basel, Switzerland) (3 caps/d = 900 IU) or its placebo. Subjects were therefore randomized into one of four supplemented groups: menhaden oil + vitamin E; menhaden oil + vitamin E placebo; olive oil + vitamin E; olive oil + vitamin E placebo.

Breath collections and biochemical measurements were performed in the morning, in the fasted state, at baseline (week 0) and at week 6 of supplementation. Blood was collected in tubes containing lithium heparinate for phospholipids; 0.1% EDTA for tocopherols, β-carotene, ascorbic acid, MDA release, and lipid peroxides; additive-free tubes for cholesterol and triglycerides; and trace element-free for selenium-dependent glutathione peroxidase (Se-GSHPx). Samples were centrifuged promptly at 3000 rpm for 10 min. The plasma was removed, aliquoted separately for each assay, and frozen (−70°C) until analysis. For phospholipid analysis, plasma was stored in chloroform containing 0.02% butylated hydroxytoluene (BHT) as an antioxidant, in an oxygen-free environment at −70°C. Plasma for the vitamin C assay was stabilized immediately with 50 g/L metaphosphoric acid (HPO_3) (0.5 mL plasma plus 4.5 mL HPO_3).

Fatty acid analysis. Total lipids were extracted with chloroform/methanol (2:1, vol/vol), containing BHT as an antioxidant (8) and dried under nitrogen gas. The residue then dissolved in 0.1 mL chloroform and applied to silica gel thin-layer plates (Whatman Silica Gel60A K6F, 20 × 20 cm; Interscience Biotechnology, Markham, Ontario, Canada) to separate the phospholipids using a nonpolar solvent system (hexane/diethyl ether/acetic acid; 80:20:1). Phospholipid bands were scraped and transmethylated with boron trifluoride–methanol at 90°C for 30 min. Fatty acid methyl esters were separated on a fused-silica capillary column coated with a 25 μm cyanopropylphenyl film (Durabond 23, 30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA) in a Hewlett-Packard (HP) 5890A gas–liquid chromatograph (Palo Alto, CA) equipped with a flame-ionization detector (9).

Vitamin analyses. Tocopherols and β-carotene were analyzed using reverse-phase high-performance liquid chromatography (HPLC) and fluorescence spectrophotometry according to the method of Hess *et al.* (10). In this method, the samples are deproteinized by adding 500 μL of ethanol. The samples are then extracted by *n*-hexane and evaporated to dryness at ambient temperature under reduced pressure. The residue is dissolved in 100 μL ethanol/dioxane (1:1) and then 150 μL of acetonitrile is added. The sample extract (100 μL) is then injected into a reverse-phase HPLC. Ascorbic acid was analyzed fluorimetrically by the method of Brubacher Vuilleumier (11). The vitamin concentrations were measured in coded samples by the vitamin research laboratories of F. Hoffmann-La Roche Ltd. (Basel, Switzerland).

Breath analysis. Breath samples were collected and analyzed for ethane and pentane as described previously (12). Briefly, subjects are first required to breathe hydrocarbon-free air for 4 min to wash contaminating hydrocarbons from their lungs. Subsequently, expired air is collected for 2 min and analyzed by gas chromatography (Shimadzu 6-AM GC; Shimadzu Seisgkusho Ltd., Kyoto, Japan) using a Porasil D column (Chromatographic Specialties Inc., Brockville, Canada).

Plasma lipid peroxide analysis. MDA determination was measured by the thiobarbituric acid (TBA) method described by Draper *et al*. (13). Plasma proteins were denatured using 10% trichloroacetic acid solution and adding 0.05% BHT solution prior to digestion on a heating block. After centrifugation, the supernatant was combined with the TBA solution (1:1, vol/vol ratio) and heated. The reaction mixture was then extracted with 1 mL of *n*-butanol. An aliquot of the extract was mixed (2:1:1) with methanol and a mobile phase (15% acetonitrile and 0.6% tetrahydrofuran in 5 mM phosphate buffer) and 20 μL was injected onto a reverse-phase C_{18} μBondpak HPLC column. The standard solution used was 1,1,3,3-tetraethoxy propane (Sigma Chemical Co., St. Louis, MO).

Plasma lipid hydroperoxides were quantitated by calorimetry at 675 nm, measuring the methylene blue formation. Commercial kits (Kamiya Biochemicals Determiner LPO kit, Thousand Oaks, CA) were used.

Se-GSHPx enzyme activity was measured by the coupled enzyme assay (14,15). Changes in absorbance at 340 nm were recorded for 5 min on a spectrophotometer (U 2000 Hitachi Ltd., Tokyo, Japan). Se-GSHPx activity was expressed as μmoles NADPH oxidized $min^{-1} \cdot$ mg $protein^{-1}$ which was measured by the biuret method (16).

Supplement lipid peroxide analysis. A sample of menhaden and olive oil supplements also underwent peroxide analysis at 0 and 18 mon of storage to establish a peroxide value. A microtitrimetric method was used to determine all substances which oxidize potassium oxide in terms of milliequivalents of peroxide per 100 g of sample (17). This analysis was performed by the research laboratories of the National Marine Fisheries Service (Charleston, SC).

Plasma lipid analysis. Serum cholesterol, high density lipoprotein (HDL)-cholesterol, and triglyceride were analyzed using a commercially available kit (Boehringer Mannheim, Montreal, Canada). Low density lipoprotein (LDL)-cholesterol was calculated (cholesterol − (HDL-cholesterol + 0.46 × triglyceride).

Statistical analyses. It was estimated that 20 subjects were required in each group, assuming a dropout rate of 15%, to achieve 80% power using a 5% significance level for detecting at least a 10% difference in lipid peroxidation measured by breath ethane output between groups. All group data are expressed as means ± SEM. The study was a factorial design with the two factors being fish oil and vitamin E. The interaction between fish oil and vitamin E was hypothesized prior to the start of the study such that fish oil would be expected to have an effect on lipid peroxidation, which would not be evident with vitamin E. The comparison of pretreatment and

posttreatment tests was undertaken by an analysis of covariance on the change between baseline (week 0) and week 6 with an adjustment for the week 0 value (18). SAS (19) was used for these analyses.

RESULTS

Of the 80 subjects enrolled, 5 voluntarily withdrew from the trial due to noncompliance and another 3 withdrew because of unpleasant side effects of the therapy. These 3 subjects, all receiving n-3 fatty acid supplements, complained of fishy taste, belching, bloating, heartburn, and nausea. No other significant side effects were reported. Compliance, assessed by plasma α-tocopherol and fatty acids, was very good (Tables 1,2).

Plasma fatty acids and lipids. The fatty acids EPA, DHA, AA, linoleic acid (LA), and oleic acid (OA), expressed as percentage composition of phospholipids, were similar between groups at baseline (Table 2). Phospholipid content of DHA and EPA ($P < 0.001$) increased, and AA and LA composition decreased significantly and to the same extent ($P < 0.025$) in both groups of subjects receiving n-3 fatty acid supplementation. OA increased significantly ($P < 0.025$) and to the same extent in both groups receiving olive oil supplementation.

Serum total triglycerides and cholesterol (HDL-, LDL-cholesterol) showed no significant change (data not shown).

Plasma vitamin concentrations. Plasma α-tocopherol concentrations were comparable between groups at baseline (Table 1). The increase in α-tocopherol in the two vitamin E-supplemented groups ($P < 0.0001$) was significant compared to the two vitamin E placebo groups. Plasma ascorbic acid and β-carotene did not change in any groups during supplementation (Table 1), suggesting that there were no dietary changes during the study.

Lipid peroxidation products in menhaden and olive oil supplements. The peroxide value was 3.27 meq/kg for the menhaden oil supplements and 2.3 meq/kg for the olive oil supplements. An 18-mon storage stability study performed by the manufacturer of the supplements showed no effect of time stored at 5°C on the peroxide value.

Lipid peroxidation measurements in subjects. Breath ethane output was comparable between groups at baseline (Table 3). Supplementation of n-3 fatty acids either with or without vitamin E did not produce any significant changes in breath ethane output. The mean difference in the change of breath ethane output during menhaden oil supplementation between those subjects receiving vitamin E and those not receiving vitamin E supplementation was -1.0 pmol $\cdot$ kg^{-1} $\cdot$ min^{-1} (95% confidence interval, −7.2, 5.2). Breath pentane output did not differ significantly between groups at baseline, and neither n-3 fatty acid nor vitamin E supplementation produced a significant change from initial levels.

Baseline measurements of plasma MDA equivalents, lipid peroxides, and Se-GSHPx were not significantly different between groups (Table 3). In the two groups supplemented with n-3 fatty acids, plasma MDA generation ($P < 0.001$) and lipid peroxides ($P < 0.05$) were significantly increased at 6 wk. Vitamin E intake added to menhaden oil supplements had no effect on MDA or lipid peroxide values. Olive oil supplemented with vitamin E or placebo produced no change in MDA or lipid peroxide levels. Neither n-3 fatty acids nor vitamin E supplementation produced any change in Se-GSHPx activity.

TABLE 1
Plasma Antioxidant Vitamins

	Groups			
	Menhaden oil + vitamin E	Menhaden oil + placebo	Olive oil + vitamin E	Olive oil + placebo
Number	18	17	19	18
Age (yr)	33 ± 2	33 ± 3	32 ± 2	31 ± 2
Initial body mass index	24.7 ± 0.5	25.0 ± 1.1	23.3 ± 0.4	25.4 ± 0.7
α-Tocopherol (μmol/L)				
Week 0	21.3 ± 0.8	21.5 ± 1.0	24.4 ± 0.7	23.6 ± 1.6
Week 6	43.3 ± 2.6	21.4 ± 1.1	52.5 ± 3.4	25.0 ± 1.4
Change (%)	102.7 ± 8.1[a]	−0.3 ± 2.5	115.1 ± 12.9[a]	7.4 ± 2.4
γ-Tocopherol (μmol/L)				
Week 0	3.14 ± 0.24	3.28 ± 0.33	2.83 ± 0.19	2.95 ± 0.28
Week 6	0.82 ± 0.10	2.83 ± 0.20	0.89 ± 0.12	3.12 ± 0.27
Change (%)	−73.4 ± 2.6[a]	−5.6 ± 7.4	−67.3 ± 5.1[a]	7.9 ± 12.0
β-Carotene (μmol/L)				
Week 0	0.43 ± 0.05	0.38 ± 0.04	0.45 ± 0.05	0.38 ± 0.06
Week 6	0.39 ± 0.05	0.42 ± 0.07	0.42 ± 0.04	0.40 ± 0.08
Change (%)	−6.2 ± 5.3	10.1 ± 6.9	−3.2 ± 5.1	5.1 ± 4.9
Ascorbic acid (μmol/L)				
Week 0	26.0 ± 2.7	29.8 ± 2.5	29.8 ± 3.0	25.1 ± 1.9
Week 6	24.9 ± 2.6	27.0 ± 3.0	28.0 ± 3.6	27.9 ± 3.5
Change (%)	−4.0 ± 6.1	−9.1 ± 7.9	−5.4 ± 7.2	11.1 ± 8.3

[a]$P < 0.0001$.

TABLE 2
Plasma Fatty Acid Composition of Phospholipids[a]

	Groups			
	Menhaden oil + vitamin E	Menhaden oil + placebo	Olive oil + vitamin E	Olive oil + placebo
Number	18	17	19	18
EPA (20:5n-3) (composition %)				
Week 0	0.99 ± 0.13	1.07 ± 0.12	1.13 ± 0.09	1.25 ± 0.14
Week 6	8.85 ± 0.56	8.31 ± 0.89	0.99 ± 0.08	1.15 ± 0.11
Change (%)	788.6 ± 83.1[b]	681.3 ± 90.1[b]	–25.2 ± 13.6	–22.9 ± 17.9
DHA (22:6n-3) (composition %)				
Week 0	3.65 ± 0.24	4.15 ± 0.42	3.95 ± 0.24	4.22 ± 0.38
Week 6	8.11 ± 0.26	8.32 ± 0.52	3.32 ± 0.10	3.70 ± 0.23
Change (%)	133.0 ± 12.5[b]	109.7 ± 13.9[b]	–12.6 ± 3.7	–2.1 ± 8.0
AA (20:4n-6) (composition %)				
Week 0	14.23 ± 0.71	13.22 ± 0.70	14.45 ± 0.55	12.66 ± 0.48
Week 6	11.02 ± 0.58	11.13 ± 0.46	13.67 ± 0.49	12.25 ± 0.40
Change (%)	–21.6 ± 3.2[c]	–13.3 ± 4.6[c]	–3.6 ± 4.0	–1.6 ± 4.0
LA (18:2n-6) (composition %)				
Week 0	25.84 ± 0.75	25.84 ± 0.87	23.99 ± 0.65	25.59 ± 1.04
Week 6	18.56 ± 0.65	20.39 ± 0.95	24.15 ± 0.68	24.67 ± 0.85
Change (%)	–27.6 ± 2.7[c]	–20.2 ± 4.2[c]	1.1 ± 2.1	–2.5 ± 3.2
OA (18:1n-9) (composition %)				
Week 0	11.86 ± 0.78	11.13 ± 0.40	11.02 ± 0.41	11.01 ± 0.38
Week 6	10.35 ± 0.58	10.12 ± 0.46	12.39 ± 0.38	12.11 ± 0.37
Change (%)	–11.9 ± 1.6	–8.0 ± 4.8	14.4 ± 4.7[c]	10.7 ± 3.4[c]

[a]Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; LA, linoleic acid; OA, oleic acid; [b]$P < 0.000$; [c]$P < 0.025$.

DISCUSSION

The results of this randomized, double-blind, placebo-controlled study showed that n-3 fatty acid supplementation increased lipid peroxidation based on plasma lipid peroxides and MDA. Menhaden oil intake had no effect on breath alkane output. High doses of vitamin E did not provide adequate antioxidant protection as the lipid peroxidation products remained elevated.

The choice of dosage (6 g of n-3 fatty acids daily) used in this trial was based on studies investigating the effect of fish oil suppplementation on cardiovascular risk factors and lipid peroxidation parameters (20–23). A dose-response curve study (20) showed that in healthy humans 6 g of n-3 fatty acids had a beneficial effect on plasma phospholipid composition and triglyceride levels. However, 6 g had no effect on functional parameters such as blood pressure, bleeding time, erythrocyte deformability, or neutrophil function. For this reason, we did not measure functional parameters in addition to lipid peroxidation. Since our study's main endpoint was lipid peroxidation, 6 g a day of n-3 fatty acids was chosen on the basis of previous reports showing an increase in lipid peroxidation (22,23).

The amount of vitamin E supplementation used was also based on previous literature. Despite 400 IU of vitamin E, some investigators have found that 2.0 g EPA and 1.3 g DHA daily can increase plasma lipid peroxides and MDA (24,25). Our own studies in smokers, an oxidatively stressed population, demonstrated that vitamin E, 800 IU per day, had a significant effect in reducing lipid peroxidation (26). Thus a dose in similar range should have had an effect on lipid peroxidation during n-3 fatty acids supplementation. Data from the literature also indicate that for each gram of linoleic acid in the diet, a minimum additional requirement of 0.5 mg of α-tocopherol is desirable (27) to prevent lipid peroxidation. Based on Muggli's equation (27), 2.24 and 2.68 mg of supplementary dL-α tocopherol is needed for each gram of dietary EPA and DHA, respectively. Thus, the amount of α-tocopherol supplementation in our study should have been between 10 and 15 IU per day, a far lower dose than what we used.

The increase in plasma MDA and lipid peroxide levels observed during fish oil supplementation suggests that the antioxidant level, whether in the plasma or in the capsules, was not sufficient to protect against free radical generation. Ingestion of lipid peroxide products from the capsules would explain the persistent elevation in these lipid peroxidation indices despite vitamin E supplementation. This could also explain the lack of induction of Se-GSHPx enzyme activity since lipid peroxidation would have occurred *ex vivo*. However, in the menhaden and olive oil supplements, the peroxide value measuring the oxidation was within acceptable limits and was comparable between the two. To prevent oxidative deterioration of n-3 fatty acids, the American Institute of Nutrition recommends the addition of a synthetic antioxidant at a level of 0.02% (by weight of oil) to experimental diet containing polyunsaturated fatty acids (PUFA) (28) which was the amount of TBHQ added to our supplements. How-

TABLE 3
Lipid Peroxidation Indices[a]

	Groups			
	Menhaden oil + vitamin E	Menhaden oil + placebo	Olive oil + vitamin E	Olive oil + placebo
Number	18	17	19	18
Breath ethane output (pmol · kg^{-1} · min^{-1})				
Week 0	12.2 ± 1.4	11.0 ± 1.0	11.3 ± 1.0	11.2 ± 1.1
Week 6	10.3 ± 1.4	11.7 ± 1.5	12.5 ± 1.7	12.8 ± 1.6
Change (%)	−15.5 ± 13.8	5.3 ± 12.1	7.1 ± 10.9	15.1 ± 19.3
Breath pentane output (pmol · kg^{-1} · min^{-1})				
Week 0	6.3 ± 1.2	5.3 ± 0.7	5.1 ± 0.6	7.5 ± 1.6
Week 6	5.4 ± 0.8	5.8 ± 1.0	4.6 ± 0.5	5.5 ± 1.1
Change (%)	−14.3 ± 12.2	9.4 ± 14.6	−9.3 ± 10.8	−16.7 ± 19.8
MDA equivalent (nmol/mL)				
Week 0	1.87 ± 0.15	1.91 ± 0.13	1.91 ± 0.12	1.81 ± 0.16
Week 6	3.13 ± 0.38	3.06 ± 0.32	1.92 ± 0.13	1.59 ± 0.13
Change (%)	67.3 ± 15.3[b]	60.7 ± 15.9[b]	2.6 ± 2.2	−5.8 ± 5.2
Lipid peroxides (nmol/mL)				
Week 0	316.2 ± 17.4	274.0 ± 25.4	367.9 ± 19.2	346.6 ± 20.9
Week 6	407.0 ± 24.8	324.1 ± 17.5	359.8 ± 22.7	348.0 ± 24.2
Change (%)	34.6 ± 10.9[c]	21.5 ± 8.8[c]	2.6 ± 2.1	0.4 ± 1.0
Se–GSHPx (μmol NADPH · min^{-1} · mg $protein^{-1}$)				
Week 0	4.5 ± 0.2	4.7 ± 0.3	4.6 ± 0.3	4.4 ± 0.2
Week 6	5.2 ± 0.2	4.1 ± 0.2	4.5 ± 0.3	4.6 ± 0.3
Change (%)	15.1 ± 5.1	−14.6 ± 7.1	−1.9 ± 4.1	2.1 ± 7.8

[a]Abbreviations: Se-GSHPx, selenium-dependent glutathione peroxidase; MDA, malondialdehyde; [b]$P < 0.001$; [c]$P < 0.05$.

ever, a recent study by Gonzalez *et al*. (29) suggested that this may not be sufficient to totally prevent oxidative deterioration, at least in animal diets high in fish oil. In that study, the addition of even 100 times this level (2% TBHQ), although decreasing the level of oxidation products, failed to totally prevent lipid peroxidation. These experiments were performed with purified animal diets and may not be applicable to the supplements used in our study. However, although the peroxide values measured in our supplements did not increase when tested, storage condition may not have been optimal once the supplements were given to the subjects. This could have induced lipid peroxidation within the supplements. Whether ingestion of lipid peroxidation products could have significantly increased plasma levels in humans is, however, not clear. Animal studies (29) have shown a significant increase in thiobarbituric acid reactive substances (TBARS) concentrations of different organs and whole body when mice were fed fish oil diet without antioxidants. The addition of high levels of antioxidants to the diet actually reduced these levels.

The increase in plasma MDA and lipid peroxides due to n-3 fatty acid supplementation with or without vitamin E intake was reported in small studies. Some reported that high intakes of fish oil concentrate led to increased TBARS irrespective of the vitamin E intake (24) while others reported a decrease in TBARS after fish oil supplementation with additional vitamin E (25). Both studies compared end point values to baseline and were not randomized. Another study (7) investigated the effect of long-term fish oil supplementation in women. There was a significant increase in plasma lipid peroxides while plasma vitamin E concentrations did not change. Similar results for plasma vitamin E levels were found in males (30). A double-blind, crossover study (31), conducted in 12 healthy volunteers, showed a decrease in plasma vitamin E and an increase in MDA with fish oil supplemented with 0.3 IU/g of vitamin E while levels of both plasma vitamin E and MDA remained normal with 1.5 IU/g. Peroxide values of both oils remain similar during storage, suggesting that lipid peroxidation occurred *in vivo*. Other studies in animals showed an increase in lipid peroxidation along with a reduction in antioxidant defense systems (32–35). Possible effects from lipid peroxidation products within the food cannot be completely ruled out (29,36).

No change in breath ethane output was detected in this study. Breath ethane output has received less attention in human studies as a measure of lipid peroxidation, likely because pentane represents the majority of PUFA (n-6) in the body (37). However, breath ethane output is a well-recognized index of lipid peroxidation (38–40) and has been reported to be elevated in smokers (41) and children with liver disease (42). But to our knowledge, other then the present report, there are no human studies published on dietary intake of PUFA and breath alkane output. On the other hand, in animals, increased production of ethane was demonstrated with high intake of n-3 fatty acids from cod liver oil (43). In our study with humans, this effect could not be demonstrated with menhaden oil supplementation. More extreme changes in n-3 vs. n-6 fatty acid intake may have been necessary in order to detect a change in ethane output. We attempted to control for

other factors known to influence breath alkane output such as the PUFA/saturated fatty acid ratio of the diet (39), exercise levels (44), and alcohol consumption (45,46) in order to reduce the inter- and intrasubject variability. However, unlike animal studies (43) done in a laboratory environment, this study was not performed in a clinical investigation unit, and thus strict control of these variables was not possible.

Vitamin C and β-carotene were measured in the plasma to ensure that concentrations remained stable throughout the study. Since these vitamins have an antioxidant effect, changes occurring because of sudden increase in intake could have influenced our results.

In conclusion, this study has shown that supplementing the diet with 6 g of n-3 fatty acids daily resulted in increased lipid peroxidation as measured by plasma MDA equivalents and lipid peroxides. However, breath ethane output did not increase and, in humans, may not be a method sensitive enough to measure lipid peroxidation due to changes in PUFA intake. Vitamin E supplementation (900 IU/d) did not protect against lipid peroxidation during n-3 fatty acid supplementation. The possibility of increased lipid peroxide products within the capsules, while stored by the subjects, could not be excluded.

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REFERENCES

1. Harris, W.S. (1989) Fish Oil and Plasma Lipid and Lipoprotein Metabolism in Humans: A Critical Review, *J. Lipid Res. 30,* 787–807.
2. Terano, T., Hirai, A., and Hamazaki, T. (1983) Effect of Oral Administration of Highly Purified Eicosapentaenoic Acid on Platelet Function, Blood Viscosity and Red Cell Deformity in Healthy Human Subjects, *Atherosclerosis 46,* 321–331.
3. Flaten, H., Hostmark, A.T., Kierulf, P., Lystad, E., Trygg, K., Bjerkedel, T., and Osland, A. (1990) Fish Oil Concentrate: Effects on Variables Related to Cardiovascular Disease, *Am. J. Clin. Nutr. 52,* 300–306.
4. Dyerberg, J., Bang, H.O., Stoffersen, E., Moncada, S., and Vane, J.E. (1978) Eicosapentaenoic Acid and Prevention of Thrombosis and Atherosclerosis? *Lancet 2,* 117–119.
5. Piche, L.A., Draper, H.H., and Cole, P.D. (1988) Malondialdehyde Excretion by Subjects Consuming Cod Liver Oil vs. a Concentrate of n-3 Fatty Acids, *Lipids 23,* 370–371.
6. Yagi, K. (1987) Lipid Peroxidation and Human Diseases, *Chem. Phys. Lipids 45,* 337–351.
7. Meydani, M., Natiello, F., Goldin, B., Free, N., Woods, M., Schaefer, E., Blumberg, J.B., and Gorbach, S.L. (1991) Effect of Long-Term Fish Oil Supplementation on Vitamin E Status and Lipid Peroxidation in Women, *J. Nutr. 121,* 484–491.
8. Folch, J., Lees, M., and Stanley, G.H.S. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissue, *J. Biol. Chem. 226,* 497–509.
9. Cunnane, S.C. (1988) The Profile of Long Chain Fatty Acids in Serum Phospholipids: A Possible Indicator of Copper Status in Humans, *Am. J. Clin. Nutr. 48,* 1475–1478.
10. Hess, D., Keller, H.E., Oberlin, B., Bonfanti, R., and Schuep, W. (1991) Simultaneous Determination of Retinol, Tocopherols, Carotenes and Lycopene in Plasma by Means of High-Performance Liquid Chromatography on Reversed Phase, *Internat. J. Vit. Nutr. Res. 61,* 232–238.
11. Brubacher, G., and Vuilleumier, J.P. (1974) Vitamin E, in *Clinical Biochemistry: Principles and Methods* (Curtius, H.C., and Roth, M., eds.) pp. 989–997, Walter de Gruyter, New York.
12. Lemoyne, M., Van Gossum, A., Kurian, R., Ostro, M., and Jeejeebhoy, K.N. (1987) Breath Pentane Analysis as an Index of Lipid Peroxidation: A Functional Test of Vitamin E Status, *Am. J. Clin. Nutr. 46,* 267–272.
13. Draper, H.H., Squires, E.J., Mahmoodi, H., Wu, J., Agarwal, S., and Hadley, M. (1993) A Comparative Evaluation of Thiobarbituric Acid Methods for the Determination of Malondialdehyde in Biological Materials, *Free Radical Biol. Med. 15,* 353–363.
14. Paglia, D.C., and Valentine, W.N. (1961) Studies on the Quantitative and Qualitative Characterization of Erythrocyte Glutathione Peroxidase, *J. Lab. Clin. Med. 70,* 158–169.
15. Lawrence, R.A., and Burk, R.F. (1976) Glutathione Peroxidase Activity in Selenium Deficient Rat Liver, *Biochem. Biophys. Res. Commun. 71,* 952–953.
16. Grant, G.H., Silverman, L.M., and Christenson, R.H. (1976) Amino Acids and Proteins, in *Fundamentals of Clincial Chemistry* (Tietz, N.W., ed.) 3rd edn., pp. 291–342, W.B. Saunders Co., Philadelphia.
17. Seaborn, G.T., Gooch, J.A., Van Dolah, F.M., and Galloway, S.B. (1977) *Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oil,* 2nd edn., U.S. Department of Commerce, Charleston, 123 pp., NOAA Tech Mem., NMFS-SEFC-329.
18. Laird, N. (1983) Further Comparative Analysis of Pretest–Posttest Research Designs, *The American Statistician 37,* 329–330.
19. *SAS/STAT User's Guide Release 6.03 Edition* (1988) SAS Institute Inc., Cary, NC, p. 549.
20. Blonk, M.C., Bilo, H.J.G., Nanta, J.J.P., and Popp-Snijders, C. (1990) Dose-Response Effects of Fish-Oil Supplementation in Healthy Volunteers, *Am. J. Clin. Nutr. 52,* 120–127.
21. Kaare, H.B., Bjerve, K.S., Straume, B., Gram, I.T., and Thelle, D. (1990) Effect of Eicosapentaenoic and Docosahexaenoic Acids on Blood Pressure in Hypertension, *N. Engl. J. Med. 322,* 795–801
22. Meydani, M., Natiello, F., Goldin, B., Free, N., Woods, M., Schaefer, E., Blumberg, J.B., and Gorbach, S.L. (1991) Effect of Long-Term Fish Oil Supplementation on Vitamin E Status and Lipid Peroxidation in Women, *J. Nutr. 121,* 484–491
23. Kinsella, J.E. (1991) Dietary n-3 Polyunsaturated Fatty Acids of Fish Oils, Autoxidation *ex vivo* and Peroxidation *in vivo*: Implications (Review), *Advan. Exper. Med. Biol. 289,* 255–268.
24. Brown, J.E., and Wahle, K.W.J. (1990) Effect of Fish-Oil and Vitamin E Supplementation on Lipid Peroxidation and Whole-Blood Aggregation in Man, *Clin. Chim. Acta 193,* 147–156.
25. Harats, D., Dabach, Y., Hollander, G., Ben-Naim, M., Schwartz, R., Berry, E.M., Stein, O., and Stein, Y. (1991) Fish Oil Ingestion in Smokers and Nonsmokers Enhances Peroxidation of Plasma Lipoproteins, *Atherosclerosis 90,* 127–139.
26. Hoshino, E., Shariff, R., Van Gossum, A., Allard, J., Pichard, C., Kurian, R., and Jeejeebhoy, K.N. (1990) Vitamin E Suppresses Increased Lipid Peroxidation in Cigarette Smokers. *J. Parent. Ent. Nutr. 14,* 300–305.
27. Muggli, R. (1989) Dietary Fish Oils Increase the Requirement for Vitamin E in Humans, in *Health Effects of Fish and Fish Oils,* Chandra, R.K. (ed.) ARTS Biomedical Publishers & Distributors, St. John's, Newfoundland.
28. American Institute of Nutrition (1980) Second Report of the *ad hoc* Committee on Standards of Nutritional Studies, *J. Nutr. 110,* 1726.
29. Gonzalez, M.J., Gray, J.I., Schemmel, R.A., Dugan, L., and Welsch, C.W. (1992) Lipid Peroxidation Products Are Elevated

in Fish Oil Diets in the Presence of Added Antioxidants, *J. Nutr. 122,* 2190–2195.
30. Sanders, T.A.B., and Hinds, A. (1992) The Influence of a Fish Oil High in Docosahexaenoic Acid on Plasma Lipoprotein and Vitamin E Concentrations and Haemostatic Function in Healthy Male Volunteers, *Brit. J. Nutr. 68,* 163–173.
31. Haglund, O., Luostarinen, R., Wallin, R., Wibell, L., and Saldeen, T. (1991) The Effects of Fish Oil on Triglycerides, Cholesterol, Fibrinogen and Malondialdehyde in Humans Supplemented with Vitamin E, *J. Nutr. 121,* 165–169.
32. Saito, M., and Nakatsugawa, K. (1994) Increased Susceptibility of Liver to Lipid Peroxidation After Ingestion of a High Fish Oil Diet, *Internat. J. Vit. Nutr. Res. 64,* 144–151.
33. Farwer, S.R. (1994) The Vitamin E Nutritional Status of Rats Fed on Diets High in Fish Oil, Linseed Oil or Sunflower Seed Oil, *Brit. J. Nutr. 72,* 127–145.
34. L'Abee, M.R., Trick, K.D., and Beare-Rogers, J.L. (1991) Dietary (n-3) Fatty Acids Affect Rat Heart, Liver and Aorta Protective Enzyme Activities and Lipid Peroxidation, *J. Nutr. 121,* 1331–1340.
35. Javouhey-Donzel, A., Guenot, L., Maupoil, V., Rochette, L., and Rocquelin, G. (1993) Rat Vitamin E Status and Heart Lipid Peroxidation: Effect of Dietary Alpha-Linolenic Acid and Marine n-3 Fatty Acids, *Lipids 28,* 651–655.
36. Fritsche, K.L., and Johnston, P.V. (1988) Rapid Autooxidation of Fish Oil in Diets Without Added Antioxidants, *J. Nutr. 118,* 425–426.
37. Wade, C.R., and Van Rij, A.M. (1985) *In vivo* Lipid Peroxidation in Man as Measured by the Respiratory Excretion of Ethane, Pentane, and Other Low-Molecular-Weight Hydrocarbons, *Ann. Biochem. 150,* 1–7.
38. Riely, C.A., Cohen, G., and Lieberman, M. (1974) Ethane Evolution: A New Index of Lipid Peroxidation, *Science 183,* 208–210
39. Pryor, W.A., and Godber, S.S. (1991) Noninvasive Measures of Oxidative Stress Status in Humans, *Free Radical Biol. Med. 10,* 177–184.
40. Lawrence, G.D., and Cohen, G. (1982) Ethane Exhalation as an Index of *in vivo* Lipid Peroxidation: Concentrating Ethane from a Breath Collection Chamber, *Anal. Biochem 122,* 283–290.
41. Sakamoto, M. (1985) Ethane Expiration Among Smokers and Non-Smokers, *Nippon Eiseigaku Zasshi (Jpn. J. Hyg.) 40,* 835–840.
42. Refat, M., Moore, T.J., Kazui, M., Risby, T.H., Perman, J.A., and Schwarz, K.B. (1991) Utility of Breath Ethane as a Noninvasive Biomarker of Vitamin E Status in Children, *Ped. Res. 30,* 396–403.
43. Hafeman, D.G., and Hoekstra, W.G. (1977) Lipid Peroxidation *in vivo* During Vitamin E and Selenium Deficiency in the Rat as Monitored by Ethane Evolution, *J. Nutr. 107,* 666–672.
44. Dillard, C.J., Litov, R.E., Savin, W.M., Dumelin, E.E., and Tappell, A.L. (1978) Effects of Exercise, Vitamin E and Ozone on Pulmonary Function and Lipid Peroxidation, *J. Appl. Physiol. 45,* 927–932.
45. Litov, R.E., Irving, D.H., Downey, J.E., and Tappel, A.L. (1978) Lipid Peroxidation: A Mechanism Involved in Acute Ethanol Toxicity as Demonstrated by *in vivo* Pentane Production in the Rat, *Lipids 13,* 305–307.
46. Litov, R.E., Gee, D.L., Downey, J.E., and Tappel, A.L. (1981) The Role of Peroxidation During Chronic and Acute Exposure to Ethanol as Determined by Pentane Expiration in the Rat, *Lipids 16,* 52–63.

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