

POLYUNSATURATED FATTY ACID-INDUCED ANTIOXIDANT INSUFFICIENCY

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The consumption of essential fatty acid dietary supplements has risen sharply in recent years due to increasing awareness of epidemic deficiencies and a rising tide of scientific evidence of adverse clinical effects caused by deficiency. However, just as the development of essential fatty acid deficiency is an insidious process, so is the free radical pathology induced by excessive intake of polyunsaturated fatty acids (PUFAs). Quantitative concentration determinations of arachidonic acid (AA) and eicosapentaenoic acid (EPA) in plasma, malondialdehyde, and thiobarbituric acid-reactive substances (TBARS), and vitamin E in serum were made on 478 individuals. Serum TBARS values of .90, 1.07, and 1.28 nmol/mL were found for populations in low, middle, and high quartiles of plasma AA and EPA. Evidence is presented for a strong linear relationship of lipid peroxide (LPO) values and the AA and EPA sum. The elevation of LPO is found even when serum vitamin E is normal. Five cases with widely varying medical histories illustrate slight to extreme elevations of LPO when either AA or docosahexaenoic acid (DHA) is elevated. We conclude that PUFA-induced lipid peroxidation is common among patients who supplement flax and fish oils with inadequate antioxidant protection. Clinical management of fatty acid and antioxidant supplementation is aided by testing for fatty acid balance and measuring markers of oxidant damage.

Animal models have clearly shown the potential for increased oxidative damage from vitamin E deficiency induced by high polyunsaturated fatty acid (PUFA) intake. Muscle weakness due to necrotizing myopathy is a characteristic sign of increased tissue peroxidation, but there are many other risks.¹ A substantial body of evidence has demonstrated the toxic potentiation of arachidonic acid (AA) under certain conditions. In cells expressing cytochrome P4502E1, AA produces depletion

of cellular glutathione and marked elevation of malondialdehyde and 4-hydroxy-2-nonenal. This concentration-dependent, toxic effect of AA is prevented by the addition of antioxidants.² Potential toxic effects of AA are especially of concern in alcoholic liver injury where salicylates have been shown to enhance the resulting mitochondrial damage.³ Diets high in AA induce liver injury in alcoholics by inducing a mitochondrial membrane permeability transition. Such effects are related to the ease of oxidation of PUFAs and the tendency to deplete tissue antioxidant status. The mechanism is similar for all classes of PUFAs, but it is of most concern for the 20-carbon eicosanoid precursor fatty acids, AA, and eicosapentaenoic acid (EPA), because the concentrations of these members, of all fatty acids with more than 2 double bonds, are generally the highest in both depot fat and cell membrane phosphatides of most tissues. Diet and dietary supplements of fatty acids also are most likely to have effects on these fatty acids.

Discussions of excessive PUFA consumption have centered on the potential for vitamin A overdosing with high-dose cod liver oil supplementation.⁴ Many studies of high-dose fish oil supplementation have shown beneficial short-term effects. Arthritic patients who took 130 mg/kg/d of fish oils had fewer tender joints, and some were able to discontinue nonsteroidal anti-inflammatory drugs.⁵ For the average 70 kg person, this intake of 9 g/d is high enough to raise plasma EPA levels significantly. Antioxidant status was not evaluated in this study. EPA levels also may be raised by supplementation of flax oil and subsequent desaturation and elongation in human tissues.

Short-term benefits of high essential fatty acid (EFA) intake may be found concurrent with insidious effects leading to long-term toxicities if antioxidant status is

insufficient. With the growing public and physician awareness of potential benefits of dietary supplementation with EFA, it is important to develop methods for routine clinical evaluation of induced antioxidant insufficiency. Methods for quantitative concentration determinations of multiple fatty acids have been developed in the authors' laboratory, and these measurements have been combined with assays of serum antioxidant vitamins and lipid peroxides. The quantitative fatty acid assay avoids shortcomings of previous methods of reporting percentages that can obscure significant PUFA relationships due to large variations in other major fatty acid components such as palmitic and oleic acids. Malondialdehyde is a principle stable product of PUFA oxidation in vivo. The measurement of total thiobarbituric acid-reactive substances, which we will call lipid peroxides (LPO), is one of the most reliable assays of lipid peroxidation.⁶ The test has been used in previous investigations of antioxidant status changes with cod liver oil intake,⁷ and for dose-response relationships of oxidation products with dietary n-3 fatty acids in rats.⁸

We have observed an increasing number of cases being submitted for analysis in which there are elevated levels of AA or EPA concurrent with elevated serum LPO. This study was designed to investigate associations of lipid peroxidation and antioxidant vitamin levels with plasma levels of fatty acids in a general outpatient population.

MATERIALS AND METHODS

Specimens

Simultaneous specimens were drawn for routine screening of plasma for fatty acid imbalances, and of serum for vitamins A and E, β carotene, coenzyme Q-10, and LPO. The patients were being screened for metabolic abnormalities related to nutrient insufficiencies in chronic degenerative diseases or childhood developmental disorders. Dietary intake and supplemental nutrients were not restricted before testing. Specimens were received as chilled plasma (stabilized in ethylenediaminetetraacetic acid [EDTA]) and serum obtained from serum separator vacutainers, and shipped to the laboratory by overnight express.

Serum LPO

Measurement of serum LPO was performed by high-performance liquid chromatographic (HPLC) analysis of thiobarbituric-acid-reactive compounds in serum. Organic solvent extracts were applied to reversed phase columns, and vitamins were detected by ultraviolet (UV) absorp-

ance at characteristic wavelengths according to a method previously described.⁹

Serum Antioxidants

Vitamins A and E plus β carotene and coenzyme Q-10 were extracted from serum into organic solvent. Analysis was performed by HPLC with UV detection as described by Sowell and colleagues.¹⁰ Results are expressed in units of mg/L.

Fatty Acid Profiles

Calibration mixtures of the fatty acids were prepared from pure, certified fatty acid standards. Blood specimens for fatty acid analysis were drawn into EDTA-treated vacutainers and the cells were separated by centrifugation. Plasma was transferred to transport tubes and shipped with frozen packs by overnight express. Fatty acids were measured by performing total transesterification reactions in anhydrous acidic methanol as described by Lepage.¹¹ Fatty acid methyl esters in the extract were analyzed by GC/MS (gas chromatograph/mass spectrophotometer, Model 6890 GC with model 5972 MSD under Chemstation Software control from Hewlett-Packard Instruments, Palo Alto, Calif). The software converted peak areas into micromolar concentrations of individual fatty acids.

Statistical Analysis

The data were stored in a laboratory information management database (Sequel Server 2000, version SP2, Microsoft Corporation, Redmond, Wash). Sets of concurrently analyzed plasma fatty acids, serum vitamins and serum LPO that had been accessioned between November 2001 and February 2002 were extracted into a Microsoft Excel spreadsheet where charts and statistical analysis were performed using functions available in that software.

Results

The patient population of 487 cases contained 221 men and 266 women, aged 2 to 82 years. The data were investigated for significant association of LPO levels with age because there was such a large spread of ages in the patient population. The scattergram of age versus serum LPO is shown in Figure 1 with an overlaid trend line. The expected slight rise of LPO with age is seen. The lower ages were not excluded because the tendency for short-term oxidative damage is unaffected by age, and some of the children were known to have been receiving PUFA supplements.

The patient population was divided into quartiles by fatty acid concentration, and LPO levels were compared at the extremes. Average LPO levels in individuals at low versus high quartiles of AA or EPA are shown in Table 1, compared with the central 50% of the population. Average LPO values were .91, 1.09, and 1.20 nmol/mL for the low, middle, and high populations of AA concentration, respectively. The cutoff points of AA concentration fell at 401 and 633 μ M for the outer quartiles of this population. The Fisher *t* test of probability for differences between means being within error limits was performed, and the resultant probability factors were all well below .05, indicating that the differences were real.

A similar analysis was performed based on EPA concentration, as shown in Table 1. The effect was slightly more pronounced for EPA than for AA, with the low quartile average slightly lower and the high quartile slightly higher than found for AA. These data indicate that EPA may have a stronger tendency to undergo peroxidation than AA in humans. Because the effect was similar for both of these PUFAs, the analysis was repeated for the total of the 2 fatty acids, and effects of antioxidant vitamins on the high quartile of this population were

examined. The entire population is shown in Figure 2 as a scattergram of serum LPO versus the sum of AA plus EPA. The overlaid trend line shows the steady rise of LPO production as total PUFA levels increase.

It might be expected that serum vitamin E levels would be inversely related to LPO. However, this patient population showed a regular, direct relationship between the antioxidant levels and peroxidation products. Both vitamin E and coenzyme Q-10 levels rose steadily as PUFA concentrations and LPO levels increased. We suspect that the use of antioxidant dietary supplements is responsible for the observed relationships. This finding makes the detected LPO relationship even more significant. A population with combined high PUFA levels and low vitamin E or coenzyme Q-10 status would be expected to have even greater rises in peroxidation rates as PUFA levels increase.

We used the current data to investigate the simultaneous influence of high PUFA and low vitamin E in this population. The population with AA plus EPA above 670 μ M was further divided into tertiles according to vitamin E levels. Because the group with AA plus EPA >670 μ M has higher vitamin E levels, it is not optimal for testing the question. Indeed, we found that the high-PUFA

TABLE 1
LIPID PEROXIDE AVERAGES IN SUBPOPULATIONS BASED ON PUFA LEVELS*

Lipid peroxides by AA quartiles					
AA (μ M)	n	LPO (nmol/mL)	t test		
<401	121	.91	<.05		
401-633	244	1.09	<.05		
>633	122	1.20	.05		
Lipid peroxides by EPA quartiles					
EPA (μ M)	n	LPO (nmol/mL)	t test		
<10.5	121	.89	<.05		
10.6-39.9	244	1.05	<.05		
>40	122	1.28	<.05		
Lipid peroxides and antioxidant vitamins by AA+EPA quartiles					
AA+EPA (μ M)	n	LPO (nmol/mL)	t test	Vitamin E (mg/L)	Coenzyme Q-10 (mg/L)
<430	121	.90	<.05	11.4	.82
431-670	244	1.07	<.05	13.7	1.05
>670	122	1.28	<.05	16.9	1.27

*PUFA indicates polyunsaturated fatty acids; AA, arachidonic acid; LPO, lipid peroxides; EPA, eicosapentaenoic acid.

population with lower vitamin E had only slightly higher LPO (1.30 vs. 1.24 nmol/mL) than the high-PUFA population with higher vitamin E. No statistical difference was found between these 2 groups.

Although the average serum LPO levels shown in Table 1 are only in the range of 1.2 to 1.3 nmol/mL, the data points in Figure 2 show many individuals at LPO levels above 1.5 nmol/mL. Such levels are associated with increased risk of general peroxidation damage and specific malondialdehyde adduct formation that is associated with heart disease and liver degeneration.¹² Malondialdehyde-modified proteins are highly immunogenic and have been implicated to precipitate autoimmune responses.¹²

CASES

Five cases are shown in Table 2 illustrating the increasing severity of LPO elevation with either AA or EPA excess, with or without low serum vitamin E. Case 1 shows a mild elevation of EPA with low-normal AA and mid-normal serum vitamin E. The LPO is 1.1 nmol/mL, slightly above the laboratory limit of <1.0 nmol/mL. In case 2, mildly elevated AA with low-normal vitamin E is associated with LPO of 1.3 nmol/mL. Note that the low EPA in this case indicated a possible need to increase dietary intake of flax or fish oils to improve the n-3/n-6 balance. This is a case in which corrective antioxidant intervention is important as fatty

acid balance is being restored.

A slightly more pro-oxidant result is found in case 3 in which LPO is 1.4 nmol/mL. Here we see AA slightly elevated and EPA in the mid-normal range, with vitamin E concentration barely in the normal range. Case 4 is the only one in this group with a vitamin E level below the low cutoff. However, this antioxidant insufficiency is combined with elevated AA and mid-normal EPA. The AA plus EPA level is 804 μM, putting this case well to the right of the population shown in Figure 2, and the LPO at 1.5 nmol/mL is significantly elevated. Case 5 represents the extreme fatty acid situation of a patient with hypertriglyceridemia who is being treated with fish oils. Both AA and EPA are significantly elevated, and vitamin E is in the low-normal range with a resultant LPO of 2.6 nmol/mL.

All of the brief descriptions of medical history shown in Table 2 suggest potential benefit from treatment with essential fatty acids. The laboratory data allow them to be monitored in a manner that is most likely to permit both short-term symptomatic improvements from balancing PUFA status and long-term avoidance of compromised function due to oxidative damage. The antioxidant status can be adjusted easily by more aggressive antioxidant supplementation and dietary modification, and the degree of special customization that is needed will be revealed on follow-up test profiles.

TABLE 2
FIVE CASES OF ELEVATED AA OR EPA WITH INCREASING LEVELS OF LPO*

Case No.	Age	Sex	Description	AA (μM) [300-700]†	EPA (μM) [20-80]	Vitamin E (mg/L) [12-50]	LPO (nmol/mL) [<1.0]
1	55	M	Prostate cancer patient, used flax oil supplements	399	90 ‡	22.7	1.1
2	58	M	Dizziness, tinnitus, night sweats	754	13	13.3	1.3
3	55	F	Desired antiaging supplements; followed a low carbohydrate, high protein diet	737	42	12.7	1.4
4	63	M	Prostate cancer, 15-year history of hypertension	769	35	<i>11.8</i>	1.5
5	74	M	Cardiovascular disease, used fish oils for hypertriglyceridemia	761	186	14.4	2.6

*AA indicates arachidonic acid; EPA, eicosapentaenoic acid; LPO, lipid peroxides.
 †Numbers in brackets are laboratory reference values.
 ‡Numbers in bold are above laboratory reference values; those in italics are below.

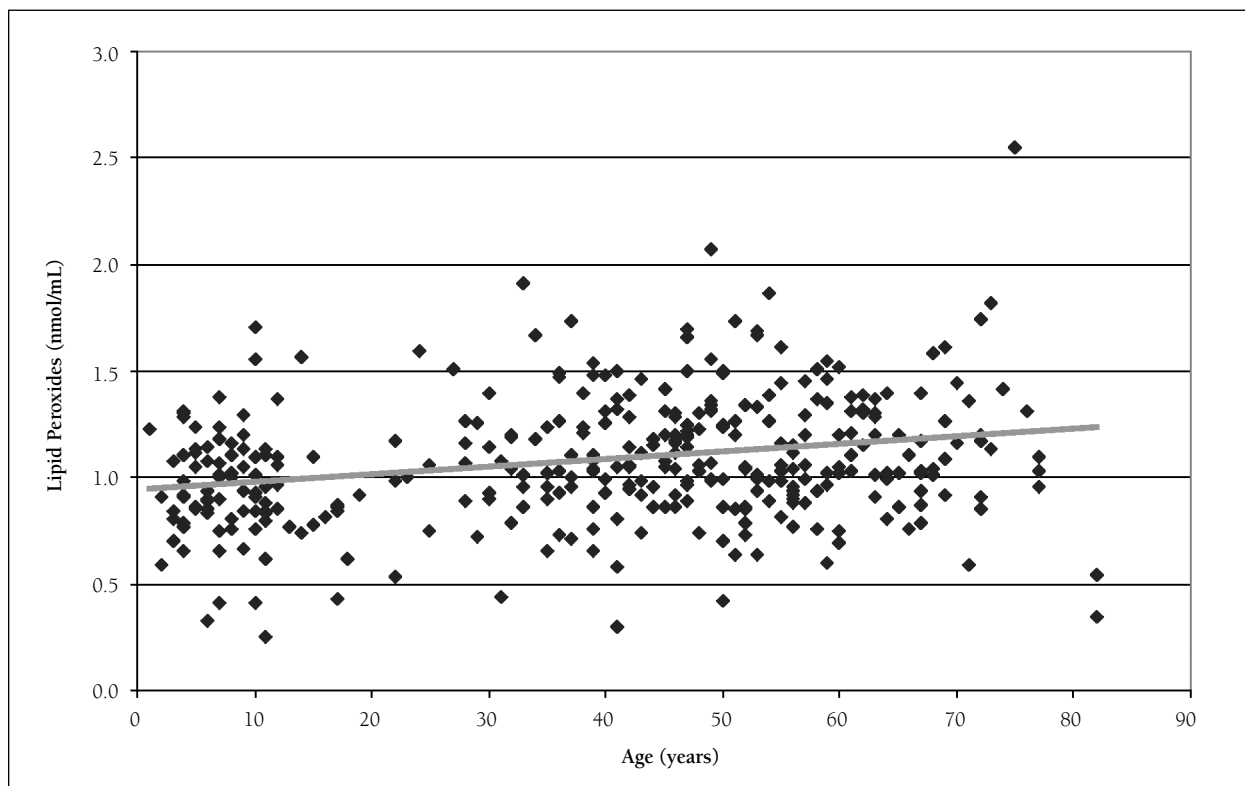


FIGURE 1
SERUM LIPID PEROXIDES PLOTTED AGAINST AGE SHOW A SLIGHT RISE IN AVERAGE RATES OF EXCRETION IN OLDER INDIVIDUALS.

COMMENT

These results show the potential for increased lipid peroxidation risk for patients in whom elevated plasma AA or EPA concentrations are found. Such patients may be self-treating with diet or high PUFA oils or they may be under clinically supervised programs of intervention to restore fatty acid or lipid balance. Whether the plasma PUFA increase is due to high fat, low carbohydrate diet or PUFA supplementation, measurements of serum antioxidants and a marker of antioxidant insufficiency allow the detection and management of risk from elevated rates of oxidative damage. It is very difficult to use only clinical observations to discover fatty acid imbalances and increased rates of lipid peroxidation insults. Both phenomena produce multiple, subtle, nonspecific tissue effects that have few predictable short-term symptomologies.

The clinical practice issue must be correctly identified. The potential negative health effects of high PUFA intake may be avoided by concurrent adequacy of antioxidant nutrients, especially the membrane-protective vitamin E. Susceptibility of erythrocytes to hydrogen peroxide hemolysis in newborn babies with physiological

jaundice is reduced by adding balanced PUFA and vitamin E to formulas.¹² Studies of EPA and docosahexaenoic acid (DHA) supplementation have shown simultaneous beneficial effects on blood lipids and detrimental pro-oxidant effects on antioxidant status. Diets enriched with EPA or DHA had positive triglyceride-lowering and cholesterol-lowering effects in rats while plasma and hepatic vitamin E levels were significantly decreased, and hepatic glutathione synthesis was stimulated.¹³

Elevated AA is especially of concern because of inflammatory responses and other specific cell control effects. In addition to the risk of general free radical pathology associated with elevated LPO levels, specific neurotransmitter regulatory issues arise with elevation of AA. Active oxygen radicals derived from AA mediate inhibition of γ -aminobutyric acid (GABA) receptors. High levels of AA favor this inhibitory effect that can lead to increased neuronal excitability and excitotoxic cell death in conditions of ischemia and seizures. This GABA receptor effect is specifically reduced by the antioxidant effects of N-acetylcysteine.¹⁴

Diabetes confers greatly heightened concern over the effects of lipid peroxidation damage. A high percentage of

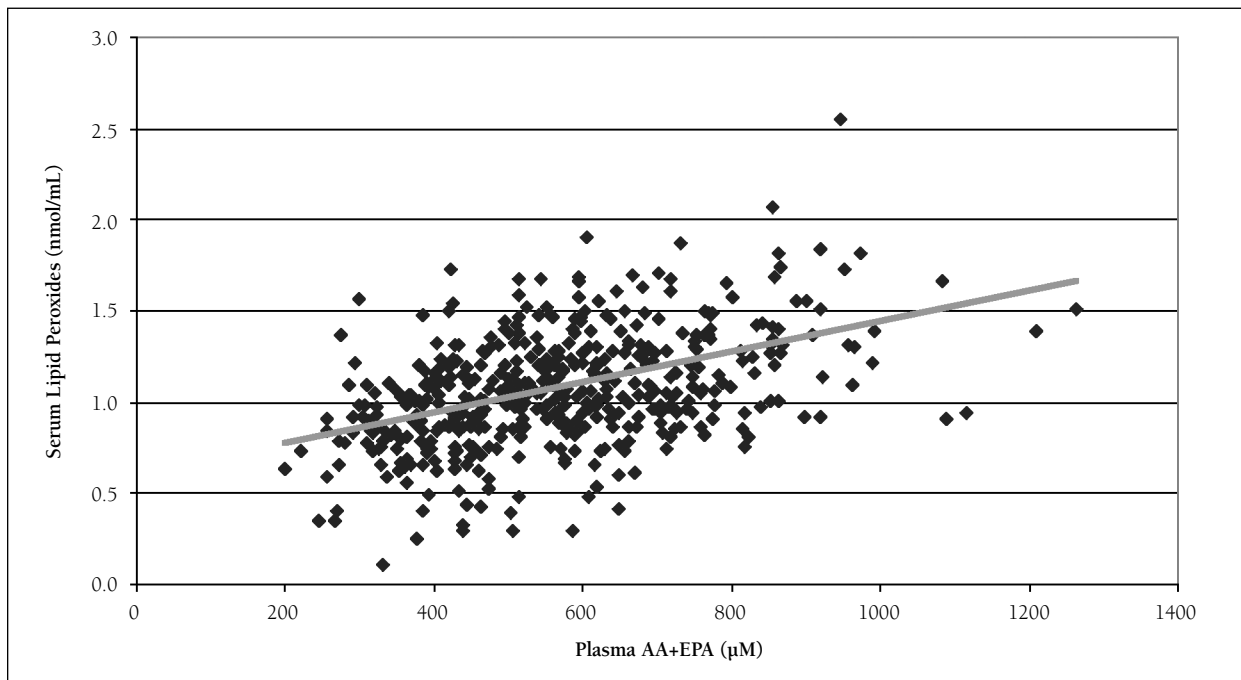


FIGURE 2
SERUM LIPID PEROXIDE CONCENTRATIONS ARE PLOTTED AGAINST THE SUM OF ARACHIDONIC AND EICOSAPENTAENOIC ACIDS IN PLASMA, SHOWING THE POSITIVE RELATIONSHIP OF OXIDATION PRODUCT EXCRETION AT HIGHER LEVELS OF POLYUNSATURATED FATTY ACIDS.

diabetic patients die of kidney failure, and the damage is related to eicosanoid metabolites of AA. Vitamin E has been shown to specifically reduce the formation of thromboxane A₂ in kidney microsomes in streptozotocin-induced diabetic rats.¹⁵ Similar effects are found in ethanol-induced liver damage where vitamin E, curcumin, and N-acetylcysteine were all found effective in lowering AA-induced prostaglandin formation.¹⁶ The use of EPA for controlling serum lipoprotein levels should be combined with antioxidant support. Impaired status of vitamin C and glutathione in patients with coronary artery disease may be a manifestation of PUFA peroxidation effects.¹⁷

Association of higher levels of plasma gamma linolenic acid and erythrocyte DHA with coronary artery luminal narrowing could be another effect of PUFA-induced vitamin E deficiency and other endothelial sequelae of lipid peroxidation.¹⁸ PUFA-fed animals have increased peroxidation products and altered activities of enzymes that control oxygen radical concentrations and these effects are worsened by elevated thyroid hormone status.¹⁹

We conclude that patients with elevated levels of serum PUFA are at increased risk of oxidative damage due to lipid peroxidation. Laboratory evaluation of the status of specific antioxidant vitamins and markers of peroxida-

tive damage along with profiling of plasma fatty acids allow significant improvements in patient management. Corrections may include lowering PUFA intake, adding antioxidant nutrients (vitamin E, N-acetylcysteine, curcumin, vitamin C, or others), or increasing other antioxidant categories such as the trace elements selenium, zinc, copper, and molybdenum.

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REFERENCES

1. Diehl JF. Vitamin E deficiency in rabbits receiving a high PUFA diet with and without a non-absorbable antioxidant. 1. Incorporation of [1-14C]glycine into skeletal muscle proteins. *Z Ernährungswiss.* 1986;25(2):103-113.
2. Chen Q, Galleano M, Cederbaum AI. Cytotoxicity and apoptosis produced by arachidonic acid in HepG₂ cells overexpressing human cytochrome P-4502E1. *Alcohol Clin Exp Res.* 1998;22(4):782-784.
3. Wu D, Cederbaum AI. Cyclosporine A protects against arachidonic acid toxicity in rat hepatocytes: role of CYP2E1 and mitochondria.

- Hepatology*. 2002;35(6):1420-1430.
4. Grubb BP. Hypervitaminosis A following long-term use of high-dose fish oil supplements. *Chest*. 1990; 97(5):1260.
 5. Kremer JM, Lawrence DA, Pettrillo GF, et al. Effects of high-dose fish oil on rheumatoid arthritis after stopping nonsteroidal antiinflammatory drugs. Clinical and immune correlates. *Arthritis Rheum*. 1995;38(8): 1107-1114.
 6. Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic Biol Med*. 1990;9(6):515-540.
 7. Piche LA, Draper HH, Cole PD. Malondialdehyde excretion by subjects consuming cod liver oil vs a concentrate of n-3 fatty acids. *Lipids*. 1988;23(4): 370-371.
 8. De Schrijver R, Vermeulen D, Daems V. Dose-response relationships between dietary (n-3) fatty acids and plasma and tissue lipids, steroid excretion and urinary malondialdehyde in rats. *J Nutr*. 1992;122(10):1979-1987.
 9. Nielsen F, Mikkelsen BB, Nielsen JB, Andersen HR, Grandjean P. Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. *Clin Chem*. 1997;43(7):1209-1214.
 10. Sowell AL, Huff DL, Yeager PR, Caudill SP, Gunter EW. Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, alpha-carotene, trans-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwave-length detection. *Clin Chem*. 1994;40(3):411-416.
 11. Lepage G, Roy CC. Direct transesterification of all classes of lipids in a one-step reaction. *J Lipid Res*. 1986;27(1):114-120.
 12. Villalaz RA, Toner N, Chiswick, ML. Dietary vitamin E and polyunsaturated fatty acid (PUFA) in newborn babies with physiological jaundice. *Early Hum Dev*. 1981;5(2):145-150.
 13. Demoz A, Asiedu DK, Lie O, Berge RK. Modulation of plasma and hepatic oxidative status and changes in plasma lipid profile by n-3 (EPA and DHA), n-6 (corn oil) and a 3-thia fatty acid in rats. *Biochim Biophys Acta*. 1994;1199(3):238-244.
 14. Saxena NC. Inhibition of GABA(A) receptor (GABAR) currents by arachidonic acid in HEK 293 cells stably transfected with alpha1beta2gamma2 GABAR subunits. *Pflugers Arch*. 2000;440(3):380-392.
 15. Kwag OG, Kim SO, Choi JH, Rhee IK, Choi MS, Rhee SJ. Vitamin E improves microsomal phospholipase A2 activity and the arachidonic acid cascade in kidney of diabetic rats. *J Nutr*. 2001;131(4):1297-1301.
 16. Akkrishnan VR, Menon VP. Potential role of antioxidants during ethanol-induced changes in the fatty acid composition and arachidonic acid metabolites in male Wistar rats. *Cell Biol Toxicol*. 2001;17(1):11-22.
 17. Tamer L, Sucu N, Polat G, et al. Decreased serum total antioxidant status and erythrocyte-reduced glutathione levels are associated with increased serum malondialdehyde in atherosclerotic patients. *Arch Med Res*. 2002;33(3):257-260.
 18. Blaha V, Solichova D, Cernohorsky D, Bratova M, Vyroubal P, Zadak Z. Bioanalysis of PUFA metabolism and lipid peroxidation in coronary atherosclerosis. *J Pharm Biomed Anal*. 2000;22(3):563-572.
 19. Varghese S, Lakshmy PS, Oommen OV. Changes in lipid peroxidation and antioxidant enzyme activities by triiodothyronine (T3) and polyunsaturated fatty acids (PUFA) in rat liver. *Endocr Res*. 2001;27(4):409-416.

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