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Original Scientific Article

Fatty Acid Composition and Oxidative Stress Parameters in Plasma after Fish Oil Supplementation in Aging

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Abstract. Fish oil affects oxidative stress parameters and changes in phospholipids fatty acids profiles in plasma, erythrocytes and tissues. We examined the effects of fish oil supplementation in young and old male Wistar rats (3 and 22 months old) on plasma phospholipids fatty acids profiles and blood oxidative stress parameters. Twenty young and twenty aged Wistar rats were randomly divided into four groups (ten animals each): two control groups and two supplemented groups treated for 6 weeks with fish oil capsules containing 45 mg eicosapentanoic acid (EPA) and 30 mg docosahexanoic acid (DHA). Fish oil supplementation changed the percentage of long chain fatty acids (FAs): the elevated percentage of eicosatrienoic acid (ETA, 20:3), eicosapentanoic (EPA, 20:5), docosapentanoic acid (DPA, 22:5), *n*-3 fatty acids and decreased arachidonic acid (AA, 20:4). However, there were no age-related changes in total SH groups, the percentages of palmitic (16:0), palmitoleic (16:1), oleic (18:1 (*n*-9)) and linoleic acid (18:2) in plasma phospholipids and MUFA and they were neither reversed nor prevented by fish oil supplementation. Results showed that fish oil supplementation increased SOD and CAT activities in erythrocytes, and PON activity in the blood plasma of both young and aged rats. Furthermore, fish oil supplementation decreased lipid peroxidation (MDA) and nitrite levels in both young and aged rats implying better antioxidant protection and a lower level of oxidative pressure after fish oil supplementation. Our results suggest that fish oil supplementation is beneficial regarding better antioxidant protection in both young and aged rats, while applied treatment differs in plasma phospholipids FAs composition.

Keywords: fish oil, aged rats, oxidative stress, fatty acids

INTRODUCTION

Aging is a natural, complex, and multifactor biological process. Many of the studies conducted on cultured human cells and animals have revealed that aging is associated with an impairment of bioenergetic functions, increased oxidative stress, attenuated ability to respond to stresses, and increased risk of contracting cancers and age-related diseases[1] The free radical theory of aging states that age-related degenerative processes are to a large extent a consequence of damage induced by free radicals.[2,3]

Several changes are associated with aging, including a reduced capacity to use oxygen along with an impaired cardio circulatory capacity and respiratory adaptation, deterioration of the nervous system, and degeneration in the muscle mass.[4] Dietary fats in mammals provide essential fatty acids for membrane synthesis, protein modification and signaling compounds.[5] High dietary polyunsaturated fatty acids series (*n*-3 PUFA) increase erythrocyte membrane susceptibility to peroxidation and lipid products in liver or kidney[6] as well as antioxidant enzyme activity in the blood.[7] Despite lacking mitochondria, erythrocytes are a target for free radical exposure due to the auto-oxidation of hemoglobin under high oxygen pressure. Dietary fats greatly modify erythrocyte membrane composition affecting their susceptibility to oxidation.[8]

The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is a normal process in the life of aerobic organisms; in fact, ROS and RNS are continually produced as a consequence of aerobic metabolism: up to 5 % of oxygen reacting with the respiratory chain is incompletely reduced to ROS.[9] Aging process is remarkably slow, indicating that even the

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aged animals or elderly have adopted a nearly stable state of redox homeostasis.[3]

The inverse correlation found between the lifespan and *n*-6/*n*-3 ratio in membrane phospholipids, suggest different effects of *n*-6 and *n*-3 PUFA in living systems.[10,11]

The aim of our study was to examine age-related fatty acids composition of plasma phospholipids and antioxidative enzyme status in the blood after fish oil supplementation in Wistar rats and to clearly define changes in those parameters induced by age, treatment or both.

MATERIALS AND METHODS

Animals and Diets

Experiments were carried out on young (3 months old) and aged (22 months old) male Wistar rats, individually housed in stainless steel cages with wired floors, in a room under controlled conditions (12 h light–dark cycles, temperature 22 ± 2 °C). Forty Wistar rats were randomly selected for the experimental (n = 10, b.w. 283 ± 5 g) or the control groups (n = 10, body weight 278 ± 7 g) for young and the experimental (n = 10, b.w. 318 ± 4.05 g) or the control group (n = 10, b.w.313 ± 6.15 g) for aged rats; thus, the groups were matched in terms of age and body weight. All experiments were conducted following our Institutional guidelines for animal research and principles 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 animals were treated with fish oil for 6 weeks. The intervention and control group were on the same diet. However, each morning the rats were fed only by one briquette, which was saturated for the intervention group only with 200 μl of fish oil (daily dose 13.5 mg EPA + 9.0 mg DHA/kg b.w.) Thirty minutes after breakfast, both the intervention and the control group were provided with the same amount of their usual diet and water ad libitum until 8 pm.

Blood samples (6–8 cm^3) from all rats were obtained before and after the end of intervention via aorta *abdominalis* puncture and collected in tubes containing Na-citrate (3.8 % *w*/*v*) as anticoagulant. Erythrocytes were separated by centrifuge (1300 g) and washed in saline solution three times, and the enzyme activities were determined.

Antioxidative Parameters Determination

Antioxidative potential in erythrocytes and plasma was determinated via CAT and SOD activities, MDA (malondialdehide concentration), nitrites concentration, SH groups determinations and PON1 activity.

The CAT activity in erythrocytes was determined following standard procedures.[12] One unit of CAT activity was defined as the activity required for degradation of 1 mol H_2O_2 for 60 s at 25 °C and pH 7.0. Activity was expressed as U/gHb. The SOD activity in erythrocytes was determined using Calbichem's Superoxide Assay Kit which utilizes 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorine, recording at 525 nm.[13] SOD activity was also expressed as U/g Hb. MDA in erythrocytes was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS)[14,15] and expressed in nM MDA/g Hb. The total Hb content was measured by the cyanmethemoglobin method.[16] Nitrite ion (NO_2) levels in plasma were analyzed using the ELISA method and Griess reagent.[17,18] Absorbance was measured on a micro plate reader (Plate reader Mod. A1; Nubenco Enterprises Inc, Paramus, NJ, USA) at a wavelength of 545 nm.Obtained results were expressed as l M/l. Free sulfhydryl groups were assayed in plasma according to the method described by Ellman *et al.*[19] The concentration of total SH groups was expressed as μM/L.

The PON1 activity in the plasma was assayed by a slightly modified method of hydrolysis of paraoxon.[6] Briefly, the plasma sample was added to 0.1 M Tris–HCl buffer, pH 8.0 containing 2.0 mM paraoxon (*O*,*O*-diethyl-*O*-*p*-nitrophenylphosphate; Sigma Chemical Co, London, UK) as a substrate, 2.0 mM $CaCl_2$ and 1 mM NaCl. The formation of *p*-nitrophenol was monitored bichromatically at 410/480 nm at 37 °C on Olympus AU600, (Dallas, TX, USA). The PON1 activity was expressed in international units (U/l) as the amount of substrate hydrolyzed per minute and per liter of plasma (lmol/min/l).

Enzyme activities (CAT, SOD, PON1) were measured on CECIL CE 2021 UV/VIS spectrophotometer (BMG LabTech GmbH, Offenburg, Germany), while electrophoresis was performed on a vertical device Mini Ve Hoffer, (LKB 2117; Bromma, Uppsala, Sweden).[19,20]

Fatty Acid Analysis

Plasma phospholipids were extracted using chloroform/methanol mixture (2:1, *v*/*v*) with 50 mg/100ml 2, 6-di-tert-butyl-4-methylphenol (BHT) added as antioxidant.

The phospholipids 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 by sulfuric acid (1 mol/dm^3) in methanol (heated 85 °C for 2 h). After 30 minutes, samples of

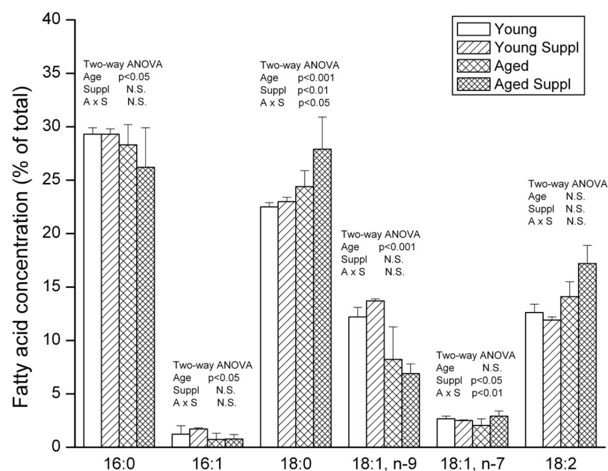

Figure 1. The percentage of palmitic acid (16:0), palmitoleic (16:1), stearic (18:0), vascenic (18:1,n7) and linoleic acid (18:2) in plasma phospholipids of young and aged rats supplemented by fish oil. Results were tested by two-way ANOVA with age (A) and supplementation (S) factors; F values and p significance were showed.

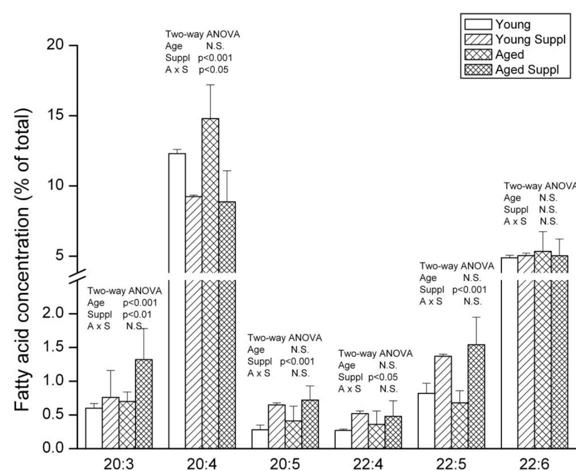

Figure 2. The percentage of ETA (20:3), AA (20:4), EPA (20:5), DTA (22:4), DPA (22:5) and DHA (22:6) in plasma phospholipids of young and aged rats supplemented by fish oil. Results were tested by two-way ANOVA with age (A) and supplementation (S) factors; F values and p significance were showed.

esters were centrifuged, upper phases were put into tubes and evaporated with technical 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 was programmed from 130 to 190 °C at the heating rate of 3°C/min. Comparing sample peak retention times with authentic standards (Sigma Chemical Company) and/or the (PUFA)-2 standard mixtures (Restec) identified individual FAs methyl esters.[21,22]

Statistical Analysis

Data are expressed as the means ± SD. Statistical significance was tested with two-way ANOVAs using the age of animals and treatment as factors on logarithmic or trigonometric transformed data. The trends were considered as significant if $p < 0.05$. The values were post hoc compared by Tukey HSD test.

RESULTS

The results of the study are expressed as age-related or not aged-related and dependent or not dependent on fish oil supplementation.

Regarding phospholipids fatty acids profiles, there were no age-related changes in 18:1, *n*-7 (vascenic acid), 18:2 (LA), 20:4 (ETA), 20:5 (EPA), 22:4 (DTA), 22:5 (DPA) and 22:6 (DHA) (Figures 1 and 2). Aging did not influence SFA and PUFA; however age-related changes were found on overall MUFA and *n*-6/*n*-3 ratio. (Figures 3 and 4).

Fish oil supplementation changed the percentages of stearic acid (18:0), vascenic acid (18:1,*n*-7), ETA (20:3), AA (20:4), EPA (20:5) and DPA (22:5); however, supplementation did not change the percentages of palmitic acid (16:0), palmitoleic acid (16:1), oleic acid (18:1, *n*-9), 18:2 (LA), and docosahexanoic acid (22:6) (Figures 1 and 2). A decrease in *n*-6/*n*-3 ratio and an increase in *n*-3 fatty acids with no changes in *n*-

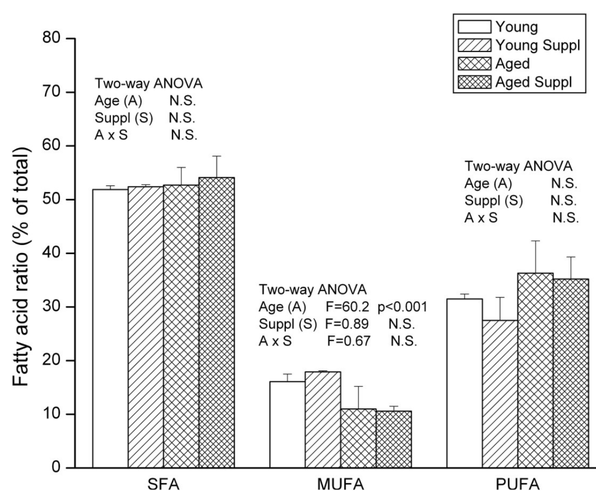

Figure 3. SFA (saturated fatty acids), MUFA (monounsaturated fatty acids) and PUFA (polyunsaturated fatty acids) percentage in plasma phospholipids of young and aged rats supplemented by fish oil. Results were tested by two-way ANOVA with age (A) and supplementation (S) factors; F values and p significance were showed.

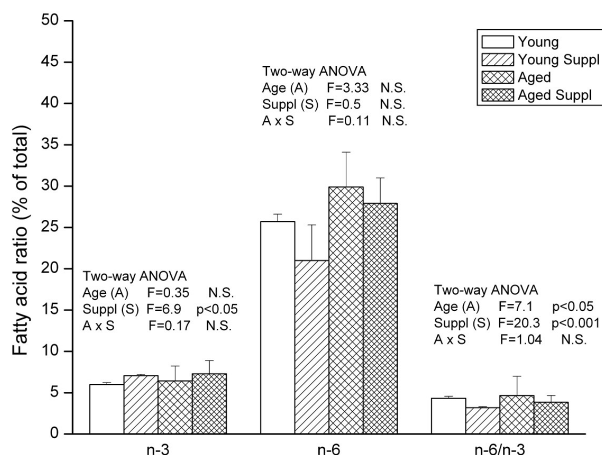

Figure 4. Fatty acids ratio (n-6/n-3) in plasma phospholipids of young and aged rats supplemented by fish oil. Results were tested by two-way ANOVA with age (A) and supplementation (S) factors; F values and p significance were showed.

6 fatty acids were further consequences of supplementation.

Results showed that fish oil supplementation led to increased activities of SOD and CAT in erythrocytes, and PON activity in serum of both young and aged rats (Figure 5; significant supplementation effect $p < 0.001$). Furthermore, supplementation decreased MDA and nitrite levels in both young and aged rats (Figure 6; significant supplementation effect $p < 0.001$). A decrease in nitrites concentration was more profound in aged rats (significant $p < 0.001$ interaction A × S effect in ANOVA, Figure 6). Fish oil supplementation had no effect on the level of total SH groups (Figure 6).

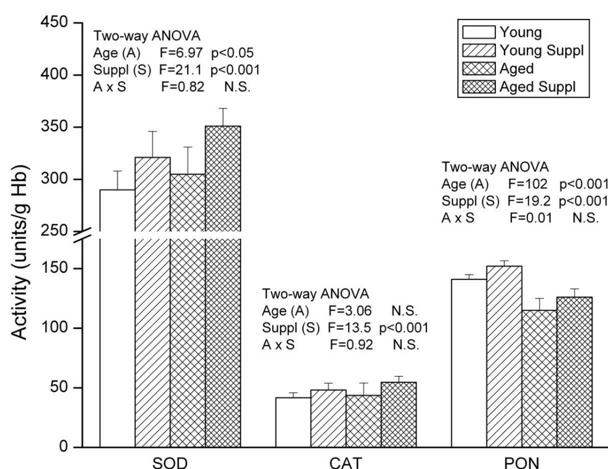

Figure 5. SOD (superoxide dismutase) and CAT (catalase) activity in erythrocytes and PON (paraoxonase) activity in the blood plasma of young and aged rats supplemented by fish oil. Results were tested by two-way ANOVA with age (A) and supplementation (S) factors; F values and p significance were showed. (*PON activity, mol/min/L)

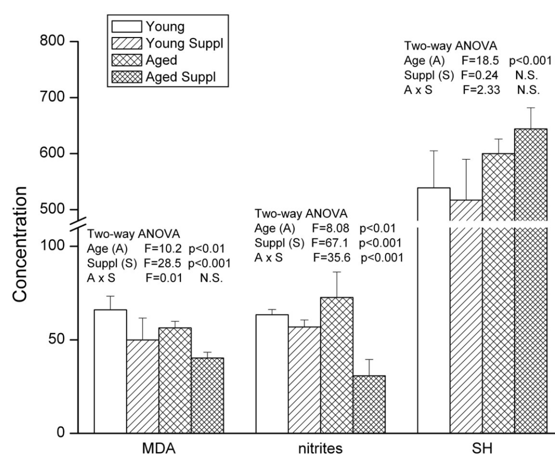

Figure 6. The concentration of MDA (malondialdehyde), nitrites and total SH (thiol) groups in the blood plasma of young and aged rats supplemented by fish oil. Results were tested by two-way ANOVA with age (A) and supplementation (S) factors; F values and p significance were showed. (*MDA, U/gHb; *SH groups, μM/L;*nitrites, mol/L)

Our results suggest that fish oil supplementation was beneficial regarding better antioxidant protection and a lower level of oxidative stress.

DISCUSSION

The relationships between body size, energy metabolism and lifespan have been analysed elsewhere (Speakman *et al.* 2005).[23] The conclusion in Speakman's study was that there must be other factors to account for species' differences in maximum lifespan. One of the factors is that membrane composition correlates with maximum lifespan in mammals and birds.[23] In the determination of longevity, FAs composition is an important factor for three basic reasons: 1) membrane FAs varies among species, 2) FA differ in their susceptibility to peroxidation and 3) many products of lipid peroxidation are powerful ROS.[7]

Polyunsaturated fatty acids of n-3 and n-6 families play multiple roles in mammals. They are rapidly incorporated into cell membranes and influence many biological functions from membrane fluidity, activation of intracellular pathways directly or via production of eicosanoids, to gene expression and cell differentiation.[24] Fish oil which we used in the supplementation treatment consists of two polyunsaturated fatty acids EPA and DHA.

EPA acts as a precursor for prostaglandin-3 inhibitor of platelet aggregation, thromboxane-3 and leukotriene-5 groups (all eicosanoids), partially blocking the conversion of n-6 FA to harmful eicosanoids, thereby reducing the cardiovascular risk and inhibiting tumour genesis.[11,25] DHA plays a role in the development of brain

tissue and is important in maintaining cognitive health in the human population.[24] DHA regulates cell transport and synaptic functions.[26] *n*-3 PUFAs increase the activity of lipoprotein lipase and improve reverse cholesterol transport. Its imunomodulatory effects are involved in the suppression T-lymphocytes activation.[27,28]

There are variations in FAs composition among species. Correlations between birds and mammals in *n*-6 and *n*-3 were discussed by some authors.

Although the membrane FAs composition is related to the body mass of birds and mammals, their FAs composition differs. In birds, balance *n*-6/*n*-3 is shifted to *n*-6 PUFA which correlates with the relatively long lifespan of birds.[7]

In our study the percentage of EPA significantly increased after fish oil supplementation, while the percentage of DHA did not change. However, overall *n*-3 plasma phospholipids increased after supplementation, while *n*-6/*n*-3 ratio decreased significantly. Possible explanation for this could be the starting percentage of EPA which was lower than the starting percentage of DHA. Supplementation increased EPA levels and its incorporation more efficiently than DHA.

Supplementation elevated the percentage of stearic acid (18:0), ETA, (20:3), EPA (20:5), DPA (22:5) (Figures 1 and 2), *n*-3 fatty acids (Figure 4) and decreased AA (20:4) (Figure 2). However, no age-related changes in the percentage of palmitic acid (16:0), palmitoleic (16:1), oleic acid (18:1 (*n*-9)), linoleic acid (18:2) (Figure 2) and MUFA profiles (Figure 3) were either reversed or prevented by fish oil supplementation. FAs incorporation is also tissue dependent and sometimes it is more efficient in tissues or erythrocytes than in plasma.

Incorporation of PUFA into phospholipids leads to conformational changes and lowered availability of double bonds for lipoperoxidation.[29]

Contrary to our findings, examinations of Hsu *et al.*[10] showed that dietary supplementation with *n*-3 PUFA had no effect on lipid membrane peroxidation or had a positive effect on glutathione level and antioxidative enzymes activity. The question that remains unclear is if the increased sensitivity of membranes to lipid peroxidation (or the increased quantity of lipid peroxidation products) is a result of increased ROS activation from phagocytes or dysfunction of AOS in the blood or both.[10] Erythrocytes are attacked with ROS considering auto oxidation of hemoglobin influenced by high oxygen pressure in the arterial blood. Increased ROS in erythrocytes leads to membrane lipid peroxidation and can damage other intracellular proteins.[2]

Intensive lipid peroxidation decreases the fluidity of biological membranes, increases ionic permeability and leads to the inactivation of enzymes in the membrane. Also, lipid peroxidation decreases membrane potential, increases permeability for hydrogen ions and increases damage of cells.

After fish oil supplementation, lipid peroxidation (MDA) and nitrites concentration decreased in both young and aged rats (Figure 6; significant supplementation effect $p < 0.001$). The decrease in nitrites concentration was more profound in aged rats (significant $p < 0.001$ interaction A × S effect in ANOVA, Figure 6). Fish oil supplementation had no effect on the level of total SH groups (Figure 6).

In the study of Gamba *et al.* fish oil supplementation was unable to protect against the natural age-induced modification in renal hemodynamics and was unable to reduce lipoprotein concentration in patients with chronic glomerular disease.[30] In young animals, renal hemodynamics is not controlled by eicosanoids, but with age it becomes dependent on vasodilatory prostaglandins (PG) and the renal vasoconstrictor effect of thromboxane A_2.

In the study of Oarada *et al.*, Wistar rats received EPA or DHA by gavage.The treatment increased EPA and DHA, and itdecreased AA and *n*-6/*n*-3 ratio in plasma phospholipids membranes.[8]

It should be mentioned that NO can play a multifaceted role in the regulation of the mitochondrial ROS generation in young and aged Wistar rats.[31]

In the study of Lukiw *et al.*, plasma nitrates concentration was increased in aged and SH rats.[26] Early decline in eNOS activity and protein expression was not found in WKY but was found in SH rats.[17] In the aging process, a decreased eNOS activity was observed in WKY, while iNOS increased in the same group of animals. The higher basal levels of NO_2/NO_3 may result from higher expression of iNOS in SH rats[17] and probably in aged Wistar rats, too. Impaired NO bioavailability in aging may contribute to age-related alterations in the mitochondrial mass in blood vessels.[32] RNS markers (NOx and nitrotyrosine) in the blood and some tissues increased from young to middle-aged rats but decreased from middle aged to old aged rats.[33,34]

In the study of Hamden *et al.*, aging significantly decreased SOD, CAT and GSH-Px and increased LDH, GGT AST, ALT in the liver[35] Prooxidative/antioxidative balance in erythrocytes is important for other tissues considering erythrocytes as mobile detoxified elements in circulation.[36,37]

Our results showed that fish oil supplementation led to increased SOD and CAT activities in erythrocytes, and PON activity in the plasma of both young and aged rats (Figure 5; significant supplementation effect $p < 0.001$).

Primary high reactive intermediers occur during lipid peroxidation. Their degradation leads to aldehydes which react with amino-groups of proteins changing their structural and functional properties.[38,39] Sarsilmaz *et al.* 2003 showed that treatment with *n*-3 FA decreased

lipid peroxidation in corpus striatum in rats. Dietary supplementation with fish oil can increase antioxidative defense and reduce lipid peroxidation.[38] Supplementation of *n*-3 FAs contributes to the preservation of erythrocyte membranes, their structure, fluidity and functionality in rats of different ages.[40]

CONCLUSION

Fish oil supplementation changes plasma phospholipids profiles in the blood plasma elevating ETA, EPA, DPA, *n*-3 and decreasing the percentage of AA. Age-related changes of total SH, the percentage of palmitic, palmitoleic and oleic acids in plasma phospholipids and MUFA were neither reversed nor prevented by fish oil supplementation. SOD, CAT activities in erythrocytes and PON activity in the plasma were increased in both young and aged rats. Decrease of MDA and nitrites levels after fish oil supplementation was present in young and aged rats. Our results suggest that fish oil supplementation is beneficial regarding better antioxidant protection lowering the level of oxidative stress in both young and aged rats.

Abbreviations: EPA = eicosapentanoic acid, DHA = docosahexanoic acid, Fas = fatty acids, ETA = eicosatrienoic acid, DPA = docosapentanoic acid, AA = arachidonic acid, LA = linoleic acid, PUFA = polyunsaturated fatty acids, MUFA = monounsaturated fatty acids, SFA = saturated fatty acids, NO = nitric oxide, SOD = superoxide dismutase, CAT = catalase, PON = paraoxonase, MDA = malondiadehyde, ROS = reactive oxidative species, RNS = reactive nitric species, AOS = antioxidative system

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