Brief Communication:
Omega-3 Essential Fatty Acid Supplementation and Erythrocyte Oxidant/Antioxidant Status in Rats

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Abstract. Fish oil contains large amounts of essential omega-3 fatty acids, such as eicosapentaenoic and docosahexaenoic acids, which are building structures of cell membranes. The goal of this study was to elucidate the effects of dietary omega-3 fatty acid supplementation on the oxidant/antioxidant status of erythrocytes in rats. The malondialdehyde (MDA) and nitric oxide (NO) levels and the catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) activities were assayed in erythrocytes of male Wistar albino rats after 30 days of dietary supplementation with fish oil (0.4 g/kg/day). Erythrocyte CAT activity in the treated group was increased in comparison with the control group. Erythrocyte MDA and NO levels were lower in the treated group than the controls. Erythrocyte GSH-Px and SOD activities did not differ significantly in the 2 groups. Negative correlations were found between SOD and CAT activities, and between SOD and GSH-Px activities in the treated group. In conclusion, omega-3 fatty acid supplementation helps to prevent lipid peroxidation and to safeguard erythrocytes from oxidative injury. Dietary supplementation with omega-3 fatty acids might possibly protect tissues from oxygen free radical injury in the various diseases in which the oxidant/antioxidant defense mechanisms are disturbed. (received 20 October 2004; accepted 30 December 2004)

Keywords: fish oil, omega-3 essential fatty acids, oxidant/antioxidant st, lipid peroxidation, nitric oxide

Introduction

The health benefits of omega-3 essential fatty acids (ω-3 EFA) have been extensively studied. Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and α-linoleic acid (ALA) are ω-3 EFA that are important for structural and biochemical integrity of all cells, including erythrocytes. Dietary consumption of long-chain ω-3 and ω-6 EFA is essential because mammals are incapable of synthesizing fatty acids with a double bond past the 9 position. Thus, dietary intake of ω-3 EFAs has far-reaching effects on cell membrane composition and may influence erythrocyte function as well [1].

In recent years, increased oxidative stress has been implicated in the pathogenesis of numerous diseases including cancer, atherosclerosis [2], schizophrenia, and certain neurological disorders [3,4]. Harmful effects caused by reactive oxygen species (ROS) occur as a consequence of imbalance between the production and inactivation of these species. Numerous oxygenated compounds, mainly aldehydes such as malondialdehyde (MDA), are produced during the attack of free radicals on membrane lipoproteins and polyunsaturated fatty acids (PUFA). Assay of MDA is probably the most common method for the measurement of lipid

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peroxidation [5]. Important antioxidant enzymes include superoxide dismutase (SOD), which catalyzes dismutation of superoxide anion (O$_2^•$) to H$_2$O$_2$, which is then deactivated to H$_2$O by catalase (CAT) and glutathione peroxidase (GSH-Px).

There have been conflicting reports on the effects of fish oil supplementation on oxidant/antioxidant status in both animal and human studies [6-10]. There are contradictory results regarding the effects of increased proportions of ω-3 EFA in the diet on indexes of lipid peroxidation [11-13]. The influence of ω-3 EFA supplementation on the activity of antioxidant enzymes, lipid peroxidation, and nitric oxide in erythrocytes of normal rats has not been studied in detail. The goal of the present study was to determine the effects of increased dietary intake of ω-3 fatty acids on indices of lipid peroxidation and the oxidant/antioxidant status of erythrocytes in rats. MDA, an important lipid peroxidation product of ROS; nitric oxide (NO), a gaseous free radical; and the activities of antioxidant enzymes were assayed in erythrocytes of rats after 30 days of dietary supplementation with fish oil. The results were compared with corresponding measurements in erythrocytes of untreated controls.

Materials and Methods

*Animals and experimental design.* Male Wistar albino rats (n = 16, body weight = 250-300 g) were purchased from Firat University Animal Laboratory and housed in quiet rooms with 12:12-hr light-dark cycle, room temperature 20-22°C, and relative humidity 40-50%. The experiment complied with the Guide for the Care and Use of Laboratory Animals, DHEW/NIH Publication #85-23, 1985). The rats were assigned to weight-matched control and treatment groups. Omega-3 EFA (0.4 g/kg/day, Marincap, Kocak, Turkey, 5 kcal/capsule) was given to the treatment group (n = 8) for 30 days by gavage. The fatty acid composition of the Marincap capsule (500 mg) was EPA (18%) and DHA (12%). In the control group (n = 8), saline was given in the same way. Water and standard pelleted rat chow were provided ad libitum throughout the experiment. The rats were checked daily and their body weights were recorded at the beginning and end of the experiment.

After 30 days, the animals were anesthetized by ether and venous blood was drawn into Na-EDTA containing tubes. Erythrocyte lysate was prepared as previously described [14]. Briefly, the plasma and buffy coat were removed after centrifugation at 1000 x g for 10 min at 4°C. The sedimented erythrocytes were washed 3 times with 10-fold isotonic NaCl solution to remove plasma remnants. After each wash, the tubes were centrifuged at 1000 x g for 10 min at 4°C. The sedimented erythrocytes were then stored at -70°C until analysis.

**Erythrocyte MDA.** Erythrocyte MDA levels were determined by reaction with thiobarbituric acid (TBA) [15]. MDA and MDA-like substances react with TBA to produce a pink pigment. The erythrocyte sample was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid to precipitate proteins. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was measured at 532 nm. The results were expressed as µmol MDA/g Hb, based on a calibration curve prepared with dilutions of a standard solution of 1,1,3,3-tetramethoxypropane.

**Erythrocyte NO.** NO has a half-life of only a few seconds, because it is rapidly oxidized to nitrite (NO$_2^-$) and subsequently to nitrate (NO$_3^-$), which provide an index of NO production. The method for erythrocyte nitrite and nitrate levels was based on the Griess reaction [16]. Erythrocyte samples were initially deproteinized with Somogyi's reagent. Total nitrite (nitrite + nitrate) was measured at 545 nm after conversion of nitrate to nitrite by copperized cadmium granules. A standard curve was prepared with serial dilutions (10$^{-8}$ to 10$^{-3}$ mol/L) of sodium nitrite. The standard curve was analyzed by linear regression and the resulting equation was used to calculate the total nitrite concentrations in test samples. Results were expressed as µmol/g Hb.

**Erythrocyte SOD.** Total SOD (EC 1.15.1.1) activity in erythrocytes was assayed by the method of Sun et al [17]. The method is based on the inhibition of
NBT reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the sample after 1.0 ml of an ethanol/chloroform mixture (5:3, v/v) was added to an equal volume of erythrocyte sediment and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition of the NBT reduction rate. SOD activity was expressed as U/g Hb.

*Erythrocyte GSH-Px.* Erythrocyte GSH-Px (EC 1.6.4.2) activity was measured by the method of Paglia et al [18]. The enzyme reaction, which contained NADPH, reduced glutathione (GSH), sodium azide, and glutathione reductase, was initiated by addition of H$_2$O$_2$ and the absorbance change at 340 nm was monitored by a spectrophotometer. Activity was expressed as U/g Hb.

*Erythrocyte CAT.* Erythrocyte CAT (EC 1.11.1.6) activity was determined by the method of Aebi [19] using a UV spectrophotometer. The principle of the reaction is based on determination of the rate constant “k” of H$_2$O$_2$ decomposition. By measuring the absorbance change per min, the rate constant was determined. Activities were expressed as k/g Hb.

*Hemoglobin.* The hemoglobin concentration in the erythrocyte suspension was measured by Drabkin’s reagent, which contains potassium ferricyanide and potassium cyanide [20].

*Statistical analysis.* Data were analysed by non-parametric statistics (Mann-Whitney U test) using the Statistical Package for Social Sciences (SPSS, Chicago, IL) software for Windows. Bivariate relationships were examined using Spearman’s rank correlation coefficient ($r$). A two-tailed $p < 0.05$ was considered significant. Results were expressed as mean ± SE.

*Results*

There were no significant differences of mean body weight in the treated and control groups at baseline (252 ± 12 vs 255 ± 14 g, respectively) or after the 30-day trial (262 ± 16 vs 260 ± 17 g, respectively).

Table I. Malondialdehyde (MDA) and nitric oxide (NO) levels and superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activities in erythrocytes of control rats and of rats that received 30 days of dietary $\omega$-3 EFA supplementation. Results are expressed as mean ± SE.

<table>
<thead>
<tr>
<th>Parameter (and units)</th>
<th>Control rats (n = 8)</th>
<th>$\omega$-3 EFA-treated rats (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/g Hb)</td>
<td>2839 ± 399</td>
<td>2422 ± 135</td>
</tr>
<tr>
<td>GSH-Px (U/g Hb)</td>
<td>33.63 ± 3.33</td>
<td>34.57 ± 2.23</td>
</tr>
<tr>
<td>CAT (k/g Hb)</td>
<td>196.37 ± 18.52</td>
<td>298.30 ± 14.86$^a$</td>
</tr>
<tr>
<td>MDA (µmol/g Hb)</td>
<td>0.056 ± 0.010</td>
<td>0.024 ± 0.003$^b$</td>
</tr>
<tr>
<td>NO (µmol/g Hb)</td>
<td>0.281 ± 0.014</td>
<td>0.170 ± 0.017$^c$</td>
</tr>
</tbody>
</table>

$^a$ p < 0.0001; $^b$ p < 0.002; $^c$ p < 0.005 vs controls.

The results of this study are summarized in Table I. The erythrocytes of rats that received dietary supplementation with fish oil had significantly higher mean CAT activity ($p < 0.0001$), but lower mean NO ($p < 0.002$) and MDA levels ($p < 0.005$), compared to controls. The mean activities of erythrocyte SOD and GSH-Px did not differ significantly in the treated and control groups.

Negative correlations were observed between erythrocyte CAT and SOD activities ($r = -0.857$, $p = 0.014$), and between erythrocyte SOD and GSH-Px levels ($r = -0.786$, $p = 0.036$) in the treated group.

*Discussion*

Environmental factors, including diet, may affect the antioxidant enzyme activities in susceptible organs [21]. The antioxidant enzyme activities found in erythrocytes do not necessarily reflect the total antioxidant defense system of the whole organism. Erythrocytes have high CAT, SOD, and GSH-Px activities compared to other cells, which presumably reflects the enhanced exposure of erythrocytes to molecular oxygen.

In this study, a statistically significant increase of erythrocyte CAT activity was observed in rats fed $\omega$-3 EFA. Under physiological conditions, the erythrocyte antioxidant enzymes appear to be in balance. If one of the factors that affect the oxidant/
antioxidant status within erythrocytes is decreased or increased, the alteration may lead to minor changes in antioxidant enzyme activities. On the other hand, the decreased levels of erythrocyte NO and MDA levels, as a result of ω-3 EFA supplementation, clearly show the protective effect of ω-3 EFA against lipid peroxidation and oxidative stress directed by NO in erythrocytes. Contrary to Song and Miyazawa [9], our results indicate that dietary ω-3 EFA supplementation leads to increased resistance to lipid peroxidation and increased erythrocyte antioxidant capacity. Our study supports an idea suggested by Barbosa et al [22] that ω-3 EFA supplementation may have free radical scavenger activity. Administration of ω-3 EFA may stimulate vitamin E incorporation into membranes to avoid lipid peroxidation resulting from increased membrane ω-3 EFA content [23]. Treatment with ω-3 EFA has been reported to decrease lipid peroxidation in the corpus striatum and to increase antioxidant enzyme activities in the hippocampus and corpus striatum of rats [24,25]. These studies are all consistent with our findings and they suggest possible benefits of ω-3 EFA treatment of some diseases related to excessive oxidative stress.

NO formation is catalyzed by different types of nitric oxide synthases (NOS), enzymes present in a variety of cells including erythrocytes [26]. Since NO is formed by conversion of L-arginine to L-citrulline, decreased erythrocyte NO levels after ω-3 EFA supplementation may reflect decreased entrance of L-arginine into the erythrocytes. The generation of NO in erythrocytes can produce peroxynitrite (ONOO⁻) by interacting with superoxide radicals (O₂•⁻). The peroxynitrite molecule is a strong oxidizing agent that can interact with thiol groups of proteins to cause cellular injury. Its decomposition to the potent hydroxyl radical (•OH) can further damage cell structures, including the erythrocyte membrane.

This is the first study in which ω-3 EFA prevented cellular oxidative stress by diminishing NO generation and reducing lipid peroxidation in erythrocytes. Although fish oil polyunsaturated fatty acids would be expected to increase lipo-peroxidation [27], some studies have shown in animal models [28] and humans [29,30] that fish oil ω-3 fatty acids act as free radical scavengers. Fisher et al [30] administered fish oil ω-3 EFA for 6 wk to healthy volunteers and found a decrease in chemiluminescence of polymorphonuclear leukocyte inflammatory potential.

In the light of our results, it seems possible that the administration of ω-3 EFA may have ameliorating effects on cellular damage by 2 mechanisms: First, ω-3 EFA may increase the levels of CAT within the peroxisome and in the cytoplasm, resulting in enhanced defense against free oxygen radicals. Second, ω-3 EFA may be replaced by polyunsaturated fatty acid components of the erythrocyte membranes that had been attacked by superoxide anions, hydrogen peroxide, and hydroxyl radicals [31,32]. The decreased hemolysis of rabbit erythrocytes fed a fish oil enriched diet [33] supports our hypothesis of protective effects of ω-3 EFA.

In conclusion, the results of the present study indicate that ω-3 EFA enhances erythrocyte CAT activity, and thereby improves the antioxidant defense system and attenuates the oxidative stress that causes lipid peroxidation in erythrocytes. To explore the effects of ω-3 EFA on erythrocyte oxidative stress, lipid peroxidation, and antioxidant enzyme activities, further studies are needed to compare the effects of acute, subacute, and chronic ω-3 EFA supplementation.

References

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