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Original Article

Effect of fish oil on *glutathione* redox system in multiple sclerosis

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Abstract: Multiple sclerosis (MS) is a chronic, inflammatory and autoimmune disease of the central nervous system. Dysregulation of glutathione homeostasis and alterations in glutathione-dependent enzyme activities are implicated in the induction and progression of MS. Evidence suggests that Omega-3 polyunsaturated fatty acids (PUFAs) have anti-inflammatory, antioxidant and neuroprotective effects. The aim of the present work was to evaluate the effect of fish oil on the activity of glutathione reductase (GR), content of reduced and oxidized glutathione, and GSH/GSSG ratio in MS. 50 patients with relapsing-remitting MS were enrolled. The experimental group received orally 4 g/day of fish oil for 12 months. Fish oil supplementation resulted in a significant increase in n-3 fatty acids and a decrease n-6 fatty acids. No differences in glutathione reductase activity, content of reduced and oxidized glutathione, and GSH/GSSG ratio were found. Conclusion: Glutathione reductase activity was not significantly different between the groups; however, fish oil supplementation resulted in smaller increase in GR compared with control group, suggesting a possible effect on antioxidant defence mechanisms.

Keywords: Fish oil, glutathion, multiple sclerosis

Introduction

Multiple sclerosis (MS) is a chronic, inflammatory and autoimmune disease of the central nervous system, characterized by blood brain barrier breakdown, perivascular inflammation, axonal and oligodendrocyte injury, and breakdown of the myelin sheath [1]. The inflammatory process seen in multiple sclerosis is due to an excess production of pro-inflammatory cytokines, that leads to increased secretion of reactive oxygen species, and reduction of antioxidant defense mechanisms [2, 3]. Reduced glutathione (GSH) is one of the most important agents of the endogenous antioxidant defence system. GSH can act as a cofactor of glutathione peroxidase (GPx) by which catalyses the reduction of hydroperoxides. Oxidized glutathione (GSSG), a product of this reaction, is then recycled into GSH by glutathione reductase (GR). GPx and GR activities in MS remain unclear, as some studies show decreased activity, while others indicate normal or increased activity of MS [4]. Some studies have demonstrating the important role of n-3 long-chain polyunsaturated fatty acids (n-3 PUFAs) on MS. The mechanism of action for n-3 PUFAs is suggested to be attributed to immunomodulation, and antioxidant action [5]. n-3 PUFAs decrease the production of inflammatory mediators (eicosanoids, cytokines, and reactive oxygen species) and the expression of adhesion molecules. They act both directly (eg, by replacing arachidonic acid as an eicosanoid substrate and inhibiting arachidonic acid metabolism) and indirectly (eg, by altering the expression of

inflammatory genes through effects on transcription factor activation). n-3 PUFAs also give rise to antiinflammatory mediators (resolvins and protectins) [6]. Effects of resolvins and protectins include reducing neutrophil trafficking, cytokine and reactive oxygen species regulation, and lowering the magnitude of the inflammatory response [7]. The aim of this study was to evaluate the effect of fish oil on the activity of glutathione reductase, content of reduced and oxidized glutathione, and GSH/GSSG ratio in relapsing-remitting multiple sclerosis.

Material and methods

Subjects

This study was a clinical trial on a randomized, double blind placebo-controlled group. Patients were recruited exclusively from the multiple sclerosis clinic of the Neurology Department of the Unidad Médca de Alta Especialidad (UMAE), Hospital de Especialidades (HE), Centro Médico Nacional de Occidente (CMNO), IMSS, Guadalajara, JAL, Mexico. Age of participants was 18-55 years. Patients had clinically definite and magnetic resonance image supported MS, at least one relapse in the year before entry into the study, and a baseline EDSS score of 0-5 and were treated with subcutaneous 250 μg interferon beta-1b (Betaseron, Bayer) every other day at least one year before the trial [8, 9].

Patients were excluded if they were taking another supplement; had progressive forms of MS; had history of severe depression; had history of acute liver or renal dysfunction; had history of tobacco, drug, or alcohol abuse; had intolerance, contraindication, or allergy to fish oil; and had customary antioxidant intake. Patients were followed up for at least 1 year. Patients were evaluated at the clinic every 3 months until each patient had reached the 1 year endpoint.

This study was conducted in accordance with the updated Declaration of Helsinki [10] and was approved by the Research Committee of the Social Security Institute of Mexico (Protocol number: R-2010-1301-8). Informed consent was obtained from all patients prior to study enrollment, according to the ethical code of the institution. Identification numberswere assigned to assure patient confidentiality.

Randomization and blinding

Patients were randomly assigned in a 1:1 ratio to receive oral fish oil (4 g/day) or placebo, with a computer-generated randomization sequence (blocks of 2-4). To ensure masking between the fish oil and the placebo, capsules were identical in appearance, packaging, and labeling. Physicians and patients were blind to the intervention. An independent physician evaluated the EDSS score and collected the samples at each clinical visit.

Intervention

Patients received 4 g/day Omega Rx capsules (Dr. Sears zone diet) containing 0.8 g EPA, 1.6 g DHA,and excipient (gelatin, glycerin, water purified, tocopherol, canola oil, sunflower oil, natural rosemary flavor and citric acid) or placebo (Perfect Source Natural Products) orally (4 capsules per day).

Clinic visits were scheduled every 3 months to assess glutathione reductase, content of reduced and oxidized glutathione, and GSH/GSSG ratio in erythrocytes.

Fasting blood samples were taken at 0, 3, 6, 9, and 12 months. While the fatty acids profile was analyzed at 0, 6, and 12 months An independent physician assessed the occurrence of side effect at the Neurology Department. In this study, a relapse was defined as new or recurrent neurological abnormalities that were separated by at least 30 days fromthe onset of the preceding event, lasted at least 24 hours, and occurred without fever or infection.

Outcomes measurements

Peripheral venous blood was collected into sampling tubes without EDTA, blood was centrifuged at 3500 rpm for 5 minutes to separate the serum. For erythrocytes, venous blood samples were collected in tubes with EDTA. Erythrocytes were washed three times in PBS (1.4 mM KH_2PO_4, 8 mM Na_2PO_4, 140 mM NaCl, 2.7 mM KCl; pH 7.3) and was centrifuged at 3500 rpm for 5 minutes. The samples was stored at -80°C until analysis.

Glutathione reductase activity was determined by following the oxidation of NADPH to $NADP^+$ during the reduction of oxidized glutathione (GSSG) [11]. Total glutathione was assayed by

Table 1. Serum profile of phospholipid fatty acids of patients before and after the supplementation with fish oil or olive oil

Fatty acid	Baseline		6 months		12 months	
	Fish oil	Olive oil	Fish oil	Olive oil	Fish oil	Olive oil
C14:0	0.34±0.08	0.33±0.09	0.93±1.51	0.89±1.42	0.35±0.07	0.37±0.09
C15:0	0.24±0.1	0.25±0.09	0.91±1.45	0.87±1.52	0.51±0.13	0.49±0.10
C16:0	28.07±2.02	28.12±1.28	29.24±1.96	29.16±1.86	28.71±1.74	28.83±1.77
C18:0	14.91±1.39	14.54±1.16	29.24±1.97*	14.36±0.96	16.57±1.59	15.4±1.40
C20:0	0.41±0.36	0.32±0.2	0.25±0.10	0.28±0.14	0.27±0.05	0.28±0.04
C22:0	0.25±0.1	0.25±0.1	0.52±0.22	0.55±0.32	0.48±0.13	0.44±0.15
C24:0	0.29±0.26	0.26±0.19	0.27±0.17	0.27±0.22	0.19±0.10	0.16±0.08
C14:1	0.21±0.05	0.24±0.07	0.62±1.09	0.51±0.96	0.08±0.03*	0.07±0.03
C16:1	0.48±0.2	0.46±0.11	0.37±0.13**	0.49±0.23	0.36±0.14**	0.45±0.15
C18:1	8.44±1.04	8.39±1.23	7.11±0.69***	8.61±1.11	7.58±0.88***	8.76±1.12
C20:1	0.3±0.15	0.32±0.15	0.36±0.10**	0.46±0.11	0.41±0.10*	0.57±0.10
C22:1	0.44±0.21	0.35±0.08	0.51±0.37*	0.88±0.94	0.3±0.18***	0.4±0.09
C24:1	0.22±0.12	0.24±0.14	0.29±0.24	0.36±0.35	0.07±0.06	0.06±0.05
C18:2n6 LA	21.31±1.82	22.84±2.71	18.58±3.11	20.12±5.30	18.27±2.58*	21.2±2.86
C18:3n6 GLA	0.04±0.03	0.04±0.03	0.03±0.01	0.04±0.02	0.02±0.03	0.03±0.03
C20:2n6	0.42±0.19	0.4±0.13	0.18±0.05	0.27±0.10	0.19±0.07	0.27±0.08
C20:3n6 DGLA	3.48±0.78	3.07±0.7	2.3±0.69***	2.94±0.73	2.4±0.88***	3.14±0.70
C20:4n6 AA	11.01±1.56	10.42±2.71	8.89±1.80***	10.08±1.90	8.86±1.36***	10.12±1.87
C22:2n6	0.47±0.19	0.37±0.13	0.34±0.19**	0.55±0.36	0.25±0.11***	0.42±0.13
C22:4n6	0.56±0.18	0.6±0.23	0.73±0.20	0.78±0.29	0.9±0.24*	0.77±0.21
C22:5n6	0.82±0.31	0.84±0.21	1.35±0.39***	0.82±0.23	1.35±0.29***	0.8±0.19
C18:3n3 ALA	0.18±0.08	0.17±0.06	0.16±0.07	0.18±0.09	0.15±0.06	0.18±0.06
C18:4n3	0.24±0.11	0.28±0.21	0.4±0.09	0.4±0.12	0.42±0.07	0.39±0.07
C20:3n3	1.39±0.62	1.52±0.75	1.19±0.31	1.31±0.64	1.23±0.51	1.06±0.29
C20:4n3	0.72±0.23	0.72±0.22	0.56±0.16*	0.69±0.15	0.59±0.26	0.68±0.22
C20:5n3 EPA	0.88±1.18	0.72±0.63	2.52±1.36***	0.44±0.34	2.68±1.47***	0.46±0.36
C22:5n3 DPA	1.08±0.29	0.96±0.15	0.56±0.16	1.08±0.23	1.5±0.42	1.26±0.43
C22:6n3 DHA	2.8±0.86	2.95±1.04	4.96±1.16***	2.6±0.85	5.33±1.08***	2.93±1.03
AA/EPA	19.67±8.48	20.89±10.26	5.84±5.82***	30.45±15.14	5.18±4.53***	29.55±13.24
EPA+DHA	3.68±1.88	3.67±1.55	7.48±2.39***	3.04±1.13	8.01±2.37***	3.39±1.34
n6/n3	5.51±1.15	5.57±1.31	3.19±1.07***	5.54±1.35	2.84±.77***	5.52±1.16
ALA+EPA+DPA+DHA	4.93±1.82	4.8±1.6	8.77±2.46***	4.31±1.24	9.65±2.39***	4.82±1.44

Values are mean ± s.d. Mann-Whitney *U* test. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

an enzymatic recycling procedure in which it is sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)' and reduced by NADPH in the presence of glutathione reductase. Formation of 2-nitro-5-thiobenzoic acid was monitored by comparing the absorbance at 412 nm with that of standard curve. For the determination of GSSG, the GSH in a sample was derevatized with 2 μl 2-vinylpyridine was assayed. After a 60 min incubation at room temperature, the assay proceeded as described for total glutathione [12]. GSH levels were calculated by subtracting GSSG from total glutathione. Fatty acid compositions of total serum were determined after lipid extraction. Lipids were extracted with chloroform/methanol (2:1 v/v) by thin-layer chromatography. Each phospholipid sample was then esterified and analyzed by gas-liquid chromatography (Agilent 7890) equipped with a DB-23 capillary column. The relative amount of each fatty acid (percentage of total fatty acids) was calculated by dividing each fatty acid by the total area for all fatty acids.

If any patient had a relapse, the treating physicians indicated intravenousmethylpredniso-

Table 2. Effect of fish oil and placebo on *glutathione* redox system

		Baseline	3 months	6 months	9 months	12 months
GSH (mM)	Fish oil	0.03±0.02	0.04±0.02	0.03±0.02	0.04±0.03	0.03±0.02
	Control	0.04±0.02	0.04±0.02	0.03±0.02	0.04±0.02	0.03±0.02
GSSG (mM)	Fish oil	0.02±0.01	0.03±0.02	0.04±0.02	0.06±0.03	0.05±0.03
	Control	0.02±0.01	0.04±0.01	0.03±0.01	0.06±0.04	0.06±0.03
GSH/GSSG	Fish oil	2.87±2.7	1.57±1.4	1.22±1.3	1.36±1.7	1.06±1.0
	Control	3.45±3.2	1.96±2.0	1.34±2.2	1.43±2.3	1.03±1.7
GR (U/L)	Fish oil	342.7±170.9	606.5±518.9	439.8±232.2	468.4±222.2	471.8±312.8
	Control	330.4±197.7	565.8±420.2	589.8±444.3	529.2±285.0	570.9±455.6

Data expressed in mean and standard deviation (SD), Mann-Whitney *U* test. Reduced glutathione (GSH), oxidized glutathione (GSSG), GSH/GSSG ratio, glutathione reductase (GR).

lone 1 g/day, for 3 days. We waited one month after the last dose of methylprednisolone for blood sampling. If a patient during the intervention had an infection that required antibiotic treatment, we waited one day after the last dose of antibiotic for blood sampling. The adverse effects of the fish oil treatment were recorded during the clinic visit. A severe adverse effect was defined as any event that causes death and requires hospitalization or prolonged hospital stay. At study entry and every three months after enrollment, blood samples were collected to ascertain liver function (aspartate aminotransferase, and alanine aminotransferase and alkaline phosphatase); kidney function (urea, creatinine, and uric acid); blood lipids (total cholesterol, high density lipoproteins, low density lipoproteins, and very low density lipoproteins); hemoglobin; leukocytes; platelets; and glucose (data not shown).

The habitual dietary intake, including the essential fatty acids (EPA and DHA), was recorded for all participants. All the patients maintained their diet style and their exercise activity.

Adhesion and safety

Participants reported daily consumption of the supplement in a consumption posting sheet. Thepercentage adherence for each subjectwas determined by the following formula: (number of pills consumed)/(number of pills returned to the physician) × 100.

We considered it as an optimal adherence if the percentage was higher than 80%.

Statistical analysis

Data are expressed as mean ± standard deviation of mean. Friedman test to determine whether there were time differences in each group, and Mann-Whitney U test differences between two treatment groups. Statistical analyses were done on SPSS version 18. Values of $P \leq 0.05$ were considered statistically significant.

Results

Fatty acids profile

After 12 months of intervention in both groups there was a significant change in the percentage of EPA (an increase of 1.82±2.18 in fish oil group vs. a decrease of 0.30±0.53 in control group; $p \leq 0.001$) and DHA (an increase of 2.59±1.16 in fish oil group vs. a decrease of 0.13±0.58 in control group; $p \leq 0.001$). A significant decrease of AA was seen in both groups of 2.18±1.69 in the fish oil group, compared with 0.36±1.76 in the control group. Further, the n-3 fatty acids index in this group increased significantly from 4.93±1.82 to 9.65±2.39 ($p \leq 0.001$). The ratios of n-6/n-3 and AA/EPA decreased from 5.5±1.15 to 2.84±0.77 and from 19.67±8.48 to 5.18±4.53, respectively ($p \leq 0.001$ for both), in the fish oil group. In contrast, the ratio of EPA+DHA and EPA+DGLA increased 3.68±1.88 to 8.0±2.37 ($p \leq 0.001$) and from 4.36±1.03 to 5.08±1.28 ($p \leq 0.05$), respectively, in the fish oil group (**Table 1**).

Glutathione redox system

No differences in glutathione reductase activity, content of reduced and oxidized glutathione, and GSH/GSSG ratio were found (**Table 2**). However, there was a significant change in Glutathione reductase activity within subjects in the fish oil group, the difference between 6 months of baseline, 9 months of baseline, 12 months of baseline were different, $P \leq 0.01$.

Within subjects in contol group show no significant differences.

Discussion

Although the mechanisms by which act fatty acids are not fully elucidated, the effect they have on the synthesis of inflammatory mediators such as eicosanoids and cytokines has been widely reported. The n-6 fatty acid arachidonic acid (AA; 20:4 n-6) is an important regulator of pro-inflammatory mediators. The amount of eicosanoids formed depends not only on the activation of AA-releasing enzymes such as phospholipase A2 (PLA2) but also on the membrane concentration of AA [13]. AA is the progenitor of both PGE2 and LTB4 via the cyclooxygenase and 5-lipoxygenase enzymatic pathways, respectively. PGE2 and LTB4 have pro-inflammatory biological actions [14]. In addition, these eicosanoids may stimulate the release of proinflamtorias cytokines such as TNF-α, IL-1, IL-2, IL-6 and IFN-γ [6].

Fish oil is rich in the long chain *n*-3 fatty acids eicosapentaenoic acid (EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA; 22:5 *n*-3), which can displace arachidonic acid (AA; 20:4 *n*-6) from cell membranes, and, thus, can suppress production of the n-6 eicosanoid inflammatory mediators [14]. For example EPA-derived LTB5 is 10- to 100-fold less potent as a neutrophil chemoattractant compared with LTB4. Furthermore, EPA-derived eicosanoids may antagonise the action of those produced from arachidonic acid, as was demonstrated for PGD3 vs. PGD2 [15]. Also, EPA is able to reduce the production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, which are released when macrophages and monocytes are activated [6, 16]. Therefore, the balance between *n-6* and *n-3* polyunsaturated fatty acids in body is essential to regulating inflammatory processes and preventing exacerbated inflammation in inflammatory disorders, such as MS [17, 18].

In the present study, fish oil supplementation resulted in a decrease of AA concentration. In MS, increased pro-inflammatory metabolites of AA, such as LTB4, PGE2, TNFα, IL-1β and IL-6 have been related with the disease progression [19-22]. The AA concentration of the membranes is an important regulatory step in the synthesis of these molecules. In fact, the AA/EPA ratio in cell lipids determines the degree of competitive eicosanoid inhibition. The decrease observed in the AA/EPA ratio in fish oil group suggest a reduction in the production of pro-inflammatory metabolites of AA [23]. In a previous intervention trial in our population of patients with MS showed a significant reduction on Serum levels of TNF, IL-1β and IL-6 after 12 months of fish oil supplementation [24].

On the other hand, at present, have identified new lipid mediators generated from omega-3 fatty acids which play a key role in the resolution of inflammation by acting as anti-inflammatory agonists. These compounds have attracted great interest for its high bioactivity and its potent anti-inflammatory and immunoregulatory properties. Lipid mediators derived from eicosapentaenoic acid (EPA) was termed E-series resolvins (RvE), whereas that derived from DHA include the D-series resolvins (RvD) and neuroprotectins/protectins (NPD/PD). Resolvins block the production of proinflammatory mediators and regulate leukocyte trafficking to inflammatory. Specifically, resolvins limit polymorphnuclear leukocyte migration and infiltration across the endothelium. Protectins additionally possess protective actions in neural tissues and systems. Like resolvins, protectins stop polymorphnuclear leukocyte infiltration and they also limit cytokine expression such as TNF-α and IL-1β. Thus, the increase in EPA and DHA observed in this study could modulate the inflammatory response by inducing these lipid mediators [7, 25, 26].

The inflammatory process seen in multiple sclerosis is due to an excess production of pro-inflammatory cytokines, that leads to increased secretion of reactive oxygen species, and reduction of antioxidant defense mechanisms [2, 3]. Reduced glutathione (GSH) is one of the most important agents of the endogenous antioxidant defence system. GSH can act as a cofactor of glutathione peroxidase (GPx) by which catalyses the reduction of hydroperoxides. Oxidized glutathione (GSSG), a product of this reaction, is then recycled into GSH by glutathione reductase (GR). GPx and GR activities in MS remain unclear, as some studies show decreased activity, while others indicate normal or increased activity in MS patients when compared to healthy subjects [4].

In this study, no differences in glutathione reductase activity, content of reduced and oxi-

dized glutathione, and GSH/GSSG ratio were seen after 12 months of supplementation. However, fish oil supplementation resulted in smaller increase in GR compared with control group. In addition, there was a significant change in glutathione reductase activity within subjects in the fish oil group after 6 months of treatment, while within subjects in control group show no significant differences, which might suggest a possible effect of fish oil on antioxidant defense mechanisms of the cell.

Conclusions

Fish oil supplementation resulted in a high increase in EPA and DHA proportions, in turn, leading to a decrease of AA concentrations, as well as the AA/EPA ratio. These changes on fatty acids are indicative for a reduction in the production of inflammatory eicosanoids from AA and an increase of antiinflammatory mediators, such as resolvins and protectins. On the other hand, although glutathione reductase activity was not significantly different between the groups, fish oil supplementation resulted in smaller increase in GR compared with control group, suggesting a possible antioxidant effect of fish oil supplementation.

Disclosure of conflict of interest

None.

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