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ORIGINAL CONTRIBUTION

Combined fish oil and astaxanthin supplementation modulates rat lymphocyte function

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Abstract
Purpose Higher intakes of n-3 polyunsaturated fatty acids that are abundant in marine fishes have been long described as a "good nutritional intervention" with increasing clinical benefits to cardiovascular health, inflammation, mental, and neurodegenerative diseases. The present study was designed to investigate the effect of daily fish oil (FO—10 mg EPA/kg body weight (BW) and 7 mg DHA/kg BW) intake by oral gavage associated with the antioxidant astaxanthin (ASTA—1 mg/kg BW) on the redox metabolism and the functional properties of lymphocytes from rat lymph nodes.
Methods This study was conducted by measurements of lymphocyte proliferation capacity, ROS production [superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2)], nitric oxide ($NO^{\bullet}$) generation, intracellular calcium release, oxidative damage to lipids and proteins, activities of major antioxidant enzymes, GSH/GSSG content, and cytokines release.
Results After 45 days of FO + ASTA supplementation, the proliferation capacity of activated T- and B-lymphocytes was significantly diminished followed by lower levels of $O_2^{\bullet -}$, H_2O_2 and $NO^{\bullet}$ production, and increased activities of total/SOD, GR and GPx, and calcium release in cytosol. ASTA was able to prevent oxidative modification in cell structures through the suppression of the oxidative stress condition imposed by FO. L-selectin was increased by FO, and IL-1β was decreased only by ASTA supplementation.
Conclusion We can propose that association of ASTA with FO could be a good strategy to prevent oxidative stress induced by polyunsaturated fatty acids and also to potentiate immuno-modulatory effects of FO.

Keywords Antioxidant · Astaxanthin · Carotenoid · Fish oil · Leukocytes · Lymphocyte · Oxidative stress · n-3 fatty acids

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Introduction

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been extensively studied in cells of the immune system due to their ability to alter the production of anti- and pro-inflammatory interleukins and eicosanoids, thus affecting the humoral function of immune cells. In fact, EPA and DHA are associated with production of less inflammatory and, in some cases, anti-inflammatory molecules [1, 2] contrasting with arachidonic acid, which generally induces the pro-inflammatory eicosanoids production. Inclusion of EPA and DHA in the diet in the form of n-3 polyunsaturated fatty acids, PUFA–rich fish oil (FO), reduces the symptoms of diseases, as well as the use of nonsteroidal anti-inflammatory drugs in arthritis patients with severe inflammatory joint disease [3].

In vitro studies with n-3 PUFA have demonstrated decreased adhesion molecule expression on the surface of monocytes and endothelial cells [4–7]. Furthermore, feeding studies reported that FO decreased the expression of ICAM-1, L-selectin, and VCAM-1 on the surface of murine macrophages, lymphocytes, and human monocytes [7, 8]. The n-3 PUFAs induced reduction in adhesion molecule expression is accompanied by a decreased ability to bind ligand-bearing leukocytes [5, 7, 9, 10]. However, the true functional effects of such changes for inflammatory responses and diseases are still unclear.

Lymphocytes are cells that specifically recognize and respond to foreign antigens and, as such, form the core of the acquired (or specific) immune system. Lymphocytes are involved in both the beneficial and detrimental effects of the immune system. Both concentration and composition of fatty acids present in the diet can affect lymphocyte functions, through an eicosanoid-dependent and independent mechanism [11]. On the other hand, the highly unsaturated hydrophobic chains of EPA and DHA make them prone to free-radical-progressive oxidation, with severe cellular consequences as membrane disruption and cell/organelle decompartmentalization [12].

The marine carotenoid astaxanthin (ASTA) is naturally found in a wide variety of living organisms, such as microalgae, fungi, and crustaceans. Several studies have demonstrated that ASTA possesses powerful antioxidant properties, both in vitro [13, 14] and in vivo, especially as an inhibitor of LDL oxidation [15–17]. Furthermore, ASTA has also shown important roles as an anti-inflammatory [18], anti-apoptotic [19], and anti-diabetic agent [20].

Although the anti-inflammatory properties of FO are currently well recognized, some adverse effects on immune function were also reported and related to n-3 PUFAs influence on membrane fluidity and its sensitivity to oxidation [21]. Thus, it is plausible that combined treatment with FO and ASTA could prevent those adverse effects caused by FO alone or possibly enhance its anti-inflammatory properties. The present study was designed to investigate the effect of daily FO intake alone or associated with ASTA on oxidative stress parameters and the functional properties of lymphocytes isolated from rats lymph nodes. This study was conducted by measurements of B- and T-lymphocyte proliferation capacity (by MTT assay), production of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) by fluorescence/chemiluminescence techniques, nitric oxide ($NO^{\bullet}$) metabolism (by the Griess reagent), intracellular calcium release (Fura 2-AM fluorescent probe), oxidative injury to lipids (TBARS assay) and proteins (thiol and carbonyl contents), activities of major antioxidant enzymes (total and mitochondrial Mn-dependent superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase), GSH/GSSG content, and cytokines release.

Materials and methods

Chemicals and natural products

All purified chemicals were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO), except common laboratory solutions and buffers, which were obtained from Labsynth (Diadema, Sao Paulo, Brazil). Fish oil (FO) capsules were purchased from Pharmanostra (Sao Paulo, Brazil). Each FO capsule of 500 μL contains 9 kcal (38 kJ), 2.0 mg of mixed tocopherols, and 1.0 g of total fat, out of 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 BioReal AB (Gustavsberg, Sweden). 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). Based on that composition, we calculated the AstaREAL A1010 biomass per gavage volume (of 10% Tween-80 aqueous solution, v/v) and animal body weight (BW) to reach the aforementioned 1 mg ASTA/kg BW.

Animals

Adult Wistar male rats, weighing 225.6 ± 17.1 g (3 months old) at the beginning of the study, were provided by the Department of Psychobiology, Universidade Federal de São Paulo (UNIFESP), Sao Paulo, Brazil. All animals were housed in Plexiglas cages (four rats/cage) under standard laboratory conditions: 12 h light/dark cycle; lights on at 7:00 a.m.; 22±2 °C; and ad libitum access to water and Purina rat chow. The experiment was carried out in accordance with the scientific procedures 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 Committee of Ethics in Research from Universidade Federal de São Paulo approved the experimental protocol (CEP: no 1938/09).

Supplementation protocols

After room acclimatization for 1 week, four experimental groups of 16 animals each were formed: control (fed with 400 μL of 10% Tween-80 aqueous solution (v/v)); ASTA (fed with 1 mg ASTA/kg body weight (BW)); FO (fed with 10 mg EPA/kg BW and 7 mg DHA/kg BW); and FO + ASTA (fed with 1 mg ASTA/kg BW, 10 mg EPA/kg BW and 7 mg DHA/kg BW). The animals were treated orally by gavage in a constant volume of 1 mL/kg, 5 days a

week, for 45 days. A maximum volume of 400 μL was established for the gavage treatment in order to prevent regurgitation or stomach discomfort of the animals.

Fish oil 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 BW and 7 mg DHA/kg BW. An identical procedure was conducted for animal supplementation with 1 mg ASTA/kg BW. For combined FO and ASTA treatments (FO + ASTA), both components were diluted in the same stock 10% Tween-80 aqueous solution (v/v) to reach previously described concentrations.

Experimental procedure

After 45 days of treatment, fed rats were killed by decapitation between 11:00 a.m. and 01:00 p.m. Mesenteric lymph nodes were dissected, and lymphocytes were prepared as previously described [22]. After centrifugation at 400 g for 10 min, lymphocytes were suspended in RPMI 1640 medium. The number of viable cells (>95%) was determined in a Neubauer chamber using an optical microscope (NikonYS2-H), following addition of Trypan blue solution (1% w/v). In all experiments, ten animals were used per group, and at least three different experiments were carried out for each analysis.

Determination of lymphocyte proliferation capacity

Assessments of the functional capacity of the immune response can be made by measuring specific cell functions ex vivo. Lymphocyte proliferation is the increase in number of lymphocytes in response to a stimulus. The proliferation response of lymphocytes was determined using the Vybrant MTT Cell proliferation (InVitrogen) according to the manufacturer's instructions. Briefly, the MTT assay involves the conversion of the water-soluble compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the insoluble formazan. The formazan is then solubilized, and the concentration was determined by optical density at 570 nm. The cells (1.5×10^6 cell/mL) were stimulated with concavalin A (20 μg Con A/mL) or lipopolysaccharide (100 μg LPS/mL) to evaluate T-lymphocyte and B-lymphocyte proliferation capacity, respectively. Absorbance was measured at 570 nm, and the results were expressed as optical density (OD).

Measurement of ROS by dihydroethidium

Dihydroethidium (DHE) was used for the fluorimetric measurement of intracellular $O_2^{\bullet -}$ content. DHE is a lipophilic probe and readily diffuses across cell membranes. Once inside the cell, it is rapidly oxidized to ethidium (a red fluorescent compound) by $O_2^{\bullet -}$ and H_2O_2 (in the presence of peroxidase). Ethidium is trapped in the nucleus by intercalating into DNA, leading to an increase of ethidium fluorescence. The cells (1.5×10^6/mL) were preloaded with dihydroethidium (5 μM) by incubating for 15 min at room temperature in the dark. The assay was carried out in the presence and the absence of phorbol myristate acetate (PMA) (20 ng/well) used as a potent inducer of ROS production. Fluorescence was measured at 396 nm of excitation wavelength and at 590 nm of emission wavelength and analyzed in a fluorimeter (Tecan, Salzburg, Austria).

Hydrogen peroxide production

The production of H_2O_2 was measured by the method of [23], which is based on horseradish-peroxidase-dependent oxidation of phenol red by H_2O_2 to a colored compound. Briefly, lymphocytes (1.5×10^6/mL) were incubated in Dulbelco's PBS pH 7.4 and a solution of phenol red (0.28 mM) and horseradish peroxidase (5 mg/mL) (1,000 units/mg) at 37 °C for 1 h. The production of H_2O_2 was measured at rest and after stimulation with PMA (20 ng/well). The reaction was terminated by addition of 10 μL 1 N NaOH solution, and the amount of products formed was measured by spectrophotometry at 620 nm using a H_2O_2 curve concentration as a standard.

Nitric oxide production

Nitric oxide ($NO^{\bullet}$) decomposes rapidly in aerated solutions to form stable nitrite/nitrate products. In our study, nitrite concentrations were determined and used as an index of $NO^{\bullet}$ synthesis. Nitrite was quantified colorimetrically after its reaction with the Griess reagent as described [24]. Briefly, lymphocytes (1.5×10^6/mL) were cultured with lipopolysaccharide (LPS, 10 μg/well) for 4 h. LPS activates a number of intracellular signaling pathways, including NF-κB pathway, thereby allowing rapid gene induction and the expression of inflammatory mediators and inducible nitric oxide synthase (iNOS). Afterwards, spectrophotometric analysis of the total nitrite content was performed by adding 100 μL of Griess reagent (1% sulfanilic acid, 0.1% N-1-naphtyl-ethylenediamine dihydrochloride) in supernatants. Absorbance was measured at 550 nm, and nitrite concentration was determined using sodium nitrite as a standard.

Intracellular Ca^{2+} concentration

Basal levels of cytosolic Ca^{2+} were monitored by fluorescence using the Ca^{2+}-sensitive probe Fura 2-AM as previously described [25]. The loading period for 5 μM Fura 2-AM was 1 h at 37 °C in 1×10^6cells/mL in

Tyrode's solution. Afterwards, cells were washed and intracellular $[Ca^{2+}]_i$ was monitored for 20 min, and fluorescence emission at 510 nm (excitation wavelengths alternating between 340 and 380 nm) of Fura 2-AM was measured in a microplate reader (Tecan, Salzburg, Austria). Transformation of the fluorescent signal to $[Ca^{2+}]_i$ was performed by calibration with ionomycin (100 μM, maximum concentration) followed by EGTA addition (60 μM, minimum concentration) according to the Grynkiewicz equation, using the K_{diss} of 224 nM [26].

Preparation of homogenates

For measurements of enzyme activities and oxidative modification in biomolecules, cells were pelletized (5×10^6) and mixed with 1.0 mL of the assay-specific extraction buffer, vortexed briefly, and lysed by ultrasonication in a Vibra Cell apparatus (Connecticut, USA) as previously described [27]. A centrifugation step was included (10,000*g*, 10 min, at 4 °C); the supernatant was then used for further analysis. The extracts for enzyme determinations and oxidative lesions were prepared in 50 mM sodium phosphate buffer (pH 7.4).

Lymphocyte antioxidant enzyme activities

Glutathione peroxidase (GPx), glutathione reductase, and superoxide dismutase (SOD) activities were determined in lymphocytes using a microplate reader (Tecan, Salzburg, Austria) spectrophotometer at 37 °C. Catalase activity was measured by the spectrophotometric method of [28] based on the decomposition of H_2O_2. Glutathione reductase and GPx activities were measured as described by the [29] spectrophotometric method. SOD activity was measured using the method of [30].

Oxidative lesions (TBARS assay, thiol and carbonyl groups)

The measurement of TBARS was described by [31] through the formation of a colored adduct after the stoichiometric reaction between thiobarbituric acid (TBA) and several lipid-derived aldehydes, including malondialdehyde (MDA). The absorbance at 535 nm was measured after the mixture reaches room temperature, and the TBARS content was estimated by a standard curve of 10 μM 1,1,3,3-tetraethoxypropane. Thiol and carbonyl groups were evaluated as biomarkers of aminoacid oxidation in lymphocytes. Homogenate of lymphocytes was precipitated with 20% trichloracetic acid solution in ice. Reduced thiol groups were detected by the formation of colored adducts after reaction with 4 mM 5.5′-dithio-bis (2-nitrobenzoic acid) solution (DTNB). The absorbance of DTNB-treated samples at 412 nm was calculated using GSH as a standard [32]. The same procedure was used to estimate protein carbonyls. The protein carbonyls were identified by the hydrazones formed with 10 mM dinitrophenylhydrazine (DNPH) in 0.25 M HCl. Absorbance of the peak detected within the range of 340–380 nm was measured, and the carbonyl group concentration was calculated based on the molar coefficient of $\varepsilon = 2.2 \times 10^4\ M^{-1}\ cm^{-1}$ [33].

Cytokines release

Cytokines IL-6, IL-1β, and L-selectin were assayed in cell culture supernatant with ELISA kits, according to the manufacturer's instructions (Quantikine, R&D System, Minneapolis, MN, USA). Lymphocytes (1×10^6/mL) were cultured for 18 h in the presence of LPS as a stimulus (10 μg/mL). Afterwards, cells were centrifuged (1,000*g*, 4 °C, 10 min), and supernatant was collected and used for cytokines determination.

GSH/GSSG content

Lymphocytes were used for determination of glutathione status, using the method described by [34]. Both total GSH and GSSG were analyzed using 5,5′-diothiobis-2 nitrobenzoic acid (DTNB), which reacts with reduced glutathione (GSH) to form 5-thio-2-nitrobenzoic acid (TNB). The GSH/GSSG concentrations were calculated from a standard curve prepared with pure GSH/GSSG standards and were expressed as nM of GSH and GSSG.

Protein measurement

Specific enzyme activities, as well oxidative lesion measurements, were all related to protein concentrations, which were estimated by the [35] method using bovine serum albumin as a standard.

Statistical analysis

All data points are the mean values with their standard errors of at least three independent experiments. Data were analyzed by one-way ANOVA followed by the Tukey's post-test. The software employed for statistical analysis was GraphPad Prism (version4; GraphPad Software, San Diego, CA, USA).

Results

Proliferative capacity

Lymphocyte functionality was accurately evaluated in the present study by measurement of the proliferation capacity after stimulation with a specific mitogen. Figure 1 shows the MTT assay results after stimulation by Con A (a specific mitogen to T-lymphocytes) or LPS (specific stimulus to B-lymphocytes) for 48 h. FO + ASTA decreased the proliferative response to both stimuli Con A and LPS by 29 and 21%, respectively, as compared to the control. Lymphocytes of the FO group showed an increase of 15% in proliferation capacity after Con A stimulation as compared to control.

Intracellular ROS and NO production

The effects of FO treatment in isolated lymphocytes were assessed by the measurement of the key redox signaling components $O_2^{\bullet -}$, H_2O_2, $NO^{\bullet}$, and intracellular calcium. After PMA stimulation, lymphocytes from rats treated with ASTA, FO, and FO + ASTA showed a significant decrease in $O_2^{\bullet -}$ production of 13, 51, and 50%, respectively, as compared to the control group assessed by the fluorescent DHE probe (Fig. 2a). Basal $O_2^{\bullet -}$ production was also decreased in the FO group as compared with the control unstimulated group.

Regarding H_2O_2 baseline production (in unstimulated lymphocytes), no significant differences were observed between groups. However, after PMA stimulation, lymphocytes from the FO and FO + ASTA groups reduced the H_2O_2 production by 40 and 17%, respectively, as compared to the control group (Fig. 2b).

For unstimulated cells, there was no significant difference in $NO^{\bullet}$ production between the groups. After LPS stimulation for 24 h, both the FO and FO + ASTA groups showed significantly lower $NO^{\bullet}$ production than the control group by 34 and 73%, respectively (Fig. 2c).

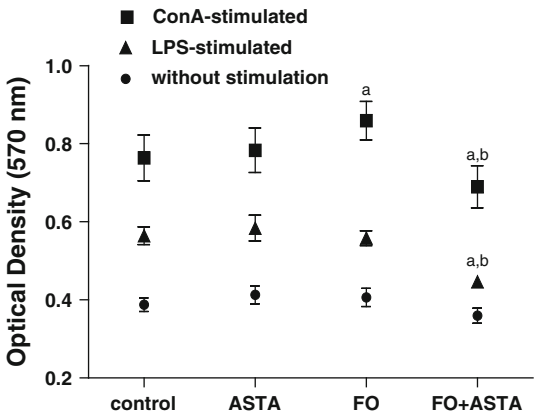

Fig. 1 Proliferation capacity of rat lymphocytes after Con A and LPS stimulation. Cells (1.5×10^6/mL) were incubated for 48 h in the absence or presence of mitogens. The results are presented as mean ± SEM (two different experiments). **a** $p < 0.05$ compared to control-stimulated group. **b** $p < 0.05$ compared to FO-stimulated group

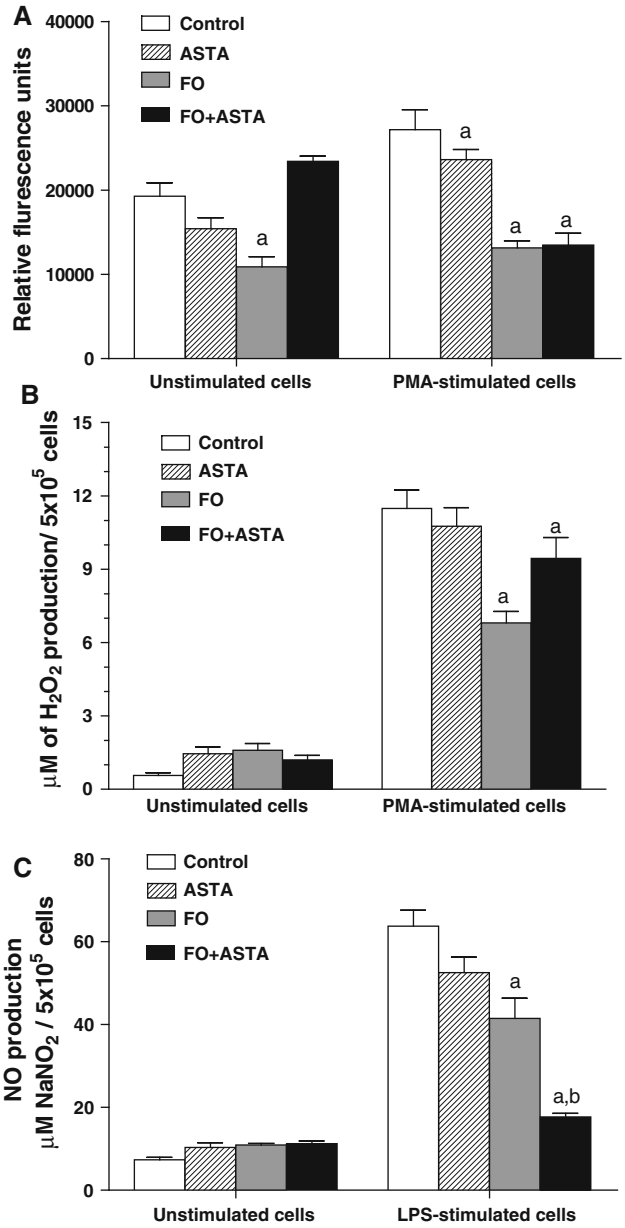

Fig. 2 **a** Superoxide anion ($O_2^{\bullet -}$), **b** hydrogen peroxide (H_2O_2), and **c** nitric oxide ($NO^{\bullet}$) production after PMA- (**a**, **b**) and LPS stimulation (**c**). Lymphocytes (1.5×10^6/mL) were freshly treated with PMA (20 ng/well) or LPS (10 µg/well). The values are presented as mean ± SEM of 10 determinations. **a** $p < 0.05$ compared to respective control group. **b** $p < 0.05$ compared to FO-stimulated group

Intracellular calcium concentration

Basal levels of Ca^{2+} monitored during 20 min are presented in Fig. 3. The content of $[Ca^{2+}]_i$ obtained from lymphocytes of ASTA-treated rats was significantly higher (by 31%) than in cells from the control group. Fish oil treatment also promoted an increase in basal $[Ca^{2+}]_i$ mobilization as compared with control lymphocytes. Combined treatment with FO + ASTA reduced intracellular Ca^{2+} levels by 10% in comparison to the FO group.

Antioxidant enzymes activities and oxidative damage in biomolecules and cytokine release

Concerning antioxidant enzyme defense, lymphocytes from the FO + ASTA group manifested a marked improvement of 40, 72, and 88% in total SOD activity when, respectively, compared to control, ASTA, and FO groups (Table 1). GPx activity was increased by 3.43-, 4.25-, and 4.52-fold in ASTA, FO, and FO + ASTA, respectively, as compared with control group. Glutathione reductase activity was decreased in the FO group, but increased in the FO + ASTA group.

As expected, the levels of TBARS were significantly elevated in the lymphocytes from FO-supplemented rats as compared to the control group, and ASTA administration did not prevent increased levels of TBARS. The daily administration of ASTA for 45 days improved thiol levels significantly by 128% compared to the control group (Table 1). FO treatment also increased content of thiol groups by 28%, but ASTA + FO combination did not result in further thiol recovery. No significant difference was observed in protein carbonyl content between groups.

FO supplementation promoted an increase in L-selectin release from lymphocytes after LPS stimulation, and the association of FO + ASTA promoted a reduction in L-selectin levels. While the pro-inflammatory cytokine IL-1β was decreased in lymphocytes from rats supplemented with ASTA, there was no change in levels of IL-6 between all groups.

GSH/GSSG content

The GSH/GSSG ratio was significantly modified by the addition of ASTA or FO, but the combined association of ASTA + FO showed a lower effect than those observed individually (Fig. 4).

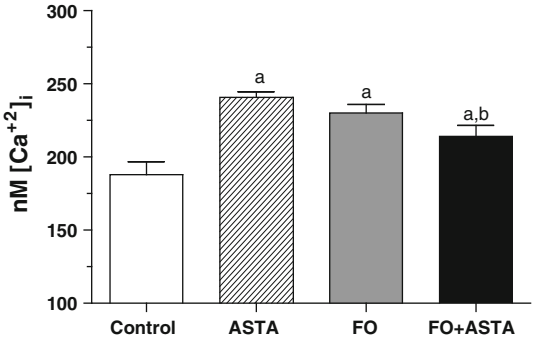

Fig. 3 Amount of basal release of calcium per minute during 20 min of monitoring ($[Ca^{2+}]_i$ nM). Cells (1×10^6/mL) were previously loaded with Fura 2-AM (5 μM) during 1 h and then freshly evaluated. Results are presented as mean ± SEM of nine determinations (area under the curve—AUC analysis). [a]Significantly different compared with cells from the control group ($p<0.05$). [b]Significantly different compared with cells from the FO group

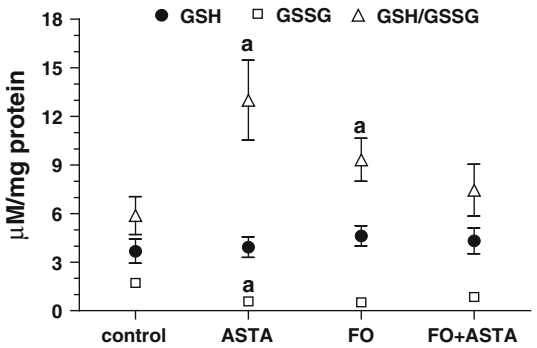

Fig. 4 GSH and GSSG content and GSH/GSSG ratio in lymphocytes from rats treated with FO, ASTA, and association of FO + ASTA. Results are presented as mean ± SEM. [a]Significantly different as compared with the control group ($p < 0.05$)

Discussion

Clearly, a well-functioning immune system is essential to health. It serves to protect the host from the effects of ever-present pathogenic organisms. There are, however, some detrimental effects of the immune system in which lymphocytes are major protagonists [36]. In some individuals, the immune system appears to recognize host antigens as "nonself" rather than as "self". This is the characteristic of so-called chronic inflammatory (or autoimmune) diseases, which leads to auto-destruction of host tissues [11]. Recently, the use of molecules from the diet (known as nutraceuticals) has been suggested as adjuvant in the treatment of many diseases, including those related to the immune system.

In the present study, we propose the association of fish oil (mainly represented by EPA and DHA) with the antioxidant and anti-inflammatory carotenoid ASTA in an attempt to minimize FO-induced oxidative stress and perhaps promote a synergic anti-inflammatory and/or antioxidant effect. Overall, association of FO + ASTA had different effects when compared with FO or ASTA alone, as demonstrated by reduced proliferative capacity of T- and

Table 1 Effect of FO and ASTA supplementation on antioxidant enzyme activities, oxidative damages in biomolecules, and cytokines release of rat lymphocytes

	Control	ASTA	FO	FO + ASTA
Total SOD (U/mg protein)	48.21 ± 5.49	39.32 ± 4.44	35.60 ± 3.48	67.56 ± 6.05[a,b,c]
Mn-SOD (U/mg protein)	47 ± 5.03	30.3 ± 4.7[a]	34.04 ± 3.55[a]	64.47 ± 7.2[a,b]
CAT (μmol/min/mg protein)	3.02 ± 0.16	1.82 ± 0.11[a]	3.05 ± 0.21	1.86 ± 0.19[a,b]
GPx (mU/mg protein)	925 ± 202	3,176 ± 353[a]	3,933 ± 378[a]	4,188 ± 1,012[a]
GR (mU/mg protein)	332 ± 49.9	310 ± 48.82	122 ± 14.63[a]	1,018 ± 95.25[a,c]
Carbonyl groups	26.85 ± 1.65	26.01 ± 0.53	25.02 ± 2.05	32.02 ± 0.25
Thiol groups	194.2 ± 5.08	443.3 ± 47.52[a]	249.6 ± 11.21[a]	223.6 ± 8.87[a]
TBARS	5.86 ± 0.33	5.74 ± 0.40	15.79 ± 2.17[a]	22.65 ± 0.85[a]
L-selectin (pg/mL)	80 ± 14	53 ± 7	147 ± 15[a,b]	102 ± 17[a]
IL-6 (pg/mL)	2.4 ± 0.13	2.27 ± 0.11	2.27 ± 0.12	2.46 ± 0.29
IL-1β (pg/mL)	3,883 ± 186	3,167 ± 65[a]	3,363 ± 93	3,337 ± 84

Results are expressed as mean ± SEM of at least 10 determinations

[a] Significantly different compared with the cells from the control group ($p < 0.05$)

[b] Significantly different compared with the cells from the ASTA group ($p < 0.001$)

[c] Significantly different compared with the cells from the FO group ($p < 0.001$)

B-lymphocytes, reduced intracellular Ca^{2+} concentration, and increased enzymatic antioxidant capacity, mainly expressed by total and Mn-SOD, GR and GPx, and L-selectin release. In addition, FO + ASTA decreased superoxide anion, hydrogen peroxide, and $NO^{\bullet}$ production. Unfortunately, ASTA treatment was unable to reverse lipid peroxidation induced by the presence of FO supplementation.

In our study, association of FO + ASTA promoted a decrease in T- and B-lymphocyte proliferation capacity after Con A and LPS stimulation, whereas we cannot observe a reduction in proliferative capacity of lymphocytes when ASTA or FO were administered alone. Previous study from our group also demonstrated a significant decrease in B- and T-lymphocyte proliferation when cells were treated with 5 μM of ASTA in vitro [37]. Lymphocyte activation is usually inhibited by fatty acids, particularly by PUFA and volatile fatty acids [38]. The effect of FA on lymphocyte function has been studied extensively, but the mechanisms involved are still unclear. However, it is widely known that treatment with tyrosine kinase inhibitors reduces lymphocyte proliferation [39]. The n-3 PUFA EPA and DHA cause a marked decrease in lymphocyte proliferation by promoting an inhibition of cell cycle progression induced by IL-2, inhibiting the MAPK pathway, and decreasing the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) [40]. These results suggest that these FA may exert their inhibitory action on ERK1/2 activation by reducing PKC activity. In addition, Denys, Hichami, and Khan [41] showed that DHA and EPA inhibit membrane recruitment of PKC-α and PKC-ε. These PKC isoforms are coupled to MAPK activation upstream of ERK1/2, which leads to a decrease in nuclear translocation of nuclear factor κB (NF-κB), resulting in an inhibition of IL-2 gene expression and cell proliferation. Other studies have demonstrated that these FA may exert suppressive effects on the phosphorylation of other MAPKs.

Studies suggested that the anticancer activity of carotenoids such as lycopene and ASTA are related to their effectiveness as antioxidants, singlet oxygen quenchers, and free radical scavengers [42]. However, the mechanisms by which carotenoids decreases the risk of cancer are not well understood [43]. ASTA is able to inhibit p38 MAPK and MEk pathway [44], down regulating the NF-κB activation and ERK1/2 and pMSK-1 pathway. Since isolated FO and ASTA did not promote an inhibition on lymphocyte proliferation, but association of both did, we can speculate that association of these compounds can cause a sufficient inhibition on protein phosphorylation involved in the process of lymphocyte proliferation. Perhaps, the amount of each compound alone was not sufficient to inhibit protein phosphorylation, but when they were associated, there was a summation of the inhibitory actions of each one, which was able to cause an inhibition of proliferative capacity. To corroborate with this hypothesis, ASTA, such as other carotenoids, is a very lipophilic compound and has low oral bioavailability. However, its bioavailability can be enhanced in the presence of fat, as the FO used in the present study [45].

A study by Okada et al. [46] showed that oral administration of 48 mg astaxanthin in smokers and nonsmokers before and after a meal significantly affects the bioavailability of astaxanthin. ASTA administration performed

immediately after the meal resulted in an increased availability of astaxanthin in plasma, as detected by HPLC. The same was observed in smokers compared to nonsmokers. In a clinical trial performed by Odeberg et al. [45], 32 healthy men received a single dose of astaxanthin (40 mg), either as a commercial food supplement or in different lipid-based formulations. All three lipid-based formulations enhanced the bioavailability of astaxanthin, with formulation containing glycerol mono- and dioleate, and polysorbate 80 B being fourfold better. In a recent study conducted by Petri and Lundebye [47], the organ distribution of high doses of astaxanthin in rats after oral application was conducted, observing time (1 and 2 weeks) and dose response (0.3, 1, and 3% of the feed). Low astaxanthin concentrations were detected in the viscera, distributed in a wide range, and not increasing from 7th to 14th day. This indicates that there was rapid elimination or catabolism and no profound long-term storage. Liver concentration was unexpectedly low, while the highest concentrations were found in spleen, kidneys, and adrenals. The main site of astaxanthin accumulation, indeed, was the hairless skin of the tail; this was associated with red coloration. The relative numbers of lymphocytes, neutrophilic granulocytes, and monocytes in blood from rats, which had received the highest dose of astaxanthin, appeared to deviate somewhat (although not significantly so) from the other groups, which appeared more homogeneous. Blood taken from rats in the highest dose group after 7 days of dietary exposure had relatively few lymphocytes, whereas blood from animals in the group taken after 14 days of exposure had relatively large numbers of lymphocytes compared with samples from the other groups. Accumulation of astaxanthin in lymphocytes was not carried out. In the present study, the total accumulation of astaxanthin from lymph nodes and lymphocytes was determined as the amount extractable by 90% (v/v) acetone (data not shown). Our results indicated that there was not a significant increase in the amount of astaxanthin between groups, although we observed an upward trend in the groups treated with astaxanthin. We emphasize that the method used is low, accurate, and we may not have observed differences between groups by the lack of sensitivity of the method used.

Based on this data, we can suggest that the effects of the combination of FO + ASTA may have effects other than those observed for the compounds alone, due to differences in bioavailability of ASTA, which can be increased by FO. If protein phosphorylation was decreased by FO + ASTA, treatment remains to be elucidated. Another mechanism, which can be suggested to explain the lower lymphocyte proliferative capacity in FO + ASTA group, might be due to the reported actions of FO on lipid rafts that are platforms of lipids on cell membrane, which once altered can modify the proliferation of these cells [36, 48–50].

The cell–cell communication is definitely a key process to immune response. This process is absolutely dependent on the oxidant–antioxidant balance. Immune cells are frequently exposed to oxidizing injury provoked by antigen stimulation of ROS as part of their normal function [51]. Reactive oxygen species (ROS) are important in the immune defense against invading pathogens, but they are also key molecules in the regulation of inflammatory reactions. ROS might not only be produced as a mechanism to eradicate invading pathogens but rather also as a means by which to fine tune the inflammatory response, depending on when, where, and at what amounts they are produced. Lymphocytes also possess the membrane-bound enzyme NAPDPH-oxidase, which when activated by PMA via PKC activation, produces superoxide in quantities sufficient to activate intracellular signaling pathways dependent on ROS. The superoxide anion is a precursor of H_2O_2, which in turn is closely related to cell proliferation. Roth and Dröge [52] found that in activated T cells, the superoxide anion or low micromolar concentrations of hydrogen peroxide increase the production of the T cell growth factor interleukin-2, an immunologically important T cell protein. Keyse and Tyrrell [53] showed that hydrogen peroxide induces the expression of the heme oxygenase (HO-1) gene, and Schreck and Baeuerle [54] reported the activation of the transcription factor nuclear factor κB (NF-κB) by hydrogen peroxide in mammalian cells.

In fact, the production of oxidative species has a key role in the processes of signaling in lymphocytes and is also recognized as essential for the proper function of these cells. In our study, $O_2^{\bullet -}$, H_2O_2, and $NO^{\bullet}$ were substantially reduced by FO treatment. Some beneficial effects of fish oil are due to their antioxidant properties. Supplementation studies providing 3.1–8.4 g EPA-DHA/dose have reported 30–55% decreases in the production of ROS (mainly $O_2^{\bullet -}$ and H_2O_2) by stimulated human neutrophils [55]. Supplementation with 6 g EPA-DHA/dose was shown to decrease H_2O_2 production by activated human monocytes [56]. In addition, omega-3 fatty acids supplementation resulted in better antioxidation status in patients on maintenance hemodialysis [57] and also protected cells from oxidative stress and apoptosis [58]. In vitro studies have shown a decrease in $NO^{\bullet}$ production by mice macrophages and cell lines after exposure to n-3 fatty acids [59, 60]. Neutrophils from patients fed with diets containing fish oil had a decreased production of ROS after stimulation [61, 62]. Generation of ROS was not affected by the presence of EPA and DHA, except at 200 μM, concentrations that also reduced monocyte viability [63]. Since the proliferative process of lymphocytes is dependent of ROS production used as signaling molecules in the cascade of activation that occur at the beginning of this process, we suggest that the reduced ROS production observed in FO + ASTA

group can be the mechanism by which these nutraceuticals are inhibiting the proliferation of lymphocytes.

It is plausible that the lower production of $O_2^{\bullet -}$ and H_2O_2 was a consequence of the observed increased activities of total SOD and GPx, which assured the improvement of the antioxidant system by ASTA and FO supplementation. Diets rich in omega-3 fatty acids increase lipid peroxidation while concomitantly raising glutathione peroxidase activity. This enzyme is particularly important in cell protection from oxidant stress under physiological conditions, converting peroxides into their alcoholic derivatives, at the expense of glutathione [64]. ASTA and other carotenoids are considered to be beneficial in the prevention of a variety of major diseases, including cardiovascular disease, cancer, and diabetes [65]. ASTA is shown to protect against lipid peroxidation [15] and DNA damage [66]. Additionally, it presents a large variety of biological activities as anti-obesity [67], anti-inflammatory [18], and antioxidant [68]. Despite ASTA was not able to revert the increased lipid peroxidation process in lymphocytes of FO-supplemented animals (assessed by TBARS assay), free protein SH content—as a parameter of oxidative damage in proteins—was clearly increased by the combined addition of ASTA and FO (Table 1). This aspect is reinforced by the observed reduction in GSSG content and GSH/GSSG ratio (Fig. 4).

Regarding Ca^{2+} homeostasis, it is worth to mention that disordered Ca^{2+} influx is often implicated in several signaling pathways, including one that leads to increased $O_2^{\bullet -}$ production by activation of NADPH-oxidase complex [69]. We observed a significant increment of basal levels of Ca^{2+} in lymphocytes from FO and ASTA groups in comparison to the control group. Association of FO + ASTA was effective in restoring Ca^{2+} levels back to baseline. The mechanism involved in the release of Ca^{2+} by the presence of ASTA and FO remains to be clarified. As previously shown, FO can either increase or decrease intracellular calcium release [70, 71]. Similarly, ASTA was shown to either increase or decrease calcium release depending on cell type and concentration/dose used [37, 72, 73]. Like other carotenoids, ASTA has a low solubility, which may be increased by the presence of long-chain fatty acids, as the FO used in the present study. In addition, it was recently shown that ASTA presents mainly esterified to saturated and monounsaturated fatty acids, but not with polyunsaturated fatty acids [74]. This fact may contribute to an increased bioavailability of ASTA in the presence of FA, such as EPA and DHA. Recently, ASTA used at 1 mM, considered a high concentration, provided better protection than the endogenous antioxidant glutathione in terms of suppressing calcium-induced turbidity of lens proteins [75]. This effect was due ASTA interaction with calcium ions to form complexes, which interferes with the hydrolysis of lens crystallins by calcium-activated calpain. Based on this data, we can suggest that the effects of the combination of FO + ASTA may have effects other than those observed for the compounds alone, due to differences in bioavailability of ASTA. Greater availability of ASTA can mean higher inhibitory effect on calcium release, perhaps by a direct chelating effect of ASTA on calcium ions. The biological significance of this event remains to be elucidated. Similar increase in intracellular Ca^{2+} concentration was obtained by our group, which evaluated the basal Ca^{2+} release in human lymphocytes and neutrophils after ASTA addition in vitro [37, 73]. Hirasawa et al. [76] showed that the stimulation of GPR120 by free fatty acids (FFAs) resulted in elevation of $[Ca^{2+}]_i$ and activation of the ERK cascade, which suggests interactions with G proteins.

Multiple adhesion molecules are involved in the interaction of lymphocytes with inflamed endothelium [77]. L-selectin (CD 62L) is expressed on all leukocytes and, among other functions, mediates the binding of naive lymphocytes to endothelial cells present at high endothelial venules in peripheral lymph nodes [78]. n-3 PUFAs are considered primarily as anti-inflammatory drugs, and in this study, supplementation with FO caused an increase in levels of L-selectin. Free L-selectin could bind to its ligands and prevent cells from interacting with the endothelium. Extra L-selectin free may mean less cell surface L-selectin, and so less capacity to bind ligand-bearing cells. These would be understood as an anti-inflammatory effect of FO + ASTA association. Regarding pro-inflammatory cytokines, the supplementation with FO was not able to reduce levels of pro-inflammatory IL-6 and IL-1β, while ASTA supplementation reduced the levels of IL-1β.

In conclusion, lymphocytes from rats supplemented with FO plus ASTA present a decreased proliferative response to mitogens, decreased production of $O_2^{\bullet -}$, H_2O_2, and $NO^{\bullet}$ in the presence of specific stimulation, accompanied by increased total/SOD, GR and GPx activities, calcium, and L-selectin release as compared with FO supplementation alone. ASTA association was also able to prevent oxidative modification in the cell structures through the suppression of the oxidative stress parameters induced by FO. We can conclude that the association of the carotenoid ASTA with FO may have a slight beneficial effect in preventing oxidative stress induced by the presence of polyunsaturated fatty acids EPA and DHA.

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Conflict of interest The authors declare they have no competing financial interests.

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