

Supplementation of postmenopausal women with fish oil rich in eicosapentaenoic acid and docosahexaenoic acid is not associated with greater in vivo lipid peroxidation compared with oils rich in oleate and linoleate as assessed by plasma malondialdehyde and F₂-isoprostanes¹⁻³

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ABSTRACT

Background: Although the replacement of dietary saturated fat with unsaturated fat has been advocated to reduce the risk of cardiovascular disease, diets high in polyunsaturated fatty acids (PUFAs) could increase lipid peroxidation, potentially contributing to the pathology of atherosclerosis.

Objective: The objective of this study was to examine indexes of in vivo lipid peroxidation, including free F₂-isoprostanes, malondialdehyde (MDA), and thiobarbituric acid reacting substances (TBARS), in the plasma of postmenopausal women taking dietary oil supplements rich in oleate, linoleate, and both eicosapentaenoic acid and docosahexaenoic acid.

Design: Fifteen postmenopausal women took 15 g sunflower oil/d, providing 12.3 g oleate/d; safflower oil, providing 10.5 g linoleate/d; and fish oil, providing 2.0 g EPA/d and 1.4 g DHA/d in a 3-treatment crossover trial.

Results: Plasma free F₂-isoprostane concentrations were lower after fish-oil supplementation than after sunflower-oil supplementation ($P = 0.003$). When plasma free F₂-isoprostane concentrations were normalized to plasma arachidonic acid concentrations, significant differences among the supplements were eliminated. Plasma MDA concentrations were lower after fish-oil supplementation than after sunflower-oil supplementation ($P = 0.04$), whereas plasma TBARS were higher after fish-oil supplementation than after sunflower oil ($P = 0.003$) and safflower oil ($P = 0.001$) supplementation. When plasma MDA concentrations were normalized to plasma PUFA concentrations, significant differences were eliminated, but TBARS remained higher after fish-oil supplementation than after sunflower oil ($P = 0.01$) and safflower-oil ($P = 0.0003$) supplementation.

Conclusions: With fish-oil supplementation, there was no evidence of increased lipid peroxidation when assessed by plasma F₂-isoprostanes and MDA, although plasma TBARS was higher than with sunflower-oil and safflower-oil supplementation. *Am J Clin Nutr* 2000;72:714–22.

KEY WORDS Postmenopausal women, F₂-isoprostanes, malondialdehyde, thiobarbituric acid–reactive substances, lipid peroxidation, fish oil, monounsaturated fatty acids, polyunsaturated fatty acids

INTRODUCTION

A large body of research supports the hypothesis that the oxidation of LDL in vessel walls plays a significant role in the development of atherosclerosis (1, 2). For this reason, factors that influence the oxidation of LDL lipids have been the subject of several investigations. Despite the favorable effects of diets that are relatively high in unsaturated fat on lipoprotein profiles (3, 4), there is concern that such diets could increase the oxidative modification of LDL, thereby negating some of their cardio-protective effects. These issues are of particular relevance to postmenopausal women, for whom cardiovascular disease is the major cause of mortality in the United States (5).

The results of in vitro studies of lipid peroxidation in homogeneous solutions suggest that oxidative susceptibility increases with the number of double bonds in a fatty acid (6, 7). Studies of slightly more complicated systems (aqueous micelles) suggested that oxidative susceptibility is influenced by additional factors, such as the polarity of lipid hydroperoxides formed from specific fatty acids (8, 9). The measurement of lipid peroxidation in vivo is of particular interest because oxidative conditions that are used to determine oxidative susceptibility in vitro may not be relevant in vivo (10). In vivo assessments of changes in lipid peroxidation related to diets rich in specific fatty acids are

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problematic for several reasons. Some fatty acids are more likely than others to form a particular decomposition product, such as malondialdehyde (MDA), whereas other oxidation products, such as F₂-isoprostanes (11), are formed during the oxidation of only one fatty acid, arachidonic acid (20:n-6).

The results of studies examining the effects of increased proportions of highly unsaturated polyunsaturated fatty acids (PUFAs) in the diet on indexes of lipid peroxidation *in vivo* are contradictory (12-14). However, much of the information regarding the effect of increased dietary PUFAs on *in vivo* lipid peroxidation is based solely on the thiobarbituric acid (TBA) assay. Although widely used, the TBA assay has been widely criticized because of its well-documented lack of specificity for MDA (15).

To determine the effect of increased intake of specific unsaturated fatty acids on several indexes of *in vivo* lipid peroxidation, we measured free F₂-isoprostanes, MDA, and thiobarbituric acid-reactive substances (TBARS) in the plasma of postmenopausal women taking daily supplements of oleate (18:1n-9)-rich sunflower oil, linoleate (18:2n-6)-rich safflower oil, and fish oil rich in eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3).

SUBJECTS AND METHODS

Subjects

Sixteen postmenopausal women aged 50-75 y were recruited from the Oregon State University campus and the surrounding community through posters and newspaper advertisements. The study protocol was reviewed and approved by the institutional review board at Oregon State University and written consent was obtained from each participant before the study began.

Menopausal status was assessed on the basis of menstrual history [absence of normal menses for ≥ 12 mo or a history of use of hormone replacement therapy (HRT), or both] for ≥ 12 mo. A thorough medical history was obtained from each participant. Therapeutic agents or nutritional supplements known to have antioxidant or lipid-altering effects were specifically excluded. All participants were required to be normolipidemic on the basis of the results of plasma lipid and lipoprotein profiles, analyzed by the Oregon State University Lipid Laboratory. Normal liver function, glucose status, and iron status were verified through medical history and the results of a fasting serum chemistry panel and a screening complete blood count, analyzed by Good Samaritan Hospital Laboratory (Corvallis, OR). All prospective participants had their blood pressures measured before entering the study to ensure normal systolic and diastolic blood pressures. Height and weight were measured and body mass index (BMI; in kg/m²) was calculated to enable selection of participants who were not obese. Women with a history of cigarette smoking were excluded. All participants agreed to refrain from taking any nutritional supplements other than calcium or vitamin D and to refrain from eating fish for the duration of the study. The participants were informed that the 15 g oil would add 567 kJ (135 kcal) daily to their diets, and they agreed to maintain a stable body weight throughout the study and to refrain from any significant change in physical activity. Because one participant dropped out after the first period for reasons unrelated to the study, only the data obtained from the other 15 participants are included in the results.

Experimental design

To assess the effects of each of the 3 oil supplements on the individual participants, a 3-period, 3-treatment, blinded crossover trial was used. During each treatment period, the participants took 15 g high-oleate sunflower oil, high-linoleate safflower oil, or fish oil rich in 20:5n-3 and 22:6n-3. Each treatment period lasted 5 wk and was followed by a 7-wk washout interval to minimize any carryover effect from the previous treatment. The initial 16 participants were randomly assigned to 1 of 6 treatment sequences, resulting in ≥ 2 participants taking supplements in each of all possible sequences. The crossover trial was designed to avoid confounding of period and treatment effects by including each treatment in each period. This design allowed for the statistical assessment of carryover effects, described in the statistical analysis section below (16). The total time of participation in the study was 27 wk.

Blood samples were taken on 2 separate days, before the start of the treatment and twice during the last 3 d of the 5-wk treatment period. The participants made a total of 14 visits to the metabolic unit to have blood samples taken or to pick up supplements. Compliance was assessed by counting leftover capsules and by evaluating changes in specific plasma fatty acid concentrations at the end of each treatment period.

Supplements

The fish oil was obtained from the National Institutes of Health Fish Oil Test Materials Program (NIH-FOTMP) in sealed opaque containers that contained one-hundred 1-g capsules. The high-oleate sunflower oil (Humpco, Memphis) and the high-linoleate safflower oil (Arista Industries, Darien, CT) were supplied in bulk and encapsulated after adjustment for antioxidant content (Professional Compounding Pharmacy, Corvallis, OR). The α -tocopherol concentration in the fish oil supplied by the NIH-FOTMP was assayed by our laboratory and found to be 1.2 mg/g oil; the γ -tocopherol concentration was 1.3 mg/g oil. The fish oil also contained tertiary butyl hydroquinone (TBHQ) added as an antioxidant to achieve the concentration of 0.17 mg/g oil. TBHQ is a food-grade, oil-soluble antioxidant that is metabolized within a few days of ingestion and excreted in the urine. Because it is not stored in tissues, it does not function as an antioxidant *in vivo* (17). After the original α - and γ -tocopherol concentrations in the sunflower and safflower oils were assayed, additional α -tocopherol, γ -tocopherol, and TBHQ (Eastman Chemical Company, Kingsport, TN) were added to match the concentrations present in the fish oil supplied by the NIH-FOTMP. Thus, all 3 oil supplements supplied ≈ 20 mg α -tocopherol equivalents in 15 g oil consumed daily.

The potential for lipid peroxidation in the oil supplements was assessed by measuring peroxide values (18) and the *p*-anisidine value, a measure of the aldehyde content of fat (19). Lipid peroxidation was assessed at the beginning of each treatment period in samples of each oil supplement, which had been kept refrigerated in opaque containers. Neither the *p*-anisidine value nor the peroxide value had increased in any of the supplements by the final period of the study. Oil supplements were given to the participants in opaque jars; each day's dose was contained in a resealable plastic bag. The participants were instructed to keep the opaque container in the refrigerator except when removing the day's supplement supply.

Dietary analysis

To determine the nutritional content of her habitual diet, each participant was instructed in the technique of keeping a 3-d



TABLE 1
Selected fatty acids supplied by 15 g/d of each oil supplement¹

Fatty acid	Sunflower oil	Safflower oil	Fish oil
		<i>g/d</i>	
14:0	ND	0.02	1.19
16:0	0.49	1.04	2.16
18:0	0.53	0.37	0.39
20:0	0.05	0.05	0.06
22:0	0.15	0.03	0.02
ΣSFA ²	1.26	1.53	3.90
16:1n-7	0.02	0.02	1.34
c-18:1n-9	12.27	1.85	0.76
18:1n-7	ND	0.15	0.37
20:1n-9	0.04	0.03	0.12
ΣMUFA ³	12.32	2.04	2.63
c,c-18:2n-6	0.58	10.46	0.21
18:3n-3	0.02	0.02	0.15
20:4n-6	ND	ND	0.12
20:5n-3	ND	0.02	1.97
22:5n-3	ND	ND	0.35
22:6n-3	ND	ND	1.44
ΣPUFA ⁴	0.59	10.49	4.56

¹Values for sunflower oil and safflower oil are mean values of samples measured in duplicate. Values for fish oil were provided by the National Institutes of Health Fish Oil Test Materials Program. ND, not detected.

²Sum of the saturated fatty acids = 13:0 + 14:0 + 15:0 + 16:0 + 18:0 + 19:0 + 20:0 + 21:0 + 22:0 + 23:0 + 24:0.

³Sum of the monounsaturated fatty acids = 16:1n-7 + *t*-18:1n-9 + *c*-18:1n-9 + 18:1n-7 + 20:1n-9 + 24:1.

⁴Sum of the polyunsaturated fatty acids = *t,t*-18:2n-6 + *c,c*-18:2n-6 + 18:3n-3 + 18:4n-3 + 20:2n-6 + 20:3n-6 + 20:3n-3 + 20:4n-6 + 20:5n-3 + 22:5n-3 + 22:6n-3.

(2 weekdays and 1 weekend day) record of all foods and beverages consumed. The participants kept 3-d diet records during each of the 3 treatment periods. The nutritional content of each participant's diet during each treatment period was analyzed by using FOOD PROCESSOR PLUS (version 6.0; ESHA, Salem, OR).

Blood collection

Venous blood samples were collected into tubes containing Na₂EDTA (1 g/L) after an overnight fast of ≈12 h. Samples were taken on 2 separate days at the beginning and the end of each 5-wk treatment period. Plasma samples were prepared within 1 h of blood collection by centrifugation at 600 × *g* for 15 min at 4°C. Blood samples were kept in the dark and on ice until centrifugation. Plasma samples were immediately divided into aliquots and stored under argon at -70°C. The plasma samples were thawed only once, at the time of assay. Frozen plasma samples were shipped on dry ice overnight from the Oregon State University Lipid Laboratory to JDM's laboratory at Vanderbilt University and to BNA's laboratory at the University of California, Berkeley, ≈3 mo after the final blood draw. Plasma vitamin E, plasma fatty acid profiles, and plasma lipid and lipoprotein profiles were assayed at the Oregon State University Lipid Laboratory at the end of each treatment period. Plasma TBARS was assayed ≈3 mo after the final blood draw.

Assays

Plasma total cholesterol concentrations were determined enzymatically by using a modification of the method of Allain et al

(20). This cholesterol assay met the National Cholesterol Education Program's performance criteria for accuracy. Plasma triacylglycerol concentrations were measured by using a modification of the method of McGowan et al (21). HDL-cholesterol concentrations were measured enzymatically after precipitation of LDL and VLDL fractions with phosphotungstic acid and magnesium chloride (22). LDL-cholesterol concentrations were calculated by using the formula of Friedewald et al (23).

To obtain fatty acid profiles of plasma and of the supplemental oils, lipids were extracted with chloroform methanol (1:2, vol:vol), according to the method of Bligh and Dyer (24). After methylation, fatty acid methyl esters were measured by gas chromatography by using heptadecanoic acid (Nu-Chek Prep, Elysian, MN) as an internal standard, as described previously (25). Individual fatty acids were reported as mmol/L of plasma. The sums of supplement and plasma saturated fatty acids (ΣSFA), monounsaturated fatty acids (ΣMUFA), and PUFAs (ΣPUFA) were calculated as shown in **Table 1**. Because fatty acids with ≥2 double bonds are thought to be more susceptible to lipid peroxidation than are SFAs or MUFAs (26), a peroxidation index (PI) was calculated as follows:

$$\begin{aligned} \text{PI} = & (\Sigma\text{PUFA with 2 double bonds} \times 1) \\ & + (\Sigma\text{PUFA with 3 double bonds} \times 2) \\ & + (\Sigma\text{PUFA with 4 double bonds} \times 3) \\ & + (\Sigma\text{PUFA with 5 double bonds} \times 4) \\ & + (\Sigma\text{PUFA with 6 double bonds} \times 5) \end{aligned} \quad (1)$$

The PI was used as an index for the susceptibility of a given fatty acid profile to lipid peroxidation.

Concentrations of α- and γ-tocopherol in the supplemental oils were measured by using normal-phase HPLC (Shimadzu, Columbia, MD) with fluorometric detection (excitation: λ292; emission: λ330) based on a standardized method published by the International Union of Pure and Applied Chemistry (27). Oil samples were diluted in hexane and injected onto a silica column (Supelcosil 5 μm; 250 × 4.6 mm; Supelco Inc, Bellefonte, PA), using hexane: isopropanol (99:1, vol:vol) as a mobile phase, at a flow rate of 1 mL/min.

Plasma α-tocopherol concentrations were measured by reversed-phase HPLC with fluorometric detection, using a modification of the method of Arnaud et al (28). Tocopherols were extracted into hexane, evaporated under nitrogen, resuspended in methanol, and injected onto a C₁₈ column (Shim-pack CLS-ODS 5 μm; 250 × 4.6 mm; Shimadzu) with 100% methanol as a mobile phase, at a flow rate of 1.5 mL/min. Concentrations were determined by using external standards. Recovery of added α-tocopherol to plasma samples averaged 92%. Application of our plasma assay to α-tocopherol standards (SRM 968b) obtained from the National Institute of Standards and Technology (NIST) Standards Reference Program (Gaithersburg, MD) yielded values within 6% of the NIST consensus values; the intra- and interassay CVs for plasma α-tocopherol averaged 6.4% and 5.6%, respectively.

Free F₂-isoprostane concentrations in plasma were determined by using gas chromatography-negative chemical ionization mass spectrometry (GC-NCIMS) as described by Morrow and Roberts (29). Briefly, a deuterated prostaglandin F_{2α} internal standard was added to plasma, and F₂-isoprostanes were extracted with C₁₈ and silica minicolumns. The extracted F₂-isoprostanes were converted to pentafluorobenzyl ester trimethylsilyl ether derivatives and quantified by using selected ion monitoring [mass-to-charge ratio

(*m/z*) 569 for F₂-isoprostanes and *m/z* 573 for the internal standard] GC-NICMS. This assay has been found to be highly accurate (96%) and highly sensitive; the lower limit of detection is in the low picogram range (30). Intra- and interassay CVs for the assay in the present study were <10%.

Plasma MDA concentrations were also measured by using GC-MS in the negative chemical ionization mode as described by Yeo et al (31). The stable isotope internal standard [²H₂] MDA and the antioxidant 2,6-*tert*-butyl-4-methylphenol (67 μmol/L) were added to the plasma samples. The samples were then incubated at room temperature in 6.6 mol H₂SO₄ for 10 min to hydrolyze the aldehydes from the proteins. Plasma MDA was converted to a stable pentafluorophenyl hydrazine derivative at room temperature, and the derivative was quantified by using the GC-MS in the negative chemical ionization mode (selective ion monitoring *m/z* 234 for MDA and *m/z* 236 for the internal standard). Intra- and interassay CVs were 6.5%. This method combines the highly specific technique of GC-MS with mild sample preparation conditions, thus avoiding cross-reactivity and heat-generated artifacts characteristic of the TBA method (32).

The TBA assay is a commonly used method of measuring MDA as an index of lipid peroxidation. Because of the lack of specificity of this assay, especially in biological systems, results are commonly expressed as TBARS rather than MDA. Plasma TBARS were measured in duplicate by using the method described by Yagi (33). To inhibit further oxidation, 2,6-*tert*-butyl-4-methylphenol (67 μmol/L) was added to plasma samples before the assay was started, just as in the GC-MS assay for MDA. Plasma lipids were precipitated along with protein through the use of a phosphotungstic acid-sulfuric acid system. After centrifugation at 1200 × *g* for 10 min at 20°C and removal of the supernate, the precipitate was resuspended and the TBA reagent (equal volumes of 0.67% TBA and glacial acetic acid) were added. The mixture was heated for 60 min at 95°C. After cooling, *n*-butanol was added, the mixture was mixed and centrifuged at 1200 × *g* for 10 min at 20°C, and the butanol layer was removed for fluorometric measurement (emission: λ553; excitation: λ515). External standards of 1,1,3,3-tetraethoxypropane were used to quantify TBARS in the plasma samples. Intra- and interassay CVs averaged 8.4% and 9.5%, respectively.

Statistical analysis

There was a 7-wk washout period after each treatment period to decrease the likelihood of carryover effects. Balanced random assignment of ≥2 subjects to all 6 possible treatment sequences allowed for the statistical assessment of carryover effects before inference regarding direct treatment effects. Carryover effects were estimated and direct treatment effects were analyzed by using a mixed between- and within-subjects analysis of variance (ANOVA) procedure described by Kuehl (16). Briefly, the between-subjects sources of variation consisted of sequence of treatment and subjects nested within sequence, whereas within-subjects sources of variation consisted of period, treatment (direct), and treatment (carryover). If treatment carryover effects were significant (*P* = 0.05), estimates of differences among treatment means could be adjusted for the carryover effects. If carryover effects were not found to be significant, the ANOVA was performed without the treatment (carryover) effect in the model. No significant treatment carryover effects were found for any of the data presented. Therefore, all least-squares means (LSMs) rep-

resent direct treatment effects, which were not adjusted for carryover. Because no significant period-treatment interactions were found, a period-treatment interaction was not included in the final model. If the ANOVA showed a significant treatment effect, *P* values for the differences between LSMs were adjusted for multiple comparisons by using Tukey's studentized range test. Results with *P* values = 0.05 for a two-sided test were considered significant. Analyses were accomplished by using the SAS general linear model procedure (version 6.12; SAS Institute Inc, Cary, NC).

Before analysis, the data were screened graphically for normality, linearity, and homogeneity of variance. A modification of Levene's test was used to ensure that the assumption of equal within-group variance was not violated (34). Studentized residuals were used to screen for multivariate outliers. Observations with studentized residuals >3, which did not resolve with transformation and showed strong influence on the model were removed from the analysis. Data are expressed as LSMs ± SEs, unless otherwise noted.

RESULTS

Characteristics of the subjects and their diets

All the participants were postmenopausal and were receiving HRT. Each participant continued her regimen of HRT, without alteration, for the duration of the study. Although the HRT regimens were not identical for each woman, they were generally equivalent to 0.625 mg conjugated estrogens/d for the women who had had hysterectomies and 0.625 mg conjugated estrogens plus 2.5 mg medroxyprogesterone/d for the women who had not had hysterectomies. The participants were not taking long-term prescription medications other than hormones and did not take nutritional supplements, other than calcium or vitamin D, throughout the study. Compliance with the supplement regimen was estimated to be 95% on the basis of the return of empty supplement containers and leftover supplement capsules. The results of the plasma fatty acid profiles for each participant during each treatment period were also consistent with high compliance.

The initial characteristics of the study participants are presented in **Table 2**. Although mean BMI was slightly greater than the desirable upper limit of 24.9, it was well under 30, the level at which morbidity and mortality associated with obesity has been found to increase rapidly (35). Average weight gain over the 9-mo study period was 0.6 kg. All participants were normolipidemic on the basis of their lipid and lipoprotein profiles (36). Fasting blood glucose concentrations and iron status indicated by hemoglobin were within normal limits (37).

Because the intake of nutrients presented in **Table 3** did not differ significantly by supplementation group, values represent the average nutrient intake from three 3-d diet records. Mean vitamin E intake was slightly below the recommended dietary allowance of 8 mg α-tocopherol equivalents (TE)/d (39), and mean folic acid intake was below the recently published dietary reference intake of 400 μg/d (38) though well within the recommendation of 180 μg/d at the time of the study.

Fatty acid intake and plasma fatty acid concentrations

The major fatty acids supplied by each supplement are listed in **Table 1**. As a result of the oil supplement, the total dietary intake of specific fatty acids differed significantly between supplementation groups (**Figure 1**). During supplementation with sunflower

TABLE 2
Initial characteristics of the subjects

	Normal range	$\bar{x} \pm SD$ ($n = 15$)
Age (y)	—	58 \pm 6
BMI (kg/m ²)	<25	25.8 \pm 3.5
Plasma cholesterol (mmol/L)	<6.20	5.23 \pm 0.55
Plasma triacylglycerol (mmol/L)	<2.30	1.49 \pm 0.47
HDL cholesterol (mmol/L)	>0.90	1.57 \pm 0.39
LDL cholesterol (mmol/L)	<4.10	2.98 \pm 0.61
Glucose (mmol/L)	3.30–6.40	4.93 \pm 0.42
Hemoglobin (g/L)	120–160	132 \pm 7

oil, total oleate intake was 79% higher than with safflower-oil supplementation ($P < 0.0001$) and 61% higher than with fish-oil supplementation ($P < 0.0001$). During safflower-oil supplementation, linoleate intake was 146% higher than with sunflower-oil supplementation ($P < 0.0001$) and 119% higher than with fish-oil supplementation ($P < 0.0001$). Because the participants were asked to exclude fish from their diets throughout the study, fish-oil supplementation was associated with significantly greater (≈ 50 – 100 fold) 20:5n-3 and 22:6n-3 contents of the diet than was sunflower- and safflower-oil supplementation ($P < 0.0001$ for all 4 comparisons). Although total intake of 20:4n-6 was relatively low during all 3 supplementation periods, it was significantly higher during fish-oil supplementation ($P < 0.0001$) because of the higher 20:4n-6 content of the fish oil.

At the end of each supplementation period, plasma fatty acid concentrations reflected fatty acid intake (Figure 1). Mean plasma 18:1n-9 concentrations were 41% higher at the end of sunflower-oil supplementation than at the end of safflower-oil supplementation ($P < 0.0001$) and 61% higher than at the end of fish-oil supplementation ($P < 0.0001$). At the end of safflower-oil supplementation, plasma 18:2n-6 was 14% higher than at the end of sunflower-oil supplementation ($P = 0.05$) and 34% higher than at the end of fish-oil supplementation ($P < 0.0001$). Plasma 18:2n-6 concentrations also differed significantly between the sunflower-oil and the fish-oil supplementation groups; 18:2n-6 concentrations were 18% higher at the end of sunflower-oil supplementation ($P = 0.03$). Plasma concentrations of 20:5n-3 and 22:6n-3 were significantly higher after fish-oil supplementation. Plasma 20:5n-3 concentrations were ≈ 10 times higher, whereas plasma 22:6n-3 concentrations were ≈ 2.5 times higher after fish-oil supplementation than after sunflower-oil and safflower-oil supplementation ($P < 0.0001$ for all comparisons). Plasma 20:4n-6, which can be synthesized from 18:2n-6, was 23% lower after fish-oil supplementation than after supplementation with sunflower or safflower oil ($P = 0.001$ for both comparisons).

Total plasma concentrations of PUFAs did not differ significantly between the different supplementation groups. However, plasma concentrations of n-6 PUFAs at the end of fish-oil supplementation were 1.1 mmol/L less than after sunflower-oil supplementation ($P = 0.003$) and 1.5 mmol/L less than after safflower-oil supplementation ($P < 0.0001$). Plasma concentrations of n-3 PUFAs at the end of fish-oil supplementation were 1.0 mmol/L higher than after sunflower-oil supplementation and 1.1 mmol/L higher than after safflower-oil supplementation ($P < 0.0001$). Plasma n-6 and n-3 PUFA concentrations did not differ significantly between the sunflower- and safflower-oil supplementation groups. At the end of fish-oil supplementation the average PI of 12.4 was significantly higher than the average PI of

9.5 at the end of sunflower-oil supplementation and the average PI of 9.7 at the end of safflower-oil supplementation ($P < 0.0001$ for both comparisons). The PI did not differ significantly between the sunflower- and safflower-oil supplementation groups.

Plasma α -tocopherol

Each of the 3 oil supplements was matched for α - and γ -tocopherol content (20 mg α -TE/d). Additionally, mean dietary vitamin E (6 mg α -TE/d) did not differ significantly between the 3 supplementation groups. Plasma α -tocopherol concentrations (Figure 2) were 13% lower after fish-oil supplementation than after sunflower-oil supplementation, a difference that showed a trend toward significance ($P = 0.07$). Plasma α -tocopherol concentrations did not differ significantly between fish-oil and safflower-oil supplementation or between sunflower-oil and safflower-oil supplementation groups. The normalization of plasma α -tocopherol to total plasma lipid concentrations has been proposed as a valuable index of vitamin E status when plasma lipid concentrations are altered. When normalized to total plasma lipid concentrations expressed as moles (estimated as plasma total cholesterol + plasma triacylglycerol), instead of plasma volume, α -tocopherol concentrations did not differ significantly between supplementation groups.

Assays of lipid peroxidation in vivo

Plasma concentrations of free F_2 -isoprostanes (Figure 3), products of 20:4n-6 oxidation, were significantly lower (15%) after fish-oil supplementation than after sunflower-oil supplementation ($P = 0.002$) and slightly lower (8%) than after safflower-oil supplementation ($P = 0.09$). Free F_2 -isoprostanes did not differ significantly between sunflower-oil and safflower-oil supplementation. When normalized to plasma 20:4n-6 (AA) concentrations, plasma free F_2 -isoprostanes did not differ significantly between supplementation groups. Unlike plasma α -tocopherol, plasma F_2 -isoprostanes were not normalized to total plasma lipid content because only lipids containing 20:4n-6 can be oxidized to form F_2 -isoprostanes.

TABLE 3
Average daily intake of selected nutrients from three 3-d diet records

Nutrient	Recommended ¹	Intake ² ($n = 15$)
Energy (kJ)	7959	7374 \pm 1916
Protein (% of energy)	15	16 \pm 3
Carbohydrate (% of energy)	—	55 \pm 9
Total fatty acids (% of energy)	≤ 30	29 \pm 6
Saturated fatty acids (% of energy)	<10	12 \pm 3
Monounsaturated fatty acids (% of energy)	—	11 \pm 3
Polyunsaturated fatty acids (% of energy)	—	6 \pm 2
Cholesterol (mg)	300	177 \pm 90
Dietary fiber (g)	20	20 \pm 8
Vitamin E (mg α -TE)	8	6 \pm 3
Vitamin C (mg)	60	138 \pm 84
Selenium (μ g)	55	57 \pm 31
Folic acid (μ g)	400	265 \pm 126
Vitamin B-6 (mg)	1.5	1.6 \pm 0.7
Vitamin B-12 (μ g)	2.4	3.6 \pm 2.8

¹Represents the 1998 dietary reference intakes for folic acid, vitamin B-6, and vitamin B-12 (38) and the 1989 recommended dietary allowances for all other nutrients (39).

² $\bar{x} \pm SD$.

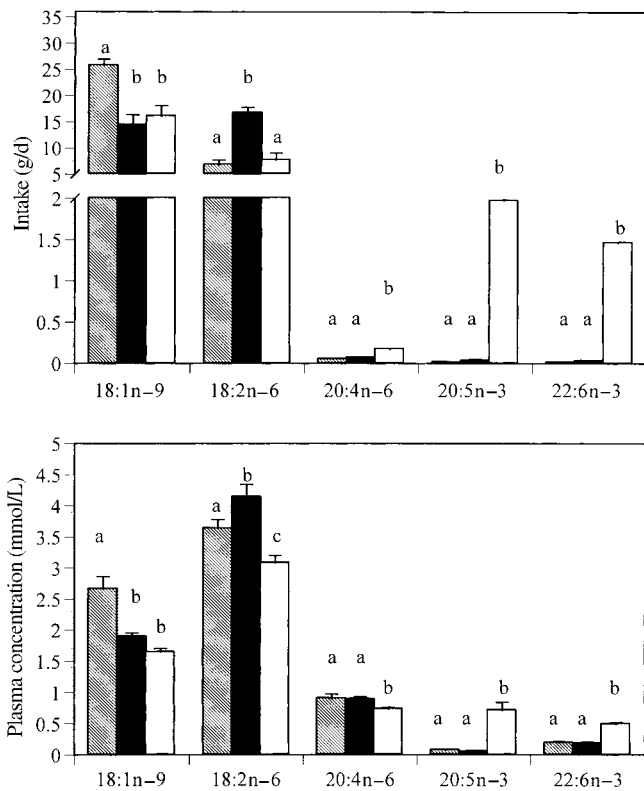


FIGURE 1. Least-squares mean (\pm SE) total intakes of specific dietary fatty acids, including dietary and supplement sources, and plasma concentrations of specific fatty acids in 15 postmenopausal women at the end of 5 wk of supplementation with 15 g high-oleate sunflower oil/d (▨), high-linoleate safflower oil (■), and high-EPA and -DHA fish oil/d (□). Values with different letters are significantly different, $P = 0.05$.

Plasma MDA concentrations (Figure 4A), measured by using the more specific method (GC-MS), showed a similar trend to the F₂-isoprostane concentrations. After fish-oil supplementation, plasma MDA concentrations were 17% lower than after sunflower-oil supplementation ($P = 0.04$) and 13% lower than after safflower-oil supplementation ($P = 0.09$). Plasma MDA concentrations did not differ significantly between the sunflower-oil and safflower-oil supplementation groups. Plasma TBARS concentrations (Figure 4B), which are often referred to as an indicator of MDA concentrations, showed a different trend than did the plasma MDA results. It is important to note that the TBARS concentrations are nearly 10 times higher than the MDA concentrations. After fish-oil supplementation, plasma TBARS were >21% higher than after sunflower-oil supplementation ($P = 0.003$) and 23% higher than after safflower-oil supplementation ($P = 0.001$). Plasma TBARS concentrations did not differ significantly between the sunflower-oil and safflower-oil supplementation groups.

MDA is thought to result mainly from the decomposition of PUFA possessing >2 double bonds (40). However, MDA has been measured during the oxidation of PUFAs with only 2 double bonds and even in very small quantities during the oxidation of MUFAs (7, 41). Normalization of plasma MDA and TBARS

concentrations to plasma PUFA content is more likely to accurately reflect the plasma sources of MDA in plasma than is normalization to total plasma lipid content. In the present study, normalization of MDA concentrations to total plasma PUFA concentrations (Figure 4C) eliminated any significant differences among supplementation groups, although MDA concentrations after fish-oil supplementation were 15% less than after sunflower-oil supplementation, showing a trend toward significance ($P = 0.09$). When TBARS concentrations were normalized to total plasma PUFA concentrations (Figure 4D), fish-oil supplementation resulted in TBARS concentrations that were 15% higher than with sunflower-oil supplementation ($P = 0.01$) and 23% higher than with safflower-oil supplementation ($P = 0.0003$). TBARS concentrations did not differ significantly between the sunflower-oil and safflower-oil supplementation groups. Normalization of plasma MDA and plasma TBARS to plasma unsaturated fatty acid concentrations (Σ MUFA + Σ PUFA) yielded similar results, as did normalization to total plasma PUFA in that MDA did not differ significantly between the supplementation groups and TBARS concentrations were significantly higher after fish-oil supplementation than after sunflower-oil or safflower-oil supplementation.

DISCUSSION

The results of the assays of in vivo lipid peroxidation did not uniformly support the idea that increased numbers of double bonds in dietary PUFAs result in increased susceptibility to lipid peroxidation. In fact, the most specific indexes of lipid peroxidation used in the present study (F₂-isoprostanes and MDA) were not higher after fish-oil supplementation than after sunflower oil and safflower-oil supplementation. Moreover, the assays purported to measure the same index of oxidative stress—malondialdehyde (the MDA and TBA assays)—suggested different

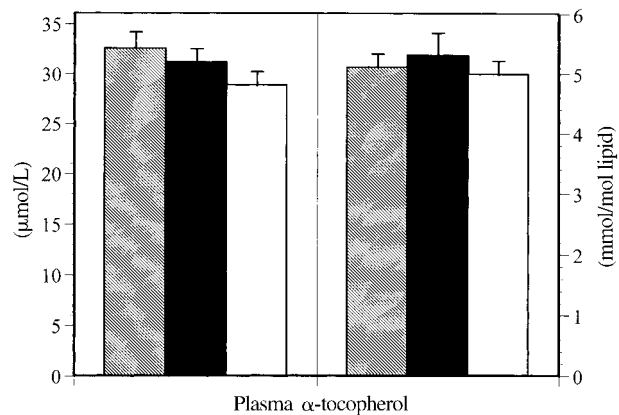


FIGURE 2. Least-squares mean (\pm SE) plasma α -tocopherol concentrations in 15 postmenopausal women at the end of 5 wk of supplementation with 15 g high-oleate sunflower oil/d (▨), high-linoleate safflower oil/d (■), and high-EPA and -DHA fish oil/d (□). The graph on the left represents α -tocopherol concentrations normalized to plasma volume; the graph on the right represents α -tocopherol concentrations normalized to total plasma lipids estimated as (plasma total cholesterol + plasma triacylglycerols). There were no significant differences between supplementation groups, $P = 0.05$.

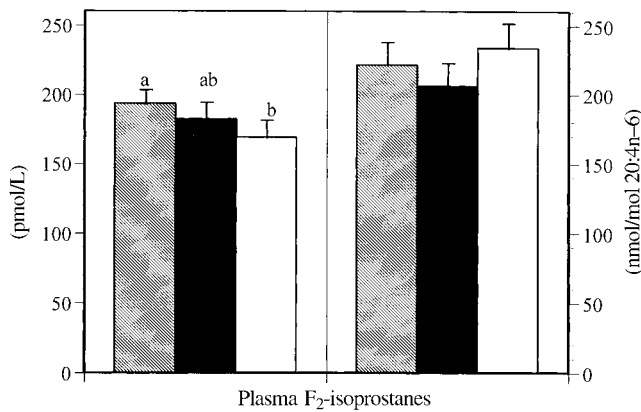


FIGURE 3. Least-squares mean (\pm SE) plasma concentrations of free F_2 -isoprostanes in 15 postmenopausal women at the end of 5 wk of supplementation with 15 g high-oleate sunflower oil/d (▨), high-linoleate safflower oil/d (■), and high-EPA and -DHA fish oil/d (□). Each bar represents least-squares mean \pm SE. The graph on the left represents F_2 -isoprostane concentrations normalized to plasma volume; the graph on the right represents F_2 -isoprostanes normalized to plasma arachidonate (20:4n-6) concentrations. Values with different letters are significantly different, $P = 0.05$.

conclusions regarding the consumption of highly unsaturated n-3 fatty acids and in vivo lipid peroxidation.

F_2 -isoprostanes are prostaglandin-like products of nonenzymatic peroxidation of AA. They are an established biomarker for oxidative stress and have been shown to correlate with conditions of increased lipid peroxidation in animals and humans (29). In humans, elevated plasma F_2 -isoprostanes have been shown in conditions associated with enhanced oxidative stress, such as chronic cigarette smoking (42), hepatorenal syndrome, and systemic sclerosis (43). Increased concentrations of F_2 -isoprostanes were found in human atherosclerotic lesions (44) and recently, increased plasma F_2 -isoprostane concentrations were associated with increased plasma total homocysteine concentrations, an independent risk factor for cardiovascular disease (45). Even when normalized to plasma 20:4n-6 concentrations, which were 18% lower after fish-oil supplementation, plasma free F_2 -isoprostanes were not significantly higher after fish-oil supplementation than after sunflower-oil and safflower-oil supplementation. Although more F_2 -isoprostanes are esterified to plasma lipids than are free F_2 -isoprostanes, only free F_2 -isoprostanes were measured in the present study. However, in all previous studies in which both plasma free F_2 -isoprostanes and F_2 -isoprostanes esterified to plasma lipids were measured, the 2 indexes were well correlated (42, 46).

The utility of the F_2 -isoprostane assay for comparing in vivo lipid peroxidation at different intakes of specific unsaturated fatty acids is limited because it does not provide direct information about the peroxidation of 20:5n-3 and 22:6n-3. It has been shown that the nonenzymatic oxidation of 20:5n-3 in vitro results in several F_3 -isoprostanes, one of which (8-epi $PGF_{3\alpha}$) can be found in small amounts in plasma (47). Moreover, F_4 -isoprostanes resulting from the nonenzymatic oxidation of the 22:6n-3 have been isolated in vitro and in vivo and found to be elevated in the spinal fluid of humans with Alzheimer disease (48). In the future,

the assessment of plasma concentrations of these 3 families of isoprostanes could provide insight into the relative contribution of individual PUFAs to in vivo lipid peroxidation.

Whether normalized to plasma volume or plasma PUFA concentration, plasma TBARS were significantly higher after fish-oil supplementation than after sunflower-oil or safflower-oil supplementation. Unlike plasma TBARS, plasma MDA concentrations were slightly lower after fish-oil supplementation than after sunflower-oil and safflower-oil supplementation when normalized to plasma volume or plasma PUFA concentration, although only the difference between fish oil and sunflower-oil supplementation normalized to plasma volume was significant. Liu et al (7) showed previously the increased sensitivity of the GC-MS assay over the TBARS assay for assessing MDA concentrations in unsaturated fatty acids oxidized in vitro. The fact that the TBARS concentrations in plasma were nearly 10 times higher than the MDA concentrations is likely due to the lack of specificity of the TBA assay for MDA and to artifactual production of MDA during the acid heating step of the TBA assay. The GC-MS technique used in the present study for measuring MDA combines the specificity of MS with relatively mild sample preparation, thus avoiding heat-generated artifact encountered with the TBA assay (32).

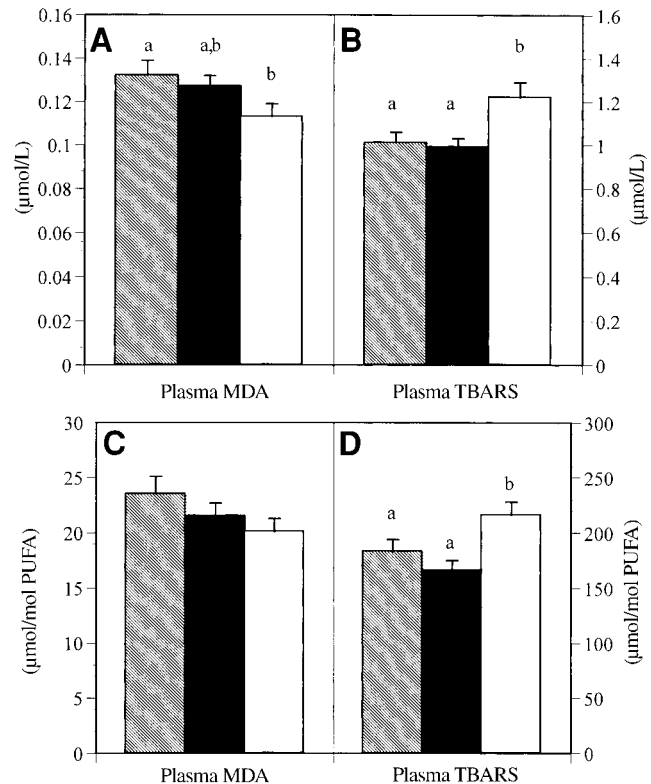



FIGURE 4. Least-squares mean (\pm SE) plasma concentrations of malondialdehyde (MDA; A) and thiobarbituric acid-reactive substances (TBARS; B) and plasma TBARS and MDA normalized to plasma total PUFA concentrations (C and D, respectively) in 15 postmenopausal women at the end of 5 wk of supplementation with 15 g high-oleate sunflower oil/d (▨), high-linoleate safflower oil/d (■), and high-EPA and -DHA fish oil/d (□). Values with different letters are significantly different, $P = 0.05$.

The disparate results of the plasma MDA and TBA assays in the present study suggest the possibility of increased TBARS other than MDA in plasma during fish-oil supplementation or the potential for increased MDA formation from 20:5n-3 and 22:6n-3 during the TBA assay, or both, under harsh conditions not encountered during the MDA assay or in vivo.

The results of several studies suggest that increased consumption of n-3 fatty acids may result in an increased potential for oxidative stress in vivo. These studies were based primarily on the results of the TBA assay (12, 13). The results of the present study and previous research at the Oregon State Lipid Laboratory did not show increased oxidative stress in vivo during fish-oil supplementation when more specific techniques than the TBA assay were used (14). For example, plasma protein carbonyl concentrations, an indicator of plasma protein oxidation, did not increase during fish-oil supplementation, despite a concomitant increase in plasma TBARS (49).

The fact that the results of the MDA and TBA assays suggested different conclusions regarding the effect of increased consumption of fish-oil on in vivo lipid peroxidation emphasizes the importance of the measurement issues raised by this research. Many of the assays available for the measurement of lipid peroxidation in vivo lose their utility when specific PUFA concentrations in plasma vary as a result of changes in dietary intake. Instead of measuring overall lipid peroxidation, different assays measure the oxidation or decomposition of specific PUFAs. For example, assays of MDA are more likely to detect the decomposition of hydroperoxides of fatty acids with >2 double bonds than the decomposition of hydroperoxides of oleate and linoleate, whereas F₂-isoprostane assays measure only specific products of 20:4n-6 oxidation. Currently, it is difficult to determine whether the results of such measurement techniques reflect actual differences in lipid peroxidation or merely differences in plasma PUFA concentrations.

In the present study, the results of more specific indexes of lipid peroxidation than the TBA assay suggested that lipid peroxidation in vivo is not increased by dietary supplementation with fish oil rich in 20:5n-3 and 22:6n-3. Thus, potentially beneficial effects of diets rich in n-3 PUFAs in preventing or ameliorating chronic conditions such as cardiovascular disease may not be offset by an increased risk of lipid peroxidation in vivo. Given that the accuracy of the TBA assay in biological samples has been questioned by several researchers (15, 26), the disagreement between the results of the MDA and the TBA assays in the present study suggests that findings of increased lipid peroxidation during fish-oil supplementation based solely on the results of the plasma TBA assay should be viewed with caution. Because no single reliable indicator of overall oxidative stress in vivo is currently available, the results of the present study highlights the need to apply more than one relevant assay when assessing the effect of increased PUFA intake on in vivo lipid peroxidation in humans. 

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