ORIGINAL ARTICLE

The effects of omega 3 fatty acid supplementation on brain tissue oxidative status in aged wistar rats

Avramovic N[1], Dragutinovic V[2], Krstic D[2], Colovic MB[3], Trbovic A[4], de Luka S[4], Milovanovic I[4], Popovic T[1]

[1] Institute for Medical Research, University of Belgrade, Serbia
[2] Institute of Medicinal Chemistry, Faculty of Medicine University of Belgrade, Serbia
[3] Department of Physical Chemistry, Vinca Institute of Nuclear Sciences, University of Belgrade, Serbia
[4] Institute for Patological Physiology, Faculty of Medicine, University of Belgrade, Serbia

Abstract

Background: The omega 3 fatty acids play an important role in many physiological processes. Their effect is well documented in neurodegenerative diseases and inflammatory diseases. Also, aging as a biophysiological process could be influenced by eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) components of fish oil. However there are not many studies showing the effect of PUFA (polyunsaturated FA) suplementation in eldery brain functions and the response to oxidative strees. The aim of this study was to investigate the effects of dietary omega-3 fatty acid supplementation on levels of lipid peroxidation and oxidant/antioxidant status of brain tissue in aged (24 months old) Wistar rats.

Methods: Animals were divided in two groups. Control group (n=8) was fed with standard laboratory food and received water ad libitum. Treated group (n=8) was also fed with standard laboratory food, water ad libitum and received fish oil capsules (EPA+DHA) for 6 weeks. Daily dose was 30mg EPA and 45mg DHA (capsules: 200mg EPA and 300mg DHA; in-house method). At the end of treatment animals were sacrificed and brains were collected and frozen on -80ºC. The levels of lipid peroxidation (malondialdehyde - MDA), activity of catalase (CAT) and activity of superoxide dismutase (SOD) were examined in cerebral cortex. Catalase activity was determined by measuring the decrease in absorbance (H_2O_2 degradation) at 240 nm for 3 min and expressed as U/mg protein. Total SOD (superoxide dismutase) activity was performed at room temperature according to the method of Misra and Fridovich. The extent of lipid peroxidation (LPO) was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA) by using the method of Aruoma et al. The incorporation of fatty acids in cellular membranes was confirmed by gas chromatography.

Results: Our results showed that lipid peroxidation significantly decreased in treated animal group, where MDA concentration was 0.38±0.001 vs. 0.43±0.001 nM/ml ($p<0.05$) in control. However SOD activity increased significantly in treated animal group 1.57±0.24 vs. 4.12±0.15 U/gHb/L ($p<0.01$) in control. CAT activity decreased in treated group but not significantly.

Conclusion: Incorporation of omega-3 fatty acids after their supplementation had beneficial effects on brain tissue. Omega-3 fatty acids increased activity of SOD and decreased lipid peroxidation. Changes in oxidative/antioxidative balance are a result of EPA and DHA effects on lipids and enzymes of antioxidative system. Hippokratia 2012; 16 (3): 214-245

Keywords: fish oil, omega 3 fatty acids, rats, aging, brain, oxidative stress

Corresponding author: Tamara Popovic, Institute for Medical Research,Laboratory for Nutrition and Metabolism, University of Belgrade, Tadeusa Koscuska 1, Tel: +381113031997, e-mail: poptam@gmail.com

Introduction

Oxidative stress is defined as imbalance between production and collection of reactive oxygen species (ROS). There is significant evidence that fish oil supplementation is beneficial in diseases with ROS imbalance, such as cancer[1,2] and arrhythmia[3]. Also, supplementation has a benefit effect on plasma triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol and uric acid concentrations[4]. Anti-inflammatory dietary mixture, containing fish oil reduces atherosclerotic lesion development in female transgenic mice[5]. Human body cannot perform de novo biosynthesis of essential FAs due to the absence of Δ-15 and Δ-12 desaturase enzymes. Therefore, essential FAs must be provided in diet[6].

Serum phospholipids (PL) FAs profile is related to dietary FAs intake up to 6 weeks, while the PL composition of FAs in erythrocyte depends on the dietary fat intake during months[7]. The FAs profile in tissues reflects not only the dietary fat intake, but FAs metabolism in

Table 1: Plasma phospholipids fatty acids profiles in control and omega-3 treated group.

Fatty acids profile (%)	Control group	Omega-3 group	Significance (p)
16:0	28.04±2.02	27.34±2.57	
16:1	1.27±0.67	0.79±0.14	
18:0	24.1±1.76	27.41±1.82*	p=0.0194
18:1 n-9	10.25±3.31	7.02±0.62	
18:1 n-7	2.42±0.53	2.81±0.56	
18:2	13.24±1.52	16.58±1.64**	p=0.0103
20:3	0.64±0.17	1.15±0.32*	p=0.0139
20:4	14.12±2.27	8.89±1.78**	p=0.0065
20:5	0.24±0.02	0.61±0.15***	p=0.0006
22:4	0.25±0.08	0.5±0.26	
22:5	0.8±0.09	1.59±0.48**	p=0.0071
22:6	4.85±0.33	5.11±1.3	

Student *T* test; *p<0.05, **p<0.01, ***p<0.001

Table 2: The enzymes of oxidative stress (SOD, CAT) and the product of lipid peroxidation (MDA).

	Control group	Omega-3group	Significance (p)
SOD (U/gHb/L)	1.7±0.24	4.18±0.16***	p=0.0001
CAT (U/gHb)	5.87±0.87	4.03±0.39	
MDA (nM/ml)	0.43±0.02	0.38±0.01*	p=0.0303

Student *T* test; *p<0.05, **p<0.01, ***p<0.001; SOD - superoxide dismutase; CAT - catalase; MDA - malondialdehyde.

the body[8]. DHA and EPA are omega-3 PUFA involved in metabolic and physiologic processes. EPA is an important component of nerve cells and serves as a precursor for the synthesis of prostaglandin-3, tromboxane-3 and leukotriene-5 and has beneficial effect in inflammation[9,10], some mental conditions such as schizophrenia[11,12] and suicidal behavior[13]. DHA, present in membrane phospholipids, plays a key role in brain development and structure[14] and maintains cognitive health especially in elderly people[15]. Furthermore, omega-3 FAs contribute to neuronal membrane fluidity and integrity, in part through the generation of docosanoids[16]. This effect is particularly prominent in the retina, where the highest concentrations of omega-3 FAs can be found[17]. Finally, DHA regulates cell transport and synaptic functions[18,19].

EPA and DHA influence on gene expression in the CNS, many of them are transcription factors[20]; it is also suggested that omega-3 FAs effect neurotransmitters and excitotoxicity[21]. However, PUFAs are not antioxidants themselves but they have shown antioxidative effect where classical antioxidants, like vitamin E, have failed[22,23]. On the other hand, it has been reported that the DHA/AA ratio could be a marker of antioxidant defense capability [24]. Mitochondrial function, measured by increased ROS, changes significantly with age, suggesting a potential mechanistic link between the cellular processes governing longevity and mitochondrial metabolism[25]. Excessive amounts of ROS is believed to shorten life span and induce age associated pathological conditions, such as carcinogenesis[26].

The aim of this study was to observe the effects of fish oil supplementation on plasma phospholipids fatty acid profiles and parameters of oxidative stress in brain tissue of aged Wistar rats. We investigated the activity of superoxide dismutase (SOD), catalase (CAT) and levels of malondialdehyde (MDA), a product of lipid membrane peroxidation.

Methods

Animals and diets. The experiments were carried out on 22 months old male Wistar rats, individually housed in stainless steel cages with wired floors, in a room under controlled conditions (12h light–dark cycles, temperature 22 ± 2°C). 16 rats were randomly assigned to experimental (n=8, body weight 350 ± 5g) or control group (n=8, body weight 355 ± 7g). All experiments were carried out according to our Institutional guidelines for animal research and principals of the European Convention for the Protection

of Vertebrate Animals Used for Experimental and others (Official Daily N. L 358/1-358/6, 18, December 1986).

The intervention and control groups were on the same diet. However, each morning the intervention group of rats was fed only by one briquette, which was saturated for intervention group only with 200μL of fish oil, containing 45mg EPA and 30mg DHA. Thirty minutes after breakfast, both intervention and control groups were provided with the same amount of their usual diet and with free access to food and water until 8:00 PM[4]. The intervention lasted for 6 weeks.

To confirm the integration of PUFA supplementation the changes in the plasma phospholipids fatty acid profile were measured in control and treated group of rats by gas chromatography (GC).

Chemicals. Thiobarbituric acid (TBA), H_2O_2 (33%), ethylenediaminetetraacetic acid (EDTA), sodium dihydrogenophosphate (KH_2PO_4), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), hydrochloric acid (HCl), 1,1,3,3 – tetramethoxypropane and n-butanol of anaytical grade used in this study were from Merck (Darmstadt, Germany). Epinephrine and bovine serum albumin were purchased from Sigma-Aldrich (Steinheim, Germany).

Tissue preparation. 0.2g of the tissue was homogenized in 1ml of the solution containing 0.05M sodium phosphate buffer (pH 7.0) and 0.4mM EDTA at 4°C.

Isolation of phospholipids. The method consists of homogenizing plasma with a 2:1 chloroform/methanol mixture and washing with 5times smaller volume of water or NaCl (0.9 g in 100 ml of water). The resulting mixture separates into two phases. The lower phase is the total pure lipid extract. In details, liver tissue (1 g) was lyophilized and chloroform/ methanol (2:1) and butylhydroxytoluene (BHT) as antioxidant were mixed. When the mixture is allowed to stand, a biphasic system was obtained. After evacuation water was added. After centrifugation upper phase was put away until complete separation of the system. Further evacuation was done with 2:1 solvent systems: methanol/benzene, acetone/ benzene, and ethanol/ benzene. Adding of chlorophorm and its evacuation and adding hexane in the sample, made it ready for thin liquid chromatography (TLC)[27].

Fatty acids analysis. The phospholipid fraction was isolated from the extracted lipids by one-dimensional (TLC) neutral lipid solvent system of hexane:diethyl ether:acetic acid (87:2:1) using Silica Gel GF plates (C. Merck, Darmstadt, Germany). The phospholipid fraction was scraped into glass tubes and phospholipid FAs methyl esters were prepared by transmethylation with sodium hydroxide (2 mol dm^{-3}) in methanol (heated at 85 °C for 1 h) and after that sulfuric acid (1 mol dm^{-3}) in methanol (heated 85 °C for 2 h). After 30 minutes, samples of esters were centrifuged, upper phase was put into tubes and evaporated with tehnical nitrogen. FAs methyl esters derivatives formed from isolated plasma phospholipids fraction were separated by Gas Chromatography (GC) using Shimadzu GC 2014 equipped with a flame ionization detector and DB-23 fused silica gel capillary column. The flame ionization detector was set at 250°C, the injection port at 220 °C, and the oven temperature programmed from 130 to 190 °C at the heating rate of 3 °C/min. Comparing sample peak retention times with authenticstandards (Sigma Chemical Company) and/orthe (PUFA)-2 standard mixtures (Restec) identified individual FAs methyl esters.

Antioxidant enzymes. Catalase activity was measured by the H_2O_2 degradation assay[28]. In brief, 50μl tissue homogenate was added to a quartz cuvette containing 2.975ml 0.05M sodium phosphate buffer (pH 7.0) and 0.4mM EDTA and 25μl of 3% H_2O_2 was added to start the reaction. Catalase activity was determined by measuring the decrease in absorbance (H_2O_2 degradation) at 240nm for 3 min and expressed as U/mg protein. One unit (U) of catalase activity was defined as 1μmol of H_2O_2 consumed/min.

Total SOD (superoxide dismutase) activity was performed at room temperature according to the method of Misra and Fridovich[29]. 10-30μl of tissue homogenate was added to 3ml of EDTA – sodium carbonate buffer (0.5M) at pH 10.2. The reaction was started by adding 100μl of epinephrine (30mM in 0.1M HCl) and the activity was measured at 480nm for 4 min. One unit of SOD is defined as the amount of enzyme that inhibits by 50% the speed of oxidation of epinephrine. Results were expressed as U/mg protein.

Lipid peroxidation assay. The extent of lipid peroxidation (LPO) was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA) by using the method of Aruoma et al.[30]. 0.5ml of thiobarbituric acid (TBA) was added to 0.5ml of tissue homogenate 0.5ml of HCl (25% in water). The mixture was placed in glass tubes, sealed with screw caps and heated in boiling water for 10 min. After cooling, the chromogen was extracted in 3ml of n-butanol and the organic phase was separated by centrifugation at 2000 x g for 10 min. The absorbance of the organic phase was read spectrophotometrically at 532nm wave lgth. The values are expressed as nmol of tba acid – reactive substance (MDA eguivalent)/mg of protein using a standard curve of 1,1,3,3 – tetramethoxypropane.

Protein concentration. Total protein was quantified by the procedure of Lowry's method[(31)] using bovine serum albumin as standard.

Statistical methods. All results are expressed as means±SD. Normality was tested using the Kolmogorov-Smirnov test. Since all parameters were normally distributed, the differences between groups were determined by using Student *t*-test. The differences were considered significant at $p \leq 0.05$.

Results

The changes in plasma phospholipids FA profile after supplementation was shown in Table 1. Percentage of 18:0 after treatment was significantly increased ($p<0.05$) compared to overall presence of all FAs. Percentage of 18:2 was also increased ($p<0.01$), as well as percentage of 20:3 ($p<0.05$). Opposed to that 20:4 was significantly

decreased ($p<0.01$). Supplementation and its efficiency were shown through the increasing in the percentage of 20:5 ($p<0.001$) and 22:5 ($p<0.01$) FAs.

As we show in Table 2, the treatment with fish oil (EPA + DHA) changed the level of lipid peroxidation in treated animals compared to control. In treated group, lipid peroxidation was significantly decreased compared to control group ($p<0.05$). To confirm that, we measured the level of lipid peoxidation using the concentration of MDA considering that reactive oxygen species degrade PUFA forming MDA. The activity of SOD was also changed with fish oil treatment. It was significantly increased compared to control group ($p<0.001$). Measured activity of CAT was not significantly changed in fish oil.

Discussion

Many studies have shown the broad specter of beneficial effects of omega-3 FAs. Salvati et al. have associated omega-3 FAs and the reduction of risk of neurological diseases. They have found that dietary omega-3 FAs have significant effect on the metabolism of pro- and anti-inflammatory eicosanoids, that influence the function, fluidity and oxidative susceptibility of neural membranes[6]. Furthermore, Choikwon et al. reported that 6 weeks of PUFA supplementation significantly decreased the infarction size in rat brains after ischemia/reperfusion injury compared to controls[32]. The levels of PUFA decrease during aging[33]. It is known that PUFAs, especially DHA and EPA, are taken up by the brain and are incorporated into the neuron membranes[32, 34, 35].

There are studies that show negative effects of omega-3 FAs. Yang et al induced ischemia/reperfusion damage in rat brains and found that FA treatment after injury aggravated neurological deficits and oxidative burden along with other parameters[36]. The origin of eicosanoids seems to be crucial as there are evidence that arachidonic acid derived eicosanoids are associated as pro-inflammatory, while those originating from DHA acid are characterized as anti-inflammatory[37].

In our study, we confirmed n-3 supplementation in the changes of plasma phospholipids FA profiles in control and treated group. We also observed that the activity of SOD increased significantly and the levels of MDA decreased significantly. However, the activity of CAT decreased insignificantly. We assume that H_2O_2, created by SOD, is collected by some other pathway. It is well known that H_2O_2 can be generated in peroxisomes, mitochondria and endoplasmic reticulum[38]. Also, H_2O_2 can be a product of cell metabolism by action of some oxidases as well as in reactions of glutathione and cateholamines. It can also be generated by the activity of monoamine oxidases (MAO) which can lead to oxidation of sulfhydrile groups of proteins and to initiate lipid peroxidation[39]. In direct action of H_2O_2 with superoxide anion radical or metal ions (Fe^{2+}) can form extremely reactive hydroxyl radical[40].

Fish oil treatment may have made the neural tissues more susceptible to lipid peroxidation and leading to beneficial effects[41]. It is well known that neural tissue is poor in antioxidant enzymes, thus it is important to increase antioxidant enzyme capacity for primary defense against free radical injury. Ozen et al. have shown decreased level of MDA followed by I/R after fish oil supplementation[42]. It agrees with our results where fish oil supplementation in aged rats decreased MDA concentration in brain tissue. However, the same authors' results showed decreased activity of SOD in I/R injury followed by fish oil supplementation compared to I/R group and control. This is not in line with our results where the activity of SOD was increased after fish oil supplementation. We suppose that I/R injury induce the creation of ROS much more than ageing itself. Cosar et al. examined fish oil, supplementation in hippocampus of diabetic rats[43]. Their results showed increased levels of MDA, SOD and CAT in diabetic rats, while after supplementation with fish oil MDA levels were significantly lower, SOD activity was significantly lower and CAT activity was lower compared to diabetic group[43]. Bas et al. showed that after occlusion of carotid artery followed by fish oil supplementation, MDA concentration was decreased compared to the intervention group without supplementation[41]. It is well known that DHA is taken up by the brain and is incorporated into the neuron membranes[32,34]. Therefore, it may be surmised that extra omega-3 PUFA may increase lipid metabolism species, especially thiobarbituric acid reactive substances. In the same study SOD activity was significantly higher in intervention group after supplementation. In Bas et al. study dietary supplementation definitely has neuroprotective effects and can ameliorate ischemic cerebrovascular diseases[41].

Conclusion

Our results support the notion that dietary fish oil supplementation may enhance resistance to free radical attack and reduce lipid peroxidation and also that omega-3 fatty acids may be effective dietary supplement in the management of various diseases in which oxidant/antioxidant balance is disturbed as in aged brain tissue.

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Conflict of interest

There is no conflict of interest.

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