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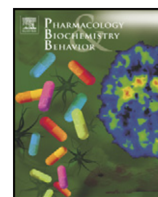

Astaxanthin limits fish oil-related oxidative insult in the anterior forebrain of Wistar rats: Putative anxiolytic effects?

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ABSTRACT

The habitual consumption of marine fish is largely associated to human mental health. Fish oil is particularly rich in n-3 polyunsaturated fatty acids that are known to play a role in several neuronal and cognitive functions. In parallel, the orange-pinkish carotenoid astaxanthin (ASTA) is found in salmon and displays important antioxidant and anti-inflammatory properties. Many neuronal dysfunctions and anomalous psychotic behavior (such as anxiety, depression, etc.) have been strongly related to the higher sensitivity of cathecolaminergic brain regions to oxidative stress. Thus, the aim of this work was to study the combined effect of ASTA and fish oil on the redox status in plasma and in the monoaminergic-rich anterior forebrain region of Wistar rats with possible correlations with the anxiolytic behavior. Upon fish oil supplementation, the downregulation of superoxide dismutase and catalase activities combined to increased "free" iron content resulted in higher levels of lipid and protein oxidation in the anterior forebrain of animals. Such harmful oxidative modifications were hindered by concomitant supplementation with ASTA despite ASTA-related antioxidant protection was mainly observed in plasma. Although it is clear that ASTA properly crosses the brain-blood barrier, our data also address a possible indirect role of ASTA in restoring basal oxidative conditions in anterior forebrain of animals: by improving GSH-based antioxidant capacity of plasma. Preliminary anxiolytic tests performed in the elevated plus maze are in alignment with our biochemical observations.

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1. Introduction

The habitual consumption of marine fish has been long associated to human mental health, even in an evolutionary scale (Broadhurst et al., 2002). The mammalian brain is particularly rich in two omega-3 polyunsaturated fatty acids (n-3/PUFAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The n-3/PUFAs are known to play a role in nervous system activity, cognitive development, neuroplasticity of nerve membranes, synaptogenesis and synaptic transmission (Bas et al., 2007). Consistent with this role, DHA has been shown to limit oxidative damage of lipids and proteins in developing and adult brains, with attenuation of neuron loss and cognitive and locomotor deficits in animal models of ischemia-reperfusion brain injury (Cao et al., 2004; Green et al., 2001). Interestingly, dietary levels of DHA and EPA are 2.5- to 100-fold higher in marine fish than in lean or fat terrestrial meats (Komprda et al., 2005).

Apart of n-3/PUFAs, sustaining normal human brain function also requires an adequate provision of dietary antioxidants, as the brain is abnormally prone to oxidative stress for several reasons: (i) intense mitochondrial activity in neurons generates high amounts of reactive oxygen species (ROS); (ii) neuronal n-3/PUFA-rich membranes are major targets for ROS; (iii) mammalian brain particularly accumulates redox-active iron ions, which catalyze the conversion of freely diffusible H_2O_2 into harmful $HO^{\bullet}$ radicals (by Fenton reaction) and the oxidation of neurotransmitters to neurotoxic metabolites; (iv) some brain regions have high concentration of easily oxidizing neurotransmitters, such as dopamine; and (v) lower activity of the H_2O_2-removing enzyme catalase (CAT) in most brain regions (Halliwell, 2006). Nevertheless, it is argued that moderate/controlled

Abbreviations: ASTA, astaxanthin; BHT, butylated hydroxytoluene; BW, body weight; CAT, catalase; CuZnSOD, copper,zinc-dependent superoxide dismutase; DHA, docosahexaenoic acid; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EPA, eicosapentaenoic acid; FRAP, ferric-reducing activity in plasma; FRC, ferric-reducing capacity; GPX, glutathione peroxidase; GR, glutathione reductase; MDA, malondialdehyde; MnSOD, manganese-dependent superoxide dismutase; n-3/PUFAs, omega-3 polyunsaturated fatty acids; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase; TEAC, Trolox-equivalent antioxidant capacity; TBARS, thiobarbituric acid reactive substances; TEP, 1,1,2,2-tetraethoxypropane.

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levels of oxidative modifications are necessary to provide the hormetic central underpinning of neuroprotective responses, triggered by a framework of redox signals including nitric oxide, hydrogen peroxide and other mitochondrial metabolites (Calabrese et al., 2010).

Astaxanthin (ASTA; Fig. 1) is an orange-pinkish carotenoid extensively found in marine organisms, including algae, crustaceans and salmonid fishes. Humans regularly obtain ASTA from their diet, and health benefits of ASTA are suggestively attributed for its antioxidant and anti-inflammatory activities (Otton et al., 2010; Bolin et al., 2010). Different from many other dietary carotenoids, ASTA is not provided with pro-vitamin A activity but alternatively shows important UV-photoinhibitory effects (preventing skin lesions), and cardioprotective, antihypertensive and anti-tumorigenic roles in humans (Guerin et al., 2003).

Several epidemiological studies have indicated that supplementation with n-3/PUFAs may reduce the risk for a variety of neurological/psychiatric diseases (Emsley et al., 2008). Oxidative stress plays a role in the development and progression of anxiety by increasing risk of neurodegeneration in animal and human brains (Floyd, 1999). However, controversial studies arose the hypothesis that unbalanced n-3/PUFA provision could also affect physicochemical properties of the neuronal membrane (fluidity, permeability, hydrophobicity, etc.), thereby impacting on speed of signal transduction and effectiveness of neurotransmission (Bas et al., 2007; King et al., 2006; Appleton et al., 2008; Cunnane et al., 2009). Consequently, neuronal membrane becomes more sensitive to oxidative injury if not properly counterbalanced by antioxidant defenses that sustain the optimal dose–response hormetic ratio (Calabrese et al., 2010). Thus, based on the strong link between oxidative stress and neurological/psychiatric diseases (e.g. anxiety), the aim of this work was to study the combined effect of natural sources of the powerful antioxidant ASTA (1 mg ASTA/kg) and n-3/PUFA-rich fish oil (providing 10 mg EPA/kg and 7 mg DHA/kg) on the redox balance in the anterior forebrain and plasma of supplemented Wistar rats with putative implications in the anxiety behavior.

2. Materials and methods

2.1. Chemicals and natural products

All purified chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), except common laboratory solutions and buffers, which were obtained from Labsynth (Diadema, SP, Brazil). The biochemical kit for plasma/serum iron determination was purchased from Doles Reagentes e Equipamentos para Laboratórios Ltda (Goiania, GO, Brazil). Fish oil capsules were purchased from Pharmanostra (Sao Paulo, SP, Brazil). Each fish oil (FO) capsule of 500 μL contains 9 kcal (38 kJ), 2.0 mg of mixed tocopherols, and 1.0 g of total fat, which 30% are from saturated fats, 20% from monounsaturated fats (mostly palmitoleic and oleic acids), and 50% of polyunsaturated fatty acids (180 mg EPA and 120 mg DHA). Natural ASTA supplements (AstaREAL A1010) were obtained as a donation from the Swedish company BioReal AB (Gustavsberg, Sweden), part of the pharmaceutical Group Fuji Chemical Industry CO (Japan). AstaREAL A1010 is an astaxanthin-rich natural *Haematococcus pluvialis* product that contains 5.2–5.8% of total carotenoids, whereas 5.0–5.6% are purely astaxanthin (3.9% as monoesters, 0.9% as diesters, and 0.1% in free form), 0.02% lutein/zeaxanthin, 0.02% adonirubin, 0.02% cantaxanthin, 0.02% β-carotene, and 0.1% others.

2.2. Animals and supplementation protocols

Adult Wistar male rats, weighing (225.6 ± 17.1) g at the beginning of the study were provided by the Department of Psychobiology, Universidade Federal de São Paulo (UNIFESP), São Paulo, Brazil. All animal were housed in Plexiglas cage (4 rats/cage) under standard laboratory conditions: 12 h light/dark cycle; lights on at 7:00 a.m.; (22 ± 1) °C; and *ad libitum* access to water and Purina rat chow. The experiments were carried out in accordance with the scientific procedure recommended for studies involving animals. The animals used in this study were handled in accordance with guidelines of the committee on care and use of laboratory animals resources. The Ethics Committee for experimental animals from Universidade Federal de São Paulo approved the experimental protocol (CEP/UNIFESP no. 1938/09). After room acclimation for 1 week, the animals were treated with ASTA and/or fish oil by gavage, 5 days a week, for 45 days. A maximum volume of 400 μL was established for the gavage treatment in order to avoid regurgitation or stomach discomfort of the animals. Fish oil (FO) content of capsules was diluted in 10% Tween-80 aqueous solution (v/v) to reach final n-3/PUFAs concentrations of 10 mg EPA/kg body weight (BW) and 7 mg DHA/kg BW. An identical procedure was conducted for animal supplementation with 1 mg ASTA/kg BW using AstaREAL A1010 as the carotenoid source (5.3% of pure ASTA). For combined FO and ASTA treatments, both components were diluted in the same stock 10% Tween-80 aqueous solution (v/v) to reach previously described concentrations. Although additional antioxidants as ascorbate, tocopherols and other carotenoids were present in the both manufactured natural products, their contribution in the total antioxidant capacity of gavage solutions is minor if compared to the prevalent ASTA or n-3/PUFA components. Thus, four experimental groups of 16 animals each were formed: (i) control, fed with 400 μL of 10% Tween-80 aqueous solution (v/v); (ii) ASTA, fed with 1 mg ASTA/kg; (iii) FO (fed for 10 mg EPA/kg and 7 mg DHA/kg); and (iv) ASTA/FO, fed with 1 mg ASTA/kg, 10 mg EPA/kg and 7 mg DHA/kg.

2.3. Anxiety behavioral scores

After 45 days of supplementation, the animals were tested for anxiety behavior by measuring exploratory activity in an elevated plus-maze apparatus (Pellow and File, 1986). The used plus-maze was built of wood and consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm). The arms extended from a central platform (10 × 10 cm) raised 50 cm above the floor. Each animal was placed at the center of the maze facing a closed arm and was allowed to explore the maze for 5 min. Total time in open arms and the time of exploratory activity in open arms (time spent in each entrance) were calculated to address the anxiety behavior of experimental animals in the plus-maze.

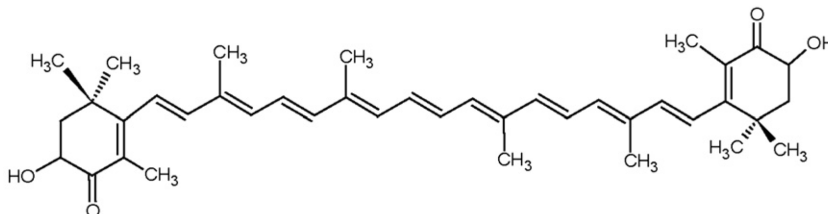

Fig. 1. Chemical structure of the marine carotenoid astaxanthin (ASTA).

2.4. Biochemical analyses

2.4.1. Plasma antioxidant capacity

In the very next day of behavioral tests, the animals were killed by decapitation between 08:00 a.m. and 11:00 a.m. and plasma was isolated from EDTA-treated blood by centrifugation at 2.5*g* (10 min, 4 °C). Plasma samples were immediately frozen in dry ice and stocked in freezer −80 °C for further analysis. The antioxidant capacity of animal plasma was tested by two distinct methods: (i) Trolox-equivalent antioxidant capacity (TEAC), with modifications described by Van den Berg et al. (1999); and (ii) ferric-reducing activity in plasma (FRAP; Benzie and Strain, 1996). Briefly, the reactant mixture for FRAP assay contains 10 mM of the stoichiometric iron-chelating agent 2,3-bis(2-pyridyl)-pyrazine (DPP) in 40 mM HCl (Brewer et al., 1987) and 20 mM $FeCl_3$ in 0.30 M acetate buffer (pH 3.6). To 200 μL of FRAP reactant mixture, 10–20 μL sample is added together with 40–30 μL distilled water (total volume, 250 μL). Absorbance at 593 nm was recorded for 4 min in a microplate reader SpectraMax M5, Molecular Devices (Silicon Valley, CA, USA) to determine the rate of Fe^{2+}–DPP complex formation as compared to a $Fe(NH_4)_2(SO_4)_2.6H_2O$ standard curve. The same technique was applied in cleared brain homogenates, but referred in the work as ferric-reducing capacity (FRC). Due to the key participation of $Fe^{2+/3+}$ ions in triggering oxidative stress, total iron content in plasma was also measured by biochemical kits based on the ferrozine method (detection at 560 nm; Goodwin and Murphy, 1966; Stookey, 1970).

Total glutathione content in plasma (Total GSH) was measured by previous reduction of oxidized glutathione molecules (GSSG) by the glutathione reductase recycling system (GR:NADPH), as described by Rahman et al. (2006). Then, total GSH molecules (original GSH plus previously reduced GSSG) react with 5,5′-diothiobis-2 nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB), which is accurately detected by spectrophotometry at 412 nm. The GR:NADPH recycling system was not used for measurements of the proper GSH content in samples. The GSH and GSSG concentrations in samples were calculated from standard curves (prepared with pure GSH and GSSG), and expressed as the GSH/GSSG ratios to indicate the reducing power in plasma of experimental animals.

2.4.2. Brain homogenates

The rat brains were quickly removed after decapitation and washed briefly in 0.1 M phosphate buffer, pH 7.4. Regarding anxiety-related segments and catecholaminergic innervations in animal brains (Sienkiewicz-Jarosz et al., 2003), the anterior forebrain section including frontal cortex, nucleus accumbens, and hippocampus was carefully excised (Paxinos and Watson, 2008). Animal brain sections were homogenized with 3 mL of 0.1 M phosphate buffer, pH 7.4, in a tissue grinder under ice-water bath for 5 min. The crude homogenates were centrifuged at 2.5*g* for 10 min (4 °C) for debris removal. The cleared supernatants were kept in ice-water bath and used for further biochemical analyses.

2.4.3. Antioxidant capacity of brain homogenates

The antioxidant capacities of brain samples were also tested by TEAC and FRAP methods, as described before. Accordingly, total iron content was also measured in the anterior forebrain section, but using an alternative method based on the formation of a Fe^{2+}:bipyridyl complex (Mattei et al., 2001).

2.4.4. Antioxidant enzyme activities

Catalase (CAT), superoxide dismutase isoforms (mitochondrial MnSOD, and cytosolic/extracellular CuZnSOD), glutathione peroxidase (GPX), and glutathione reductase (GR) activities were determined in brain samples by spectrophotometry techniques adapted for the microplate reader SpectraMax M5, Molecular Devices (Silicon Valley, CA, USA). Enzyme activity of CAT was measured by direct decomposition of H_2O_2 at 240 nm (Aebi, 1984). Total SOD activity in samples was measured at 540 nm by monitoring the linear first-order reduction of $O_2^{\bullet-}$ radicals by nitroblue tetrazolium (NBT) for 3 min (Ewing and Janero, 1995). In order to discriminate MnSOD and CuZnSOD activities, cytosolic/extracellular CuZnSODs were blocked in samples by adding 3 mM KCN in the reaction system. Both GPX (Mannervik, 1985) and GR (Carlberg and Mannervik, 1985) were measured based on the oxidation of β-NADPH triggered by 10 mM *tert*-butyl hydroperoxide addition in the reaction system ($\lambda = 340$ nm, molar extinction coefficient of $6.2 \times 10^3\ M^{-1}\ cm^{-1}$).

2.4.5. Indexes of oxidative injury

Protein fractions were isolated from homogenates by precipitation in 10% trichloroacetic acid in ice-water bath. After washing once with 0.30 M $HClO_4$, 5.0 mM EDTA and 0.06% 2,2′-bipyridine (w/v) solution, and twice with an organic mixture (1:1 ethyl acetate:ethanol; v/v), the protein pellet was dried in vacuum and then subsequently dissolved in 6.0 M guanidine.HCl. Reduced protein thiol groups in the guanidine-soluble fraction were detected at 412 nm by the formation of colored adducts after reaction with 4.0 mM 5,5′-dithio-bis(2-nitrobenzoic acid) solution (DTNB). Blanks were prepared by preliminary treatment with 10 mM N-ethylmaleimide solution (a specific thiol-blocking compound). The reduced thiol content was calculated using purified glutathione (GSH) as a standard (Murphy and Kehrer, 1989).

The extension of lipid peroxidation in brain samples was evaluated by the thiobarbituric acid reactive substances assay (TBARS; Fraga et al., 1988). Butylated hydroxytoluene (4% BHT in ethanol) was added to stop ongoing oxidation reactions in lipid-rich samples. The colored TBARS adducts were quantified at 535 nm after reaction with 0.25% thiobarbituric acid in 0.25 M HCl and 1% Triton X-100, at 100 °C, for 15 min (blanks lack thiobarbituric acid). Malondialdehyde prepared by acid hydrolysis of 1,1′,2,2′-tetraethoxypropane (TEP) was used as a standard.

2.5. Statistics

All data are presented as the mean values of at least triplicates with their standard errors (MEAN ± SE). Data from anxiety-behavior were analyzed using two-way ANOVA for treatment as between-subject factor ($n = 16$), and were plotted using an exclusion criterion for outlier points > 1.5*IQR, where IQR = interval between quartiles. Biochemical assays were conducted with 6 or more animals per group ($n \geq 6$), and data were analyzed by one-way ANOVA followed by Tukey's post-test. The software Origin 6.1 (v6.1052/B232; OriginLab Corporation, Northampton, MA, USA) was used for statistical analyses and graph preparation.

3. Results

3.1. Plasma indexes

Table 1 presents biomarkers of the antioxidant capacity in plasma of experimental animals. As expected, the long-term supplementation with ASTA improved the antioxidant capacity of plasma (35% in terms of Trolox-equivalents as shown by TEAC results), whereas no difference was observed in respect to fish oil treatment. Combined supplementation with ASTA/Fish oil also induced higher antioxidant capacity in plasma (42%; Table 1), sustaining the antioxidant role of ASTA in the mixture. Similar trends were also observed in respect to total iron content and FRAP scores in plasma. However, distinctively from TEAC results, ASTA/Fish oil group showed 35% less iron content in plasma than ASTA-fed animals. Regarding GSH metabolism, both ASTA and fish oil treatments significantly increased total GSH content (GSH + GSSG) and GSH/GSSG ratios in plasma of animals, revealing their antioxidant properties. Noteworthy, a synergistic effect of

Table 1
Biomarkers of antioxidant capacity in plasma of Wistar rats treated by gavage with 1 mg ASTA/kg and/or Fish oil (based on 10 mg EPA/kg and 7 mg DHA/kg) for 45 days. Both lipid-soluble substances were diluted in 10% Tween-80 aqueous solution (v/v), which was also used in control group.

Biomarker	Control	ASTA	Fish oil	ASTA/fish oil
Total iron[a]	0.58 ± 0.32	1.48 ± 0.63#	0.70 ± 0.29	0.97 ± 0.20
FRAP[b]	33.5 ± 4.1	87.4 ± 20.5*	36.6 ± 5.5	56.8 ± 7.5#
TEAC[c]	5.08 ± 1.66	6.85 ± 0.84#	5.81 ± 1.08	7.21 ± 0.93#
Total GSH[d]	0.082 ± 0.008	0.119 ± 0.013*	0.120 ± 0.011#	0.158 ± 0.014§
Ratio GSH/GSSG[e]	1.11 ± 0.07	1.45 ± 0.13*	1.95 ± 0.13§	6.93 ± 0.43§

Values are MEAN ± SE ($n \geq 6$). Statistical analysis: *$p<0.05$, #$p<0.01$, §$p<0.001$, compared to the control group.
[a] Expressed as ($\mu g\ mL^{-1}$) (parameter unit).
[b] Expressed as ($nmol\ Fe^{2+}\ min^{-1}\ mL^{-1}$) (parameter unit).
[c] Expressed as (μmol eq. Trolox mL^{-1}) (parameter unit).
[d] Expressed as ($mM\ mg\ protein^{-1}$) (parameter unit).
[e] Dimensionless index.

combined ASTA and Fish oil was apparently detected in respect to plasma GSH/GSSG ratio.

3.2. Oxidative stress parameters in anterior forebrain

Fig. 2A and B illustrates the significant decrease of both total- and Mn-dependent SOD activities in the anterior forebrain of Fish oil-fed animals (respectively 30% and 50%). Single ASTA treatment did not result in statistically significant variations. Combined supplementation with ASTA/Fish oil reproduced the observed lower activities of mitochondrial MnSOD as in Fish oil-fed rat brains, but not for total SOD activities (which accounts for both cytosolic/extracellular CuZnSODs and mitochondrial MnSOD together). The absolute total SOD activity values found here are in agreement to those extensively described in the literature (2.8 ± 0.6 U/mg prot in rat brains; Peeters-Joris et al., 1975). Peroxisomal CAT activity perfectly matched the variation trend of total SOD in forebrains of all experimental animals, confirming the adequate (and well-documented) balance between those frontline antioxidant enzymes in several biological systems. As observed for total SOD activities, CAT activities in brain homogenates of Fish oil- and ASTA/Fish oil-treated animals were approximately 35% and 30% lower than in control group (Fig. 2C). Finally, the GR activity in the forebrain homogenates of ASTA/Fish oil group was almost 30% higher than control, whereas no other group showed significant variations (Fig. 2D).

Regarding the oxidative stress parameters in the encephalic tissue, it is worthy to note that Fish oil supplementation (either alone or in the presence of ASTA) increased total iron content in anterior forebrain homogenates by around 45–50% (Table 2). Although not statistically significant for Fish oil group ($p = 0.361$), ferric-reducing capacities (FRC) were properly increased in ASTA/Fish oil samples, probably to counteract iron content increase. In addition, TEAC capacity in brains only varied in ASTA/Fish oil-fed animals (around 20%; Table 2). Furthermore, it is clearly observed in Table 2 that single Fish oil treatment imposed higher oxidative challenge to lipids (33% higher indexes of the oxidized lipid products TBARS) and proteins (35% lower reduced thiol content). Unexpectedly, GPX activities in rat forebrains were not significantly altered in any experimental group: (1.56 ± 0.22), (1.71 ± 0.10), (1.56 ± 0.25), and (1.83 ± 0.25) μmol NADPH/min/mg protein for control, ASTA ($p = 0.531$), Fish oil ($p = 0.998$), or ASTA/Fish oil groups ($p = 0.432$), respectively (Table 2).

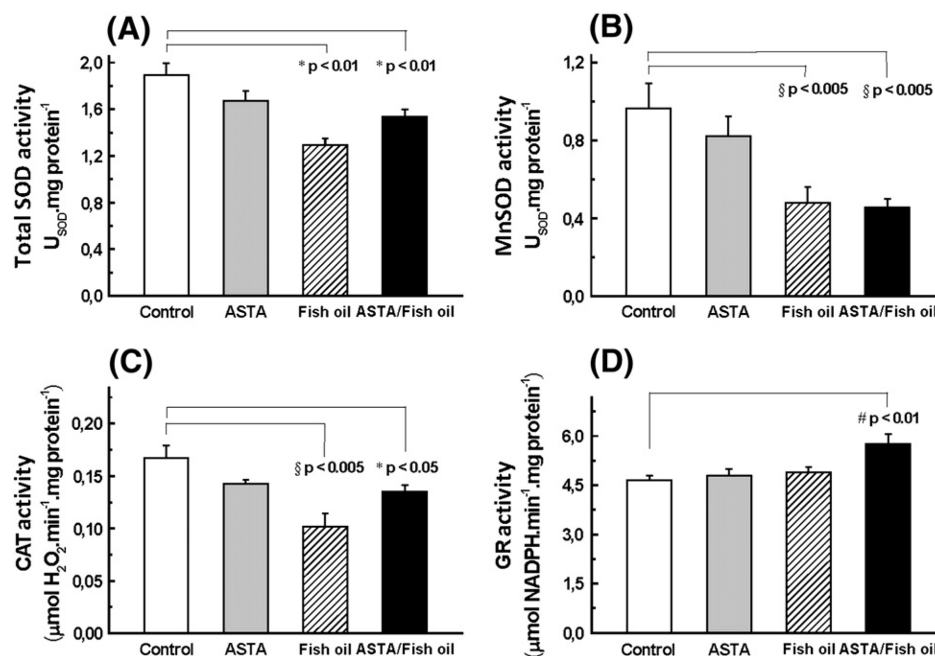

Fig. 2. Activities of antioxidant enzymes in the anterior forebrain region of Wistar rats treated by gavage with astaxanthin and/or fish oil. (A) total SOD activity (expressed in U_{SOD} mg protein^{-1}); (B) MnSOD activity (expressed in U_{SOD} mg protein^{-1}); (C) catalase activity (CAT, expressed in μmol H_2O_2 min^{-1} mg protein^{-1}); (D) glutathione reductase activity (GR, expressed in μmol NADPH.min^{-1} mg protein^{-1}). §$p<0.005$; #$p<0.01$; *$p<0.05$ ($n \geq 6$).

Table 2
Parameters of oxidative stress in the anterior forebrain section of Wistar rats treated by gavage with 1 mg ASTA/kg and/or Fish oil (based on 10 mg EPA/kg and 7 mg DHA/kg) for 45 days. Both lipid-soluble substances were diluted in 10% Tween-80 aqueous solution (v/v), which was also used in control group.

Parameter	Control	ASTA	Fish oil	ASTA/fish oil
Total iron[a]	0.161 ± 0.012	0.190 ± 0.026	0.233 ± 0.009[¶]	0.245 ± 0.017[§]
Ferric-reducing capacity[b]	1.97 ± 0.57	1.66 ± 0.39	2.52 ± 0.29	3.41 ± 0.56
TEAC[c]	0.152 ± 0.011	0.165 ± 0.011	0.164 ± 0.008	0.186 ± 0.008*
(MnSOD)/(totalSOD)[d]	0.511 ± 0.07	0.519 ± 0.07	0.371 ± 0.06[§]	0.297 ± 0.03[§]
CuZnSOD[e]	0.924 ± 0.13	0.804 ± 0.11	0.813 ± 0.13	1.08 ± 0.11*
GPX[f]	1.56 ± 0.22	1.71 ± 0.10	1.56 ± 0.25	1.83 ± 0.24
TBARS[g]	0.626 ± 0.027	0.536 ± 0.027*	0.832 ± 0.084*	0.426 ± 0.016
Protein thiols[h]	1.436 ± 0.044	1.035 ± 0.006[#]	0.945 ± 0.014[#]	1.407 ± 0.216

Values are MEAN ± SE. Statistical analysis: ¶$p<0.001$; §$p<0.005$; #$p<0.01$; *$p<0.05$, compared to the control group ($n \geq 6$).
[a] Expressed as (μg mL^{-1}) (parameter unit).
[b] Expressed as (nmol Fe^{2+} min^{-1} mL^{-1}) (parameter unit).
[c] Expressed as (μmol eq. Trolox mL^{-1}) (parameter unit).
[d] Dimensionless index.
[e] Expressed as (U_{SOD} mg $protein^{-1}$) (parameter unit).
[f] Expressed as (μmol NADPH min^{-1} mg $protein^{-1}$) (parameter unit).
[g] Expressed as (nmol MDA mg $protein^{-1}$) (parameter unit).
[h] Expressed as (nmol–SH mg $protein^{-1}$) (parameter unit).

3.3. Preliminary anxiety-behavior tests

Despite complementary behavioral experiments could be recommended to confirm our hypothesis—i.e. locomotor activity (crossing, rearing and central squares), number of fecal pellets in a central arena, marble burying behavior—a preliminary anxiety-behavior test was performed by evaluating the exploratory activity of treated animals *versus* control in the elevated plus maze. As shown in Fig. 3A, neither ASTA nor Fish oil (alone or combined) resulted in significantly different scores of total time spent in open arms, probably due to very incongruent data point distribution in supplementation groups ($p = 0.265$; $p = 0.767$; $p = 0.574$ for ASTA-, Fish oil-, and ASTA/Fish oil-fed rats versus control, respectively). On the other hand, when anxiety behavior was approached by time spent in exploratory activity during each entrance in open arms, more congruency was observed in our data points (Fig. 3B). Both Fig. 3A and B adopted the exclusion criterion for outlier points (>1.5*IQR, interval between quartiles). Despite no difference was observed between control and ASTA supplementation in rats, both Fish oil and combined ASTA/Fish oil groups showed similar significant anxiolytic properties ($p = 0.294$ between them).

4. Discussion

In order to hinder neurodegenerative processes and sustain cognitive capacities in humans, the consumption of marine n-3/PUFA-rich products has been long recommended by several medical authorities worldwide (Cunnane et al., 2009). A balanced proportion between EPA and DHA (3:2, regularly found in natural fish oils) is apparently the key factor for such health benefits, whereas many other PUFAs—such as the pro-inflammatory arachidonic acid—can, in fact, trigger harmful effects by massive ROS production from activated immune cells (Gorjão et al., 2009). In fact, a currently accepted hormesis principle sustains that every biological system (cells, tissues, etc.) adequately performs under a specific functional ratio of pro-/antioxidant events, which then depicts its optimal redox balance (Calabrese et al., 2010). Following the hormesis principle, neuronal cells adequately function under high oxidative pressure which is probably a different environment faced by erythrocytes or other cells. So, the presumption that minor oxidative modifications in brain regions necessarily culminate in neurological disturbs or neurodegenerative processes is not fully correct (obviously, depending on their extension). Herewith, the hormesis concept could be adequately

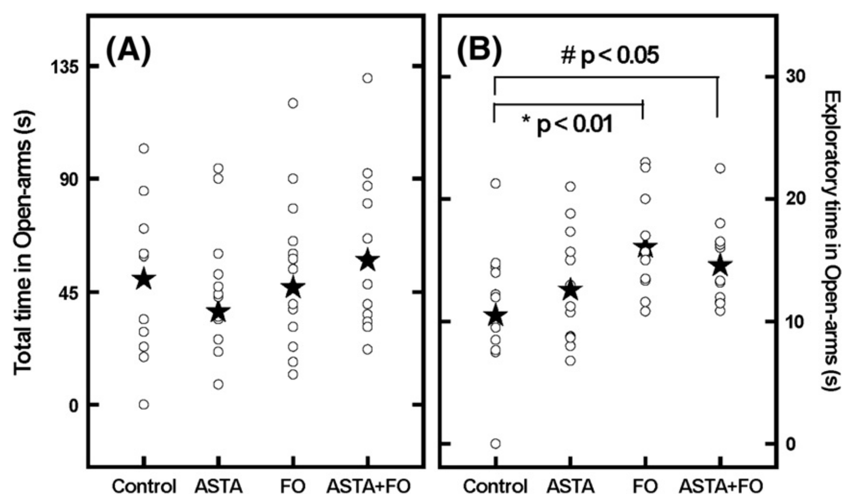

Fig. 3. Exploratory activity, as a parameter for anxiety-behavior, of astaxanthin (ASTA)- and/or fish oil (FO)-fed animals in the elevated plus maze, measured for 5 min. (A) Total time spent in open arms, in seconds; and (B) time spent during each entrance in open arms, in seconds. Star symbols (★) represent the average value of the data point distribution ($n = 16$). Exclusion of outlier points>1.5*IQR, where IQR = interval between quartiles. *$p<0.01$; #$p<0.05$.

applied to explain why such a powerful antioxidant compound as ASTA (Barros et al., 2001), only showed moderate changes in the anterior forebrain region (Table 2) whereas huge antioxidant increment was observed in plasma of the same animals (Table 1).

Fish oil supplementation caused the downregulation of the major antioxidant enzymes total SOD and CAT (Fig. 2) in the anterior forebrain of experimental animals, with special contribution from mitochondrial MnSOD (Table 2). Lower MnSOD/totalSOD ratio obtained in both Fish oil (30% lower, $p<0.005$) and ASTA/Fish oil-fed animals (40% lower, $p<0.005$)—but not in ASTA group (Table 2)—is an evidence of a mitochondrial-centered action of fish oil components, in agreement to previous evidence from other groups (Calabrese et al., 2001). Many authors attribute the antioxidant effects of n-3/PUFAs to their energy-uncoupling (thermogenic?) properties in mitochondria, which would lower the oxidative pressure on the electron-transport chain and, thus, damp down $O_2^{\bullet-}/H_2O_2$ production (Davis et al., 2008). Impaired mitochondrial energy-supply could, at some extension, deplete ATP levels and change $NAD(P)^+/NAD(P)H$ ratio, which are biochemical events tightly associated to neurotoxicity (Binienda et al., 2006). Such a secondary effect on $NAD(P)^+/NAD(P)H$ ratio could explain the observed unresponsiveness of NADPH-dependent GR activity observed here (Fig. 2).

As a result of diminished intracellular antioxidant enzyme defenses and 45% higher concentrations of free iron, exacerbated levels of lipid (33% higher TBARS) and protein oxidation (34% less thiol groups) were measured in the anterior forebrain of fish oil-fed rats without a proportional increment of antioxidant systems (checked by FRC and TEAC in Table 2). Iron homeostasis is crucial in keeping brain function, since "free" iron ions can accelerate the formation of aggressive $HO^{\bullet}$ radicals (by Fenton-reaction) and also the oxidation of catecholamines (Park et al., 2011). Despite anxiety-related catecholaminergic innervated regions are renowned as highly susceptible to oxidative stress than the remaining brain regions (Homi et al., 2002; Shelton and Brown, 2001), the rebalance of pro-/antioxidant processes in the anterior forebrain of fish oil-fed animals was apparently able to sustain putative anxiolytic effects, as preliminarily determined by increased time of open-arm exploration in the elevated plus-maze (Fig. 3B).

Astaxanthin (ASTA) possesses powerful antioxidant activity both in vitro and in vivo (Barros et al., 2001; Palozza and Krinsky, 1992; Stahl and Sies, 2005). Several authors have properly demonstrated that ASTA directly crosses the brain-blood barrier (BBB) to reach different mammalian brain regions (Choi et al., 2011; Lee et al., 2011; Lu et al., 2010; Liu and Osawa, 2009), although we still cannot confirm if ASTA particularly accumulated in the anterior forebrain of rats following our supplementation protocols. Nevertheless, ASTA supplementation alone did not profoundly alter adaptive responses in anterior forebrain of rats (enzymatic, FRC, TEAC, or iron content in brain homogenates, Fig. 2 and Table 2), but indeed resulted in significant antioxidant protection to lipid content in rat brains (TBARS levels in Table 2). Thereby, the improvement of GSH-based antioxidant capacity and FRAP/TEAC scores in plasma of ASTA/Fish oil-fed animals (Table 1) could represent an auxiliary mechanism by which ASTA limited oxidative insults in the anterior forebrain of experimental animals. However, it is worthy to note the key contribution of fish oil in the process, since ASTA supplementation alone only provided protective role in terms of oxidative modifications in such brain region (Table 2).

In summary, combined ASTA/Fish oil vs. fish oil supplementation resulted in: (i) substantial increase of antioxidant capacity in plasma (by total GSH, GSH/GSSG ratio, TEAC and FRAP) to counteract augmented "free" iron (Table 1); (ii) attenuation of the damping effect on $O_2^{\bullet-}/H_2O_2$ production, suggested by partial recovery of totalSOD and CAT activities (Fig. 2A and C); (iii) 25% and 18% higher activities of GR and CuZnSOD, respectively (GPX only showed a slight tendency to increase, $p=0.432$; Fig. 2); (iv) increased FRC and TEAC capacities in brain homogenates (not evidenced in Fish oil group; Table 2); and (v) lower indexes of lipid peroxidation and restoration of basal scores of protein oxidation in anterior forebrain of animals (Table 2).

Based on the hormesis principle, neuronal cells regularly function under high oxidative pressure (Calabrese et al., 2010). Thus, it is tempting to suggest that minor changes of the redox status in neuronal cells—such as those provoked by ASTA co-supplementation with fish oil—were not enough to grossly affect (more complex) behavioral aspects as a short-term event. However, the long-term preservation of neuronal lipid and protein fractions by combined ASTA/Fish oil supplementation could indeed represent a chronically relevant mechanism in preventing progressive neurodegenerative processes and cognitive dysfunctions. Thus, independent to the proper mechanism by which ASTA exerts its antioxidant effects in the anterior forebrain of fish oil-fed animals, the combination of such powerful ROS scavenger (ASTA) with the neuroprotective properties of fish oil not only protected such an oxidative stress-susceptible brain region against higher oxidative modifications in lipids and proteins but also sustained its hypothetical anxiolytic effects.

4.1. Future perspectives

Pharmacological modulation of cellular stress responses has emerging implications for the treatment of neurodegenerative disorders. Several clinical trials produced intriguing data suggesting that the beneficial effects of n-3/PUFAs supplementation may depend on the stage of neurological disease (different redox statuses?) and other dietary mediators, especially antioxidants (Jicha and Markesbery, 2010). A critical key to successful medical intervention is getting the dose right, since human inter-individual variation, and additional factors such as age, gender, diet, exercise, genetic profile and health status apparently reflects distinct redox statuses for each biological system. The hormetic dose–response principle may, thus, represent the proper pharmacological strategy to group all these relevant factors into a single drug formulation for pre-clinical studies, clinical trials and further disease cures.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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