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Long-term consumption of fish oil partially protects brain tissue from age-related neurodegeneration*

Długotrwałe spożywanie oleju rybnego może chronić mózg przed zmianami neurodegeneracyjnymi związanymi z wiekiem

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Summary

The purpose of this study was to determine the effect of fish oil supplementation on aging in the cerebral cortex, hippocampus and cerebellum of rats. Results of biochemical and histological analyses of brain tissue collected from young rats (age: 2 months) prior to the experiment were compared with the results obtained from the 14-month-old animals assigned to the control and supplemented group. Total polyunsaturated fatty acid (PUFA) composition and thiobarbituric acid reactive substance (TBARS) levels were assessed in the examined brain regions. Furthermore, the presence of lipofuscin and amyloid-β, as well as the number of apoptotic and proliferative cells, was determined in the brain tissue. The analyses revealed that the number of proliferative neurons was significantly higher in the fish oil treated group in the cerebral cortex, hippocampus proper and dentate gyrus. Furthermore, in animals fed the fish oil-supplemented diet, amyloid-β plaques were not observed in the examined brain regions. These results suggest that fish oil supplementation has a neuroprotective effect on the process of proliferation and may protect against spontaneous amyloidosis in the brain.

Keywords: **PUFA • lipid oxidation • PCNA • apoptosis**

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Abbreviations: **AA** – arachidonic acid; **Aβ** – β-amyloid; **AD** – Alzheimer disease; **ALA** – alfa-linolenic acid; **AP** – amyloid plaque; **APP** – amyloid protein precursor; **BDNF** – brain-derived neurotrophic factor; **CNS** – central nervous system; **DG** – dentate gyrus; **DHA** – docosahexaenoic acid; **FAME** – fatty acid methyl ester; **LA** – linoleic acid; **LP** – lipofuscin; **NPD1** – neuroprotectin D1; **PAS** – periodic acid-Schiff; **PBS** – phosphate buffered saline; **PCNA** – proliferating cell nuclear antigen; **PUFA** – polyunsaturated fatty acids; **ROS** – reactive oxygen species; **SH-SY5Y** – human derived cell line cloned from bone marrow; **TBARS** – thiobarbituric acid reactive substances; **Tg2576** – transgenic mouse models of AD.

Introduction

The central nervous system (CNS) has the second greatest concentration of lipids, immediately after adipose tissue. These brain lipids contain very high amounts of long-chain polyunsaturated fatty acids (PUFA), particularly arachidonic acid (AA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3) [8,18,36]. It has been well documented that these lipids are present in the membrane cells depending on their histological structure and function, as well as metabolism intensity of the different brain regions [2,16]. The differences between regions depend on their amount of gray or white matter. The cerebral white matter (consisting mostly of glial cells and myelinated axons) is rich in saturated fatty acids and particularly in n-9 monounsaturated fatty acid, and poor in PUFA. However, in regions with greater amounts of gray matter (made up of neuronal cell bodies), the PUFA levels are higher [8]. The process of aging results in significant differences in the amount of PUFA and other lipids when compared to their content in young individuals [4]. A significant age-dependent decrease in the levels and turnover of PUFAs has been observed in the hippocampus, cortex, striatum and hypothalamus [38, 39]. In addition to the age-related reduction in PUFA levels, aging has also been associated with progressive decline in saturated fatty acids, and reciprocal increase in monounsaturated fatty acids [24]. The data published so far have shown the positive effect of PUFA supplementation on brain aging, in both *in vitro* and *in vivo* experiments [5,4,20].

PUFA are essential for the development of the human CNS. A deficit in dietary DHA appears to contribute to inflammatory signaling, apoptosis and neuronal dysfunction, and is associated with a cognitive decline in old age-related neurological disorders. PUFA can rapidly accumulate in membrane phospholipids, and potentially affect a variety of physical properties and functions of cell membrane, as well as signaling molecules and regulators of gene expression, and patterns of lipid mediator production [34]. On the other hand, PUFA are more vulnerable to free radical oxidation than any other molecules in the body, and the sensitivity to free radical damage increases exponentially with the number of double bonds [32]. The study of Tsuduki et al. (2011) showed that these oxidative changes may be related to aging. Aging-related accumulation of oxidative species (oxygen ions and peroxides) in the CNS may contribute to membrane pathology, as well as DNA damage and mutations [30,27]. Oxidative stress in the mature brain may cause irreversible changes, such as β-amyloid formation, lipofuscin deposition, necrosis and apoptosis of neurons. Hence, data concerning the safety of long-term ingestion of fish oil, which is a rich source of PUFA, are important for proper use of this type of supplementation. Therefore, in the present study we evaluated the effects of long-term ingestion of salmon oil on oxidative stress, and its further impact on the formation of β-amyloid, lipofuscin and caspase-3 expression, and proliferation of brain cells in various regions of the brain of rats fed a diet supplemented with this oil for 12 months.

Materials and methods

Animals and diets

All experimental procedures were carried out in accordance with the policies of the Animal and Ethics Review Committee at Warsaw University of Life Sciences, Poland. Twenty-nine male Sprague- Dawley rats (Charles River Laboratories, UK) at six weeks of age, weighing 100-130 g, were housed individually in stainless steel cages at a constant temperature of 22°C, with average relative humidity of 70%, and a 12:12 light-dark cycle. Food and water was consumed *ad libitum*. Following 2 weeks of acclimatization 11 rats (young group) were euthanized by intraperitoneal injection of sodium pentobarbital, whereas the remaining 18 animals were randomly divided into two groups (2 x 9) and fed a control (CON) or supplemented (SUP) diet for the next 12 consecutive months. Both diets were semi-synthetic, prepared monthly in our laboratory, and stored at 4°C. The diet of the supplemented group was additionally enriched with salmon (*Salmonidae*) oil. The detailed composition of the diets and fatty acids content were described in our previous report [12]. Differentiation of lard content in both diets was intended to strengthen or weaken the effect of the n-3 fatty acids. Rats aged 14 months were euthanized under anesthesia, then brains were isolated, and divided into two parts (left and right). The left hemisphere of the brain was dissected into the cerebral cortex, hippocampus and cerebellum and used for biochemical analysis; the other hemisphere was

used for histological analyses. The tissues for biochemical analysis were stored at -80°C until further use, while those for histology and immunohistochemistry were fixed in 4% formaldehyde with PBS, dehydrated with ethanol and xylene, and embedded in paraffin.

Determination of lipid composition by gas chromatography

The fatty acid composition of the diet and the fatty acid content in examined brain tissue were analyzed using gas chromatography, as previously described [12]. In brief, a sample of diet (1 g) or brain homogenates (200 mg) was extracted with a chloroform- methanol (2:1) mixture. Then, the samples were saponified, and methylated to fatty acid methyl esters (FAMEs). FAMEs were separated on a 100 m x 0.25 mm I.D. x 0.2 µm CP-Sil 88 column, gas chromatograph equipped with autosampler and FID detector. Identification of peaks corresponding to FAMEs was accomplished by means of a standard mixture of 37 FAMEs. The relative concentration of individual fatty acids was expressed as the percentage of total fatty acids.

Determination of lipid oxidation

Thiobarbituric acid reactive substances (TBARS) were determined as an index of lipid peroxidation [10]. Brain lipid extract (0.5 ml in chloroform with BHA) was evaporated, then 1.5 ml of 0.05 M sulfuric acid and 1.5 ml of 1% thiobarbituric acid were added and mixed. The mixture was vortexed, incubated for 1 h in boiling water, then extracted with n-butanol. The absorbance of the samples was determined at 532 nm wavelength against a blank sample. The TBARS concentration was calculated from a standard curve using 1,1,3,3-tetraethoxypropane as the standard.

Histology

Brain samples after formalin fixation were sliced in coronal planes into 5 µm thick sections using a Leica RM2265 microtome. Sections were prepared from various levels of the cerebral cortex, hippocampus proper and dentate gyrus, cerebellum, and were stained using periodic acid-Schiff (PAS) for lipofuscin (30 areas per 1 mm^2 of each brain region) [19] and alkaline Congo red for amyloid. For the purpose of quantification, lipofuscin (LP) deposition was arbitrarily graded from grade 0 = no LP detected, 1x = small amount, to grade 2x = large amount of LP [14]. The β-amyloid deposits were categorized as amyloid plaques (AP) – detected in neuropil. The density of AP was calculated as the average number of AP per square millimeter in seven randomly selected regions. The counts were determined using 400x magnification. The quantification was made based on images taken by a digital camera (Nikon DS5-U1) connected to a Nikon ECLIPSE 90i microscope and the computer image analysis system NIS-Elements AR (Nikon Corporation, Tokyo, Japan).

Immunohistochemistry

Proliferating neuronal cells were identified using antibodies against PCNA based on a procedure previously described by Ostaszewska et al. [2008]. Tissue sections were deparaffinized with xylene and rehydrated. Endogenous peroxidase was blocked with a 3% solution of H_2O_2. The sections were incubated in Tris-buffered saline pH 8.0 for 10 min. The histological slides were incubated with PCNA antibody, clone PC10 (Monoclonal Mouse Anti-Proliferating Cell Nuclear Antigen—DAKO M0879) for 1 h at room temperature, using 1:300 dilution. The sections were subsequently incubated in a DAKO Envision System (DAKO Envision TM+/HRP K4006) until a change of color occurred. For negative control sections, the primary antibody was substituted with TRIS–BSA. PCNA-positive responses were scored for 10 fields of 0.035 mm^2 per brain section and recalculated to yield the number of PCNA-positive nucleus of neurons per 1 mm^2. Apoptotic cells were detected using rabbit polyclonal antibody anti-caspase3 (C8487; Sigma). Sections for this purpose were incubated overnight with rabbit polyclonal antibody anti-caspase3 at 4°C, and visualized using DAKO EnVision1System-HRP (DAKO K 4010, Glostrup, Denmark), while the further procedure was identical as for PCNA detection [29].

Statistical analysis

Data are presented as mean ± SD. Statistical comparisons were performed by the two-way ANOVA test to evaluate the factors "age" and "diet". Furthermore, in order to determine which pairs of samples tended to differ in regard to the age factor, Tukey's test for multiple comparisons was used (post-hoc test). A p-value <0.05 was considered to be significant.

Results

Rat body weight was measured every week. There was no difference in weight gain between rats fed the control and supplemented diet over the entire experimental period. Supplementation of the diet with fish oil also did not significantly affect the wet weight of the examined brain parts.

PUFA levels in different brain regions

Supplementation of rats' diet with fish oil for 12 months resulted in changes in lipid composition of brain tissue between the supplemented and control group (Table 1). The analysis of lipid composition, performed using gas chromatography, included n-3 fatty acids: DHA (docosahexaenoic acid; C22:6n3), DPA (docosapentaenoic acid; C22:5n3) and n-6 fatty acids: DPA (docosapentaenoic acid; C22:5n6), AA (arachidonic acid; C20:4n6) and LA (linoleic acid; C18:2n6). The sum of these fatty acids in each experimental group was considered as the PUFA content in examined brain tissue. In animals aged two months, the region with the highest amount of PUFA was

the cerebral cortex (35.02 %). The cerebellum (30.46%) and hippocampus (27.94%) had lower PUFA concentrations. In general, young rats were characterized by a higher amount of PUFA when compared to both groups of old animals (14 months). The effect of supplementation with fish oil on PUFA content in the cerebral cortex (32.52%) did not differ significantly from the control group (32.62%). A similar observation concerned the cerebellum, in which case the percentage of PUFA in the control (26.71%) as well as the supplemented group was 26.82%. The level of PUFA in the hippocampus in young animals was the highest (27.94%), whereas in old rats the percentage of PUFA was 26.91% in the control group and 24.17% in the supplemented group. The results of our work have also shown that a diet enriched with fish oil substantially enriched brain tissue primarily in DHA. However, the total amount of DHA was reduced in older rats when compared with the young animals. There was a significant reduction in the amount of AA in all examined brain regions, in samples from the supplemented group. While the main effect of diet most likely resulted from increased levels of PUFA in fish oil, the difference in saturated and mono-saturated fatty acids between the two diets could not be completely eliminated.

TBARS concentration

The level of TBARS was measured as a biomarker of oxidative membrane damage of the brain tissue. The obtained results demonstrated that there was no significant difference in TBARS concentration between the two examined dietary groups of rats at the age of 14 months (Fig. 1). However, a significant difference was observed when the comparison was made between young and old animals from both the supplemented and the control group. In young rats, the highest amount of TBARS was noted in the hippocampus (0.96 μmol/g tissue), whereas other parts of the cerebral cortex showed lower content (0.17 μmol/g tissue), similarly to the cerebellum (0.45 μmol/g tissue). In control 14-month-old animals the TBARS levels were significantly higher in the hippocampus (1.94 μmol/g tissue), cerebral cortex (0.31 μmol/g tissue) and cerebellum (0.71 μmol/g tissue) compared to the juvenile group. On the other hand, TBARS levels were found to be minimally reduced in the fish oil supplemented group in the hippocampus (1.92 μmol/g tissue), cerebral cortex (0.28 μmol/g tissue) and cerebellum (0.69 μmol/g tissue) in comparison to control rats aged 14 months, but also significantly higher when compared with young animals.

Table 1. PUFA composition (% of total fatty acids) in brain regions of examined rat groups

Fatty acid	2 CON	14 CON	14 SUP
Cerebral cortex			
C22:6n3 (DHA)	22.81±0.75[a]	19.34±0.75[c]	21.68±0.29[b]
C22:5n3 (DPA n-3)	0.04±0.01[a]	0.02±0.01[b]	0.03±0.01[ab]
C18:2n6 (LA)	0.42±0.06[a]	0.22±0.10[b]	0.37±0.03[a]
C20:4n6 (AA)	8.40±0.33[b]	9.60±0.41[a]	7.88±0.58[b]
C22:5n6 (DPA n-6)	3.35±0.25[a]	3.44±0.28[a]	2.56±0.26[b]
Σ PUFA	35,02	32,62	32,52
Cerebellum			
C22:6n3 (DHA)	20.03±0.59[a]	14.52±0.40[c]	16.49±0.34[b]
C22:5n3 (DPA n-3)	0.63±0.07[ab]	0.52±0.03[b]	0.79±0.29[a]
C18:2n6 (LA)	0.76±0.13[a]	0.33±0.03[b]	0.49±0.07[b]
C20:4n6 (AA)	7.57±0.28[a]	6.58±0.35[b]	5.59±0.31[c]
C22:5n6 (DPA n-6)	1.47±0.32[c]	4.76±0.32[a]	3.46±0.19[b]
Σ PUFA	30,46	26,71	26,82
Hippocampus			
C22:6n3 (DHA)	15.22±0.49[a]	12.29±0.06[c]	13.45±0.86[b]
C22:5n3 (DPA n-3)	0.16±0.05[ab]	0.11±0.02[b]	0.21±0.02[a]
C18:2n6 (LA)	0.58±0.07[b]	0.12±0.08[a]	0.2±0.0[a]
C20:4n6 (AA)	10.51±0.23[a]	10.45±0.25[a]	8.73±0.28[b]
C22:5n6 (DPA n-6)	1.47±0.26[b]	3.94±0.32[a]	1.58±0.41[b]
Σ PUFA	27,94	26,91	24,17

Values are mean ± SD; 2, 14- age of rats; CON- Control diet; SUP- Supplemented diet; a, b, c- means in a row with different letters are significantly different $p \leq 0,05$

Lipofuscin

Lipofuscin (LP) storage was observed in all old rats fed a control and supplemented diet, with a wide distribution in hippocampal and cerebral pyramidal neurons, and to a smaller extent in Purkinje cells of the cerebellum (Table 2). In young rats purple/brown PAS-positive pigment was also detected. The accumulation particularly affected large neurons, such as pyramidal cells (1x grade) of the hippocampus (CA1 as well as CA3 pyramidal layer). No LP was observed in the cerebral cortex and cerebellum of young rats, although LP in those animals appeared as small perinuclear and granular deposits in the hippocampus. Old (14 months) rats had more diffuse granular deposits affecting the perikaryon and proximal dendritic tree. There was no difference in the lipofuscin content in the cerebellum (1x grade) neurons of 14-month-old rats, when the comparison was made between the two dietary groups. However, a high concentration of the pigment was noted in both dietary groups of old rats in the hippocampus (2x grade) and cerebral cortex (2x grade).

Table 2. Regional lipofuscin rates and amyloid plaques density in examined rat groups

Histological marker	2 CON	14 CON	14 SUP
Cerebral cortex			
Lipofuscin	0	xx	xx
Amyloid plaques	nd	4,4*	nd
Cerebellum			
Lipofuscin	0	x	x
Amyloid plaques	nd	nd	nd
Hippocampus			
Lipofuscin	x	xx	xx
Amyloid plaques	nd	1,7	nd

2, 14- age of rats; CON- Control diet; SUP- Supplemented diet;
Lipofuscin grade: 0 = no detectable, 1x = small, 2x = large amount;
Amyloid plaques: *avarage number of amyloid plaques per square milimeter in 7 regions; nd- not detected.

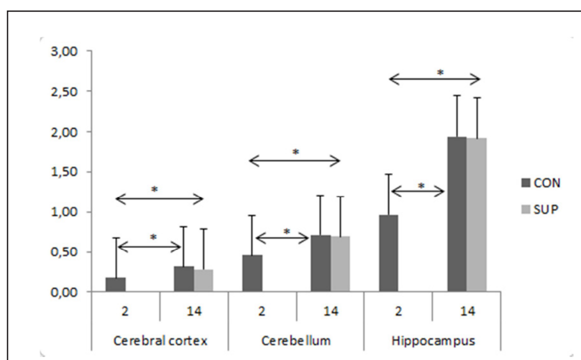

Fig. 1. TBARS concentration (μmol/g tissue) in experimented brain regions of rats. Values are mean ± SD; CON- Control diet; SUP- Supplemented diet; 2, 14- age of rats; *–significantly different between groups, p < 0,05.

β-Amyloid

In the brain of young rats there were no changes associated with β-amyloid deposition. Amyloid plaques detected by Congo red stain were observed in rats fed the control diet. This type of plaque was seen in the frontal cortex and hippocampal areas (Table 2). In addition, neuronal pigments of melanic type were identified near the area of AP. These pigments consisted of brown/black granules accumulating with age, and showed uneven distribution near the AP structure. Furthermore, we also observed cerebrovascular amyloidosis, which affected the small caliber of blood vessel walls and capillaries in examined brain regions of old control rats.

PCNA immunohistochemistry

Labeling with the antibodies against proliferative cell nuclear antigen (PCNA) showed a prominent response in all tested brain regions. We found PCNA nucleus of cells at the highest density throughout the main subregions of the hippocampus, including CA1 and CA3 areas, as well as the dentate gyrus (DG) and subgranular zone (SGZ). The cerebral cortex of young rats exhibited marked PCNA expression (71 cells/mm^2) in comparison to old rats from the control (277 cells/mm^2) and supplemented (371 cells/mm^2) group (Fig. 2). The density of PCNA-positive nucleus of cortex cells differed

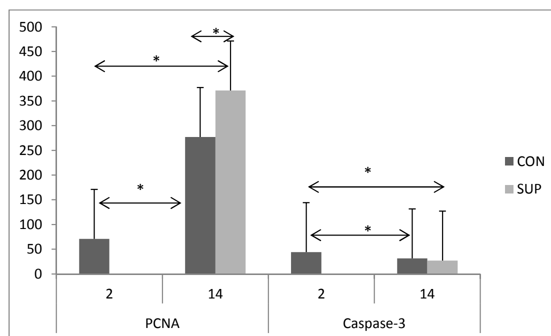

Fig. 2. PCNA and caspase-3 positive cell counts (cell/mm^2) in cerebral cortex of rat groups. Values are mean ± SD; CON- Control diet; SUP- Supplemented diet; 2, 14- age of rats; *–significantly different between groups, p < 0,05.

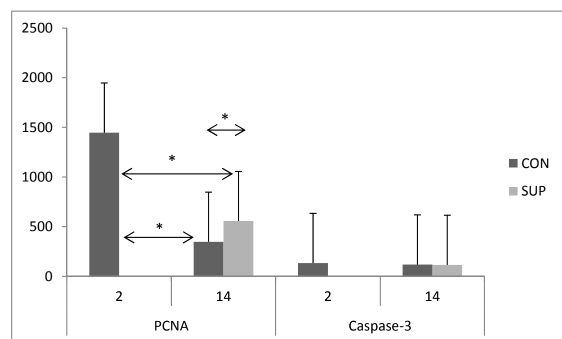

Fig. 3. PCNA and caspase-3 positive cell counts (cell/mm^2) in hippocampus proper of rat groups. Values are mean ± SD; CON- Control diet; SUP- Supplemented diet; 2, 14- age of rats; *–significantly different between groups, p < 0,05.

among the two dietary groups. Surprisingly, in the case of the hippocampus proper (CA1 and CA3) the results of the examination were similar to those obtained in the cerebral cortex (Fig. 3). A significant difference in expression of PCNA was detected when young (1446 cells/mm^2) rats were compared with old animals from both dietary groups (control group: 346 cells/mm^2; supplemented group: 556 cells/mm^2). More neuronal and glial cells labeled with PCNA were seen in the DG (Fig. 4), when analyzing young (2191 cells/mm^2) and old rats from the control group (1167 cells/mm^2) and comparing the results to the supplemented group of 14-month-old animals (2089 cells/mm^2). No effect of the diet (control old group: 366 cells/mm^2; supplemented old group: 393 cells/mm^2), or age (control young group: 343 cells/mm^2) of rats was observed in the PCNA-labeled cerebellar granular layer (Fig. 5). Generally, in the case of the CA1 and CA3 area, and the DG, the number of positive PCNA nucleus of cells was lower in animals at 14 months of age in comparison to 2-month-old rats, while in the cerebellum no difference was noted. Interestingly, the results concerning the number of PCNA-positive nucleus in the cerebral cortex differed substantially from the data obtained in other brain regions.

Caspase-3 immunohistochemistry

The caspase-3 was highly expressed in the hippocampal formation (CA1 and CA3) but no effect of diet (control old group: 118 cells/mm^2; supplemented old group: 114 cells/mm^2), or age (young group: 133 cells/mm^2) was seen in this case (Fig. 3) In the DG no significant difference in the number of caspase-3 positive cells was noted when a comparison was made between the two groups of old animals (control group: 147 cells/mm^2; supplemented group: 134 cells/mm^2), or when young rats (126 cells/mm^2) were compared with the old ones (Fig. 4). However, the analysis of the cerebral cortex showed that the caspase-3 activity was significantly higher in the young group (44 cells/mm^2) versus the old control (31 cells/mm^2) and supplemented group (27 cells/mm^2) (Fig. 2), whereas in the cerebellum (Fig. 5) the number of caspase-3-positive cells was significantly higher in old animals from the control group (58 cells/mm^2) when compared to the young rats (27 cells/mm^2). Additionally, a significant difference was noted when the comparison was made between the two dietary groups of old animals (supplemented group: 29 cells/mm^2).

Discussion

The data obtained in our study demonstrated that the cerebral cortex in young rodents had the highest amount of PUFA (35.02%) when compared to the cerebellum (30.46%) and hippocampus (27.94%). Old rats were characterized by lower levels of PUFA in tested brain regions, whereas fish oil supplementation did not affect the total amount of PUFA, but rather influenced the quantity of each PUFA in the tested brain areas. The results of this study are consistent with the observations of other researchers [38,39]. Several mechanisms are involved in reduction of PUFA in the brain: a low rate of penetration of PUFAs from the blood into the brain under oxidative stress, altered fatty acid metabolism, enzymatic and non-enzymatic peroxidation, and altered levels of desaturases [24,29]. Furthermore, this study demonstrated that the physiological aging process significantly increased TBARS levels in the three tested brain regions, with a minimal difference between control and supplemented rats at the age of 14 months. This is probably caused by changes in metabolism in the CNS observed with age. Lipid peroxidation causes the destruction and damage of cell membranes, leading to changes in membrane fluidity/permeability, and to enhanced rates of protein degradation. Thus, the ingestion of DHA, which is present in high concentrations in fish oil, should enhance the susceptibility of tissues to lipid peroxidation. The enhancement is a function of dietary DHA level, and it increases the requirement for vitamin E (lipophilic membrane antioxidant). The substitution of membrane fatty acids with DHA, which is very susceptible to lipid peroxidation, and thus potentially unstable, is thought to be the cause of enhanced susceptibility to lipid peroxidation [23]. A study by Kubo et al. [1998] showed that in the brain and testis, the lipid peroxide levels were not increased in rats fed DHA. Therefore, susceptibility to formation of lipid peroxides in the presence of DHA seems to differ between various tissues. Protection against lipid

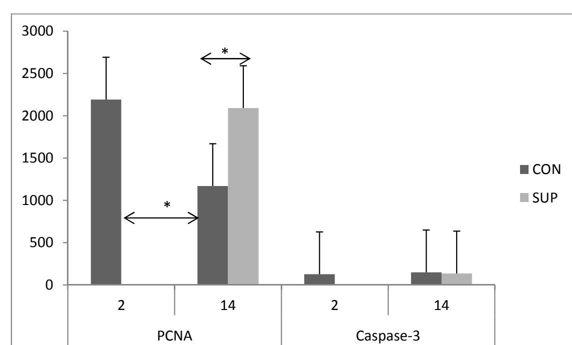

Fig. 4. PCNA and caspase-3 positive cell counts (cell/mm^2) in dentate gyrus of rat groups. Values are mean ± SD; CON- Control diet; SUP- Supplemented diet; 2, 14- age of rats; *–significantly different between groups, $p < 0{,}05$.

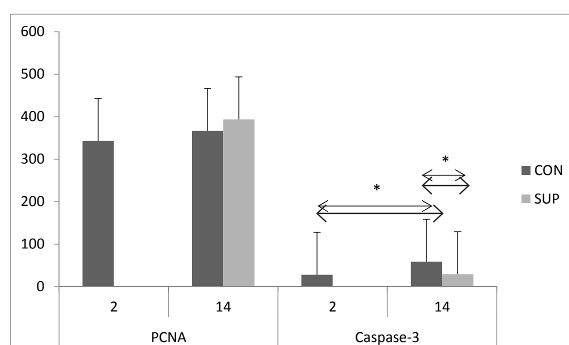

Fig. 5. PCNA and caspase-3 positive cell counts (cell/mm^2) in cerebellum of rat groups. Values are mean ± SD; CON- Control diet; SUP- Supplemented diet; 2, 14- age of rats; *–significantly different between groups, $p < 0{,}05$.

peroxidation is not achieved by antioxidants, for example vitamin E, but presumably other factors exist, which are able to suppress DHA-stimulated tissue lipid peroxide formation to a level below that expected from the peroxidizability index of the tissue. The suppressive mechanism is explained partially by an increase in the levels of lipid peroxide scavengers, such as ascorbic acid and glutathione [21].

Lipofuscin (LP) pigment accumulates progressively with age within secondary lysosomes in long-lived postmitotic cells of humans and animals [7,17]. The distribution of lipofuscin in the CNS parallels the activities of oxidative enzymes in various areas [6]. In our study we noted accumulation of lipofuscin in hippocampus pyramidal cells of young rats (at the age of 2 months). In the hippocampal and cerebellar as well as cerebral cortex neurons, the amount of age pigment did not differ between dietary groups of old animals (14 months). Since there are differences in the rate of incorporation of dietary PUFA into lipid membranes in various brain regions, it may result in differences in lipofuscin formation in these brain parts. We expected to observe an increase of lipid peroxidation in parallel with the increase of the concentration of DHA in total brain lipids. Surprisingly, in our study the detected content of TBARS was the lowest in the cerebral cortex and the highest in the hippocampus. This may suggest that PUFA does not play such an important role in the formation of LP in the brain, or the oxidation is highly regulated by the supply of endogenous antioxidants. Accumulation of age pigment is decreased by addition of antioxidants to the diet, and it increases when the diet is deficient in vitamin E [5]. Lipofuscin is formed by oxidative polymerization of lipid and protein; however, it is not clear whether the increased content of DHA in the diet, and/or peroxidizability of the dietary lipids, might be the reason for enhanced lipofuscin accumulation [6].

Formation of amyloid-β is considered as another very important marker of aging and neurodegeneration. The incidence of spontaneous amyloidosis becomes more common with aging [27]. Our results showed that the spontaneous amyloidosis increased in the groups of old rats. In young animals we did not observe these alternations [27]. Amyloid-β is deposited in the cerebral vessel walls and in the brain cortex as senile plaques accumulating on, and between, the membranes of degenerating neuronal structures, as well as in microglia and astrocytes. We found positive staining in the cerebral cortex of 14-month-old control rats, which indicates amyloid plaques (AP). Animals fed a fish oil diet did not have these alterations, which may suggest a positive effect of supplementation. The neurotrophic effect of DHA on the structure of cell membranes during the process of aging are well documented, and DHA may repress lipid raft-mediated mechanisms involving toxic $A\beta_{40-42}$ peptide generation, while favoring the mechanism of generation of the more neurotrophic sAPPα (amyloid precursor protein) [31]. DHA has been shown to suppress age-related $A\beta_{42}$ peptide shedding from human neural cells, repress Aβ peptide-related pathology in Tg2576 transgenic mouse models of AD, and stimulate nonamyloidogenic βAPP processing, which reduces both intracellular and extracellular levels of Aβ peptide in aged SH-SY5Y cells. DHA interactions with, and recruitment of, neural membrane-associated factors that modulate βAPP catabolism appear to be highly complex and interactive in the process of maintenance of normal membrane signaling and synaptic, intercellular, and extracellular secretory functions [26].

Numerous studies have shown that neurogenesis can be up-regulated and down-regulated by different psychological phenomena such as stress, environmental complexity and learning, as well as hormones [15]. In the light of these observations, it is possible that oxidative stress increases with age and may affect proliferation in brain regions. In our study, the highest level of TBARS content was noted in the hippocampus of old rats. This observation correlated with the number of PCNA nucleus of neuronal cells (a marker of proliferation), which was also the highest in the hippocampus proper and DG; however, PCNA showed lower expression in old rats from the control group in comparison to young rats. This indicates that there are other mechanisms regulating the process of neurogenesis in young animals. In the case of rats fed a fish oil-supplemented diet for 12 months, their cerebral cortex, hippocampus, and DG showed higher expression of PCNA when compared to the control group of old animals. This may indicate a neuroprotective effect of PUFA on the proliferation. As mentioned above, n-3 PUFA are incorporated into neuronal membranes, where they influence the quaternary structure of membrane proteins, some of which act as transporters and receptors. Furthermore, they alter membrane fluidity, which is important for example for modulation of serotonin binding to neuronal membranes. Serotonin stimulates neurogenesis; thus, a change in efficacy of this modulator could influence the levels of neuronal proliferation. Several studies have reported that n-3 PUFA influence levels of neurotrophins, molecules that promote neuronal survival and growth [3,9]. Among the neurotrophins, brain-derived neurotrophic factor (BDNF) levels are changed by dietary intake of n-3 PUFA, and BDNF is associated with alterations in neurogenesis and neuronal survival. Research showed that three sequential injections of ALA significantly enhanced adult hippocampal neurogenesis in mice, and increased the expression of BDNF. In this study, the treatment also increased synaptogenesis, synapse function and proteins supporting glutamatergic neurotransmission [3]. Other studies demonstrated that 12 weeks' dietary supplementation of n-3 PUFA in aged rats may partially reverse the age-related decline in neurogenesis in the dentate gyrus. The study demonstrated that the effect of PUFA may be linked to the restoration of retinoid signaling in aging. The retinoic acid receptor family includes transcription factors involved in regulating neural plasticity and neurogenesis in the hippocampus [11]. Therefore, n-3 PUFA may alter the rate of neurogenesis via the specific effects of neurotrophins, and contribute to the dynamic structure of neuronal membranes.

Proper development of the nervous system requires apoptosis, which is responsible for systematic removal of cells not only in the proliferative zones, but also among the postmitotic cells [33]. In our study, high expression of caspase-3 activity was found in the hippocampus and DG, when compared with the other tested brain regions. However, there was no significant difference between the investigated groups. This high expression of caspase-3 may correlate with the observed signs of neurogenesis, as apoptosis could serve as a control mechanism for the excessive proliferation. Furthermore, the presence of apoptotic cells in the hippocampus may be associated with increased oxidative stress (TBARS) in the groups of old rodents. It is well known that high levels of oxidative stress can cause cell death via either apoptotic or necrotic pathways [25]. Any imbalance in the normal redox state leads to toxicity via production of free radicals and peroxides (constituting reactive oxygen and/or nitrogen species), damaging proteins, lipids and DNA of the cell [35]. In our study, increased apoptosis was found in the cerebellum of control old rats when compared to the young group. This increase was not necessarily related to proliferation, because it was observed at a constant unchanged level. The high expression of caspase-3 may also be related to the levels of TBARS in this part of the brain. Furthermore, a significant difference was observed in the cerebellum when the comparison was made between the two dietary groups of old animals. In the cerebral cortex we noted decreased expression of caspase-3 in the control and supplemented animals at 14 months of age, in comparison to young rodents. Inhibition of caspase-3 expression through DHA pretreatment was previously confirmed in *in vitro* studies by Akbar et al. [2005] and Florent et al. [2006]. The beneficial neurophysiological actions of DHA occur partially through the generation of docosanoids, e.g. neuroprotectin D1 (NPD1). Neurotrophins, including pigment epithelium-derived factor, and brain-derived neurotrophic factor, stimulate NPD1 synthesis, which in turn modifies the expression of Bcl-2 family members by activating antiapoptotic proteins, reducing proapoptotic proteins, and attenuating caspase-3 during oxidative stress [26].

Conclusion

Our results suggest that the addition of fish oil to the diet is required for inhibition of the initial symptoms of neurodegeneration manifested by the formation of amyloid plaques and the reduction of neurogenesis. These findings are in agreement with other studies, indicating that PUFA administration increases hippocampal dendritic spine density and some synaptic density. The results of these studies show a positive effect of fish oil supplementation mainly in the cortex of the brain. However, we do not exclude the possible effect of the diet resulting from the difference in saturated and mono-saturated fatty acids. The data from our study show that the effects of fatty acids are not identical for all examined regions of the brain. However, the cerebral cortex often undergoes various damage in aging and neurodegeneration, so selective action of fatty acids derived from fish oil in this case is most appropriate for aging individuals.

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