Combined astaxanthin and fish oil supplementation improves glutathione-based redox balance in rat plasma and neutrophils

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The present study aimed to investigate the effects of daily (45 days) intake of fish oil (FO; 10 mg EPA/kg body weight [BW] and 7 mg DHA/kg BW) and/or natural ASTA (1 mg ASTA/kg BW) on oxidative stress and functional indexes of neutrophils isolated from Wistar rats by monitoring superoxide (O2¯), hydrogen peroxide (H2O2), and nitric oxide (NO) production compared to the progression of auto-induced lipid peroxidation and Ca2+ release in activated neutrophils. Furthermore, phagocytic capacity, antioxidant enzyme activities, glutathione-recycling system, and biomarkers of lipid and protein oxidation in neutrophils were compared to the redox status. Our results show evidence of the beneficial effects of FO + ASTA supplementation for immune competence based on the redox balance in plasma (significant increase in GSH-dependent reducing power), non-activated neutrophils (increased activity of the glutathione-recycling enzymes GPx and GR) and PMA-activated neutrophils (lower O2¯, H2O2, and NO generation, reduced membrane oxidation, but higher phagocytic activity). Combined application of ASTA and FO promoted hypolipidemic/hypcholesterolemic effects in plasma and resulted in increased phagocytic activity of activated neutrophils when compared with ASTA or FO applied alone. In PMA-activated neutrophils, ASTA was superior to FO in exerting antioxidant effects. The bulk of data reinforces the hypothesis that habitual consumption of marine fish (e.g. salmon, which is a natural source of both astaxanthin and fish oil) is beneficial to human health, in particular by improving immune response and lowering the risk of vascular and infectious diseases.

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1. Introduction

Habitual intake of marine fish and seafood has been strongly associated with human health. It is particularly considered to enhance immune competence and prevent infectious diseases [1]. Fish oil (FO) components eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) – well-known as n-3 polyunsaturated fatty acids (n-3/PUFAs) – have been studied in immune cells and proved to increase anti-inflammatory responses by stimulating interleukin production [2]. As central players in inflammatory processes, neutrophils are main protagonists of innate immune responses necessary for early host defense [3]. Regarding nutraceuticals, dietary long-chain (>C20) n-3/PUFAs are considered antioxidant compounds, which significantly diminish superoxide (O2¯) and hydrogen peroxide (H2O2) production in activated neutrophils [4,5]. On the other hand, fish oil also showed adverse effects on immune function due to the influence of PUFAs on membrane fluidity, which could result in higher sensitivity to oxidation [6,7]. Depending on the concentration, DHA and EPA can also affect the microstructure and composition of lipid rafts in the neutrophil membrane, resulting in severe changes of cell–cell molecular communication [8].

Although anti-inflammatory mechanisms in humans have been well discussed, there are still unanswered questions with respect to redox imbalances provoked in neutrophils during the pathogen-activation process. As a consensus, a wide range of reactive oxygen/nitrogen species ROS/RNS (including O2−, H2O2, and NO) are continuously produced by neutrophil oxidative burst, and pathogen cells are undoubtedly not the only targets at the inflammatory site. In fact, ROS/RNS can also (auto)-oxidize neutrophil membrane, triggering lipid peroxidation and producing lipid-derived cytotoxic aldehydes, e.g. malondialdehyde and 4-hydroxynonenal [9]. Thus, long-living/persistent neutrophils as part of an efficient immune system have to cope with huge oxidative challenges imposed by pathogen activation. Several authors have, therefore, proposed that

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dietary programs based on antioxidant supplementation could offer enhancement of immune functions, especially in terms of lifespan and slower turnover of damaged immune cells [10,11]. Accordingly, many antioxidants present in human diet have also positively affected immune functions in patients of ROS-related diseases, such as diabetes [12,13].

The marine carotenoid astaxanthin (ASTA; Fig. 1) is naturally found in a wide variety of aquatic organisms, such as microalgae, crustaceans (crabs, lobsters, and shrimp), and fishes (salmon and trout). ASTA is renowned as a powerful antioxidant [14,15], and also reported to afford benefits to human health, including protection against UV radiation (skin and eyes) [16], prophylaxis/remediation of gastric ulcer induced by Helicobacter pylori [17], and ROS–mediated neurodegenerative processes, as in Parkinson’s disease [18]. Thus, it is reasonable to assume that combined FO and ASTA supplementation could better balance pro- and antioxidant events in activated neutrophils to enhance anti-inflammatory responses and, thus, provide substantial health benefits to humans.

The present study aimed to investigate the effects of daily intake of natural sources of n-3/PUFAs (here provided as fish oil) and/or natural ASTA (obtained from the biomass of the green microalgae Haematococcus pluvialis) [19] on oxidative stress and functional indexes of neutrophils isolated from Wistar rats. This study was performed by monitoring superoxide (O₂⁻), hydrogen peroxide (H₂O₂), and nitric oxide (NO) production compared to the progression of auto-induced lipid peroxidation and Ca²⁺ release in activated neutrophils. Furthermore, our study evaluated antioxidant enzyme activities, the glutathione-recycling system and biomarkers of lipid and protein oxidation as related to the FO and/or ASTA diet, and examined the redox status in plasma as well as in isolated resting and stimulated neutrophils from experimental animals.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA), except those used in the preparation of common buffers (Labsynth, Diadema, SP, Brazil). Commercial biochemical test kits were obtained from Bioclin-Quíbas Química Básica (Belo Horizonte, Brazil) for measurements of glucose (prod. code K082), triacylglycerol (prod. code K055) and cholesterol (prod. code K083) concentrations in plasma. The fluorescent probes acetoxymethyl ester (Fura 2-AM) and 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-inden-3-undecanoic acid (C₁₁-BODIPY581/591) were purchased from Invitrogen (Grand Island, NY, USA).

2.2. Natural products

Fish oil capsules were purchased from Pharmanstra (Sao Paulo, SP, Brazil). Each fish oil (FO) capsule of 500 μL (corresponding to 9 kcal/38 kJ), contains 2.0 mg tocopherols, and 1.0 g of total fat, out of 30% which saturated fats, 20% with monounsaturated (mostly palmiroleic and oleic acids), and 50% with 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 (Gustavberg, Sweden), a subsidiary of BioReal Inc., (Hawaii, USA) and part of the pharmaceutical Group Fuji Chemical Industry CO. AstaREAL A1010 is an astaxanthin-rich natural microagal product, consisting of crushed and spray-dried aplanospores of the green microalgae Haematococcus pluvialis. AstaREAL A1010 contains 42% of crude fat, 10% of crude protein, 40% of carbohydrates, and 4% of ashes. Regarding carotenoid composition, AstaREAL A1010 contains 5.2–5.8% of total carotenoids, 5.0–5.6% of which are being pure astaxanthin (3.5% as monoesters, 0.9% as diesters, and 0.1% in free form) among others.

2.3. Animals and supplementation protocols

Adult Wistar male rats, weighing 191 ± 35 g at the beginning of the study, were provided by the Department of Psychology, Universidade Federal de São Paulo (UNIFESP), São Paulo, Brazil. All animals were housed in plexiglas cage (four rats/cage), under standard laboratory conditions: 12 h light/dark cycle; lights on at 7:00 a.m.; 22 ± 1 °C; water and Purina rat chow ad libitum. The animals used in this study were handled in accordance with the guidelines of the committee on care and use of laboratory animal resources of the Ethics Committee on Care and Use of Laboratory Animal Resources from Universidade Federal de São Paulo, which approved the study protocol (CEP/UNIFESP nº 1936/09). The animals were treated with ASTA and/or FO by gavage, 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 (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 which is 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 both manufactured natural products, their contribution in the total antioxidant capacity of gavage solutions is minor 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 with 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.

2.4. Neutrophil isolation

After 45 days of treatment, the rats were euthanized by decapitation between 11:00 a.m. and 01:00 p.m. to prevent circadian differences. Prior to decapitation (4 h), rats were intraperitoneally (i.p.) injected with 10 mL 1% (w/v) glycogen solution (Sigma type II, from oyster) in PBS to induce cell migration. Neutrophils were obtained by i.p. lavage with 20 mL PBS, followed by centrifugation in a density gradient of Histopaque 1077 [20]. The total number of leukocytes in exudate was determined in a haemocytometer. The differential cell count was obtained by optical microscopy (100×) (Nikon YS2-H) of a cytopsin cell layer stained with Giemsa. The staining allowed the identification of about 98% of neutrophils present in the exudate 4 h after injection of oyster glycogen. Neutrophils (1 × 10⁶ cell/mL) were resuspended in RPMI-1640 medium supplemented with 10% foetal calf serum, 20 mM HEPES, 2 mM glutamine, and antibiotics (streptomycin 100 U/mL and Penicillin-Streptomycin 100 U/mL).
penicillin 200 U/mL). Freshly obtained cells were used to measure phagocytic activity, ROS/RNS production, calcium release and membrane oxidation. All other experiments were performed with cells after they were frozen at –80 °C.

2.5. Phagocytic activity

Neutrophils (1 × 10⁶ cell/mL) were incubated for 60 min at 37 °C in 1 mL RPMI 1640 medium with zymosan. Particles were opsonized by incubation in the presence of control serum for 60 min at 37 °C. Cells were incubated with particles and counted after cytocentrifugation. Scores of phagocytosis were expressed by the number of cells that had one, two, three, four, or more zymosan particles phagocyted [21].

2.6. Analyses in plasma

2.6.1. Plasma preparation

Briefly, total blood volume (~5 mL) was collected in EDTA-Vacutainer tubes (5.4 mg K₂EDTA spray-dried, cod. 367835) and centrifuged for 10 min, 300g at room temperature. The clean plasma fraction was then removed and stocked in Eppendorf 2 mL-microtubes at –80 °C for further analyses.

2.6.2. Glucose, triacylglycerol and cholesterol determinations in plasma

Glucose, triacylglycerol (TAG), and cholesterol levels in plasma were all quantified by commercial test kits obtained from Bioclin-Quibasa Química Básica (Belo Horizonte, Brazil). All these assays were based on the peroxidase-catalysed oxidation of 4-aminoantipyrine by hydrogen peroxide (H₂O₂) to generate a pink color [22,23].

2.6.3. Glutathione redox status in plasma

Reduced glutathione content in plasma (GSH) was measured as described by Rahman et al. [24]. The method is based on the reaction of reduced thiol groups (such as in GSH) with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB), which is stoichiometrically detected by absorbance at 412 nm. Purified GSH and GSSG were used as standards. The Reducing Power index reveals the ratio between GSH and GSSG – thus, the reducing capacity of a biological system – and is calculated by the equation (Eq. (1)):

\[
\text{Reducing Power} = \frac{[\text{GSH}]}{([\text{GSH}] + 2[\text{GSSG}])}
\]  

2.6.4. Lipid oxidation in plasma

The extent of lipid peroxidation in plasma was determined as the concentration of thiobarbituric acid-reactive substances (TBARS) [25]. Butylated hydroxytoluene (4% BHT, in ethanol) was added to stop progressing oxidation reactions in 500 μL diluted samples. For the detection of the colored adducts, 500 μL of each sample was incubated at 100 °C for 15 min with 0.375% thiobarbituric acid in 0.25 M HCl and 1% Triton X-100. After reaching room temperature, absorbance of the reaction system was measured at 535 nm (blanks lack thiobarbituric acid) using malondialdehyde (MDA) as a standard.

2.7. Biochemical measurements in neutrophils

2.7.1. Superoxide radical (O₂⁻) production

The lipophilic and freely diffusible probe dihydroethidium (DHE) was used for the fluorimetric measurement of intracellular production of O₂⁻ [26]. Once inside cells, DHE is rapidly oxidized to ethidium (a red fluorescent compound) by O₂⁻ and H₂O₂ (in the presence of peroxidase). Ethidium is trapped in the nucleus by intercalating into DNA, leading to an increase of ethidium fluorescence. The cells (5 × 10⁵/well) were preloaded with 5 μM DHE for 15 min at room temperature in the dark. The assay was carried out in the presence and the absence of 20 ng/well phorbol myristate acetate (20 ng PMA/well), here applied as a chemical inducer of ROS production in neutrophils. Fluorescence was measured at 396 nm of excitation wavelength and at 590 nm of emission wavelength in a microplate fluorescence analyzer (Tecan, Salzburg, Austria).

2.7.2. Hydrogen peroxide production

The total production of H₂O₂ was evaluated before/after chemical stimulation (with 20 ng PMA) of a 5 × 10⁵ cell/well suspension using the horseradish peroxidase-dependent oxidation of phenol red (by H₂O₂) to form a vivid red color, which was spectrophotometrically quantified at 620 nm, using a H₂O₂ concentration curve as a standard [27].

2.7.3. Nitric oxide production

Nitric oxide production was determined by spectrophotometric analysis of total nitrite, using Griess reagent (1% sulfanilic acid, 0.1% N-(1-naphthyl)ethylene diamine hydrochloride), according to Ding et al. [28]. Supernatant of neutrophils (5 × 10⁶ cell/well) with or without lipopolysaccharide (LPS-10 μg/mL) was added to 100 μL of Griess reagent; the absorbance measured in 550 nm, and nitrite levels determined by means of a standard curve generated using NaNO₂.

2.7.4. Intracellular Ca²⁺ concentration

Intracellular changes of calcium levels (Ca²⁺) were monitored in neutrophils using the calcium-sensitive fluorescent probe Fura 2-AM. The 1 × 10⁶ neutrophil suspension was pre-loaded with 5 μM Fura 2-AM for 1 h at 37 °C in Tyrod’s solution. Afterwards, neutrophils were washed and intracellular Ca²⁺ release was monitored for 60 min at a wavelength emission of 510 nm (excitation wavelengths alternating between 340 and 380 nm) in a microplate reader (Tecan, Salzburg, Austria). Transformation of the fluorescence signal to Ca²⁺ concentration was performed by calibration with ionomycin (100 μM, maximum concentration) and EGTA (60 μM, minimum concentration) according to the Grynkiewicz equation and Kₐ₅ of 224 nM [29].

2.7.5. Membrane oxidation

The progression of membrane oxidation in PMA-activated neutrophils was monitored by fluorescence kinetics of the C₁₁-BODIPY₅₈₁/₅₉₁ probe, which is oxidized by alkoxyl and peroxyl radicals (strongly involved in lipid peroxidation progress) and loses thereby fluorescence emission in the red region [30]. Briefly, 5 × 10⁵ neutrophils were pre-incubated with 3.2 μM C₁₁-BODIPY₅₈₁/₅₉₁ for 60 min at room temperature, under mechanical agitation and in the dark. The C₁₁-BODIPY₅₈₁/₅₉₁-loaded neutrophils were placed in a microplate, and background fluorescence was adjusted to 1.0 relative fluorescence unit (RFU) and measured for 10 min (λexcitation = 545 nm, λemission = 591 nm) in a microplate reader (Tecan, Salzburg, Austria). Afterwards, 100 ng PMA/5 × 10⁵ cells were added to trigger ROS production and start auto-induced membrane oxidation, which was followed for additional 15 min. A first-order exponential decay function (Eq. (2)) was applied to obtain kinetic parameters of lipid peroxidation in the neutrophil membrane.

\[
y(\text{RFU}) = y_0 + A_1e^{-\frac{t}{k_1}}
\]

where RFU = relative fluorescence unit; y₀ = initial calculated relative fluorescence unit; A₁ = intensity factor; t = time (s); k₁ = rate constant.
2.7.6. Preparation of cell homogenates
Neutrophils (5 × 10⁶/mL) were isolated and ruptured by ultrasonication in a Vibra Cell apparatus (Connecticut, USA) in an ice-water bath using 500 µl of each assay-specific extraction solution/buffer. Afterwards, ruptured neutrophils were centrifuged for 10 min, 10,000g at 4 °C, and the supernatant was removed and kept on ice for further analysis (debris was discarded).

2.7.7. Assay of superoxide dismutase activity (SOD)
The activity of total superoxide dismutase (SOD) was measured according to Ewing & Janero [31], with modifications. The reaction system for total SOD determination included 50 mM sodium phosphate buffer (pH 7.4), 0.1 mM EDTA, 50 µM nitroblue tetrazolium (NBT), 78 µM NADH, and 3.3 µM phenazine methosulphate (PMS), here used as a chemical O₂⁻ source. Analysis of mitochondrial (manganese-dependent) MnSOD activity was performed similarly as for total/SOD, but under strong inhibition (~88–100%) of the cytosolic CuZnSOD isoform by addition of 3 µM KCN. As internal controls of the experiment, all reagents without sample were used for both total SOD and MnSOD determination. Absorbance at 550 nm was continuously monitored for over 2 min in order to evaluate O₂⁻-mediated reduction of NBT. One SOD unit is defined here as the enzyme concentration required for 50% inhibition of NBT reduction at 25 °C.

2.7.8. Assay of catalase activity
The decomposition of H₂O₂ was directly followed for 2 min by measuring the decrease in absorbance at 240 nm using a molar extinction of ε₂₄₀ = (0.0394 ± 0.0002) LmM⁻¹cm⁻¹, as described by Aeberli [32]. One catalase (CAT) unit is defined as the enzyme concentration required for the decomposition of 1 µmol of H₂O₂ per min at 25 °C. As internal control, 50 mM (pH 7.4) phosphate buffer was added to H₂O₂ without sample and monitored for 2 min.

2.7.9. Assay of the glutathione-recycling enzyme system
The activity of glutathione peroxidase (GPx) was measured according to Mannervik [33] using 2.5 U/ml glutathione reductase (GR), 10 mM reduced glutathione (GSH), 250 µM sodium azide (as a catalase inhibitor), and 1.2 mM NADPH. The reaction was triggered by addition of 4.8 mM tert-butyl hydroperoxide (a GPx substrate). The other GSH-recycling component enzyme, glutathione reductase (GR), was, in turn, measured by a direct reaction between 3.6 mM NADPH and 10 mM oxidized glutathione (GSSG). Absorbance of NADPH oxidation (in 0.2 M phosphate buffer, pH 7.4) was monitored in both cases at 340 nm for 2 min in an Ultraspec 3000 spectrophotometer (Pharmacia Biotech).

2.7.10. Oxidative stress parameters
Carbonyl groups were evaluated as parameters of amino acid oxidation in neutrophils. After pre-treatment with 10% streptomycin to remove interfering nucleic acids, the protein fraction was isolated from homogenate by precipitation with 20% trichloroacetic acid solution on ice, followed by the subsequent processes: (i) washing once with 0.3 M HClO₄, 5 mM EDTA, and 0.06% 2,2'-bipyrididine solution; (ii) washing twice with the mixture 1:1 ethyl acetate:ethanol (v/v); and (iii) removing residual organic solvent in vacuum for, at least, 30 min. Afterwards, the protein pellets were fully dissolved in 6 M guanidine hydrochloride and used for detection of the carbonyl groups after reaction with 10 mM dinitrophenylhydrazine (DNPH) in 0.25 M HCl (blanks lack DNPH). Absorbance was recorded at 380 nm, and the carbonyl group concentration was calculated using an extinction coefficient of ϵ = 2.2 × 10³ M⁻¹cm⁻¹ [34]. Reduced and oxidized glutathione (GSH and GSSG, respectively) and TBARS (biomarker of lipid oxidation) were also measured in homogenates of neutrophils, following the same procedures as described for plasma samples.

2.8. Statistical analyses
All data are presented as the mean values of, at least, triplicates with their standard errors (MEAN ± SE). Data were analyzed by one-way ANOVA followed by Tukey’s post-test, considering p < 0.05, p < 0.01, or p < 0.005. 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
All the experimental groups showed similar growth rates (BW) during the 45-day supplementation period (data not shown), although ASTA supplementation alone distinctively increased lipemia (55%) and cholesterolemia (35%, compared to control; Table 1). This effect was not observed in animals from the FO + ASTA group. Regarding redox balance, combined FO + ASTA clearly enhanced the antioxidant capacity of plasma, especially when comparing GSH/GSSG ratios (6-fold higher). Basal NO⁺ concentration in plasma was also increased in FO + ASTA animals (65%, Table 1), whereas no significant difference was observed in terms of lipid oxidation.

3.2. Non-stimulated neutrophils
The redox balance was also studied in neutrophils isolated from the experimental animals, as shown in Table 2. Concerning the frontline antioxidant enzymes (Table 2), ASTA alone provided the most significant effects, especially on the GSH-recycling enzymes GPx and GR (increased by 92% and 52%, respectively). However, GSH content and GSH-related Reducing Power (see Section 2) were abruptly depleted in neutrophils of ASTA-fed animals (both by approximately 70%; Table 2). FO treatment caused a different effect on neutrophils, since massive decreases in GPx (50%) and GR activities (65%) were accompanied by proportionally augmented levels of GSH (80%) and Reducing Power (89%). Combined FO + ASTA supplementation resulted in increased activities of total SOD (20%), mitochondrial MnSOD (22%), GPx (62%), and GR (36%). Unexpectedly, all supplementation treatments resulted in lower activities of CAT: 35% in ASTA group, and 45% in both FO and FO + ASTA animals (compared to control). Despite all changes observed in GSH content, none of the experimental groups showed statistically different indexes of oxidative modification in lipids (TBARS content) or proteins (carbonyls) compared to control neutrophils, except protein carbonyls of ASTA-fed animals (33% higher than control).

3.3. Activated neutrophils
In order to evaluate one of the most important neutrophil-functional parameters, after 45-day of treatment with FO and/or ASTA, neutrophils were challenged in vitro with opsonized zymosan particles (phagocytic capacity). The effect of ASTA and/or FO supplementation on neutrophil was also evaluated by measuring ROS/RNS production and auto-induced lipid peroxidation after chemical (PMA-) activation of neutrophils. As shown in Fig. 2, the phagocytic capacity of neutrophils was especially improved by FO supplementation (approximately 2.4-fold higher), whereas ASTA only displayed marginal effects (not significant, compared to control; p = 0.180).

In order to investigate whether FO and ASTA can exert effects on O₂⁻ production, freshly isolated neutrophils were incubated with DHE for 15 min. All supplementation protocols resulted in higher basal O₂⁻ production in non-stimulated neutrophils. This effect
was strongly related to ASTA presence, since ASTA-, FO-, and FO + ASTA-fed animals showed 53%, 17%, and 65% higher $O_2^\cdot$ basal production in neutrophils, respectively (compared to control).

Upon PMA-activation, however, a different trend was observed. FO alone or as a component of FO + ASTA supplementation significantly reduced $O_2^\cdot$ production by approximately 10% (Fig. 3).

$H_2O_2$ production in both rested and activated neutrophils followed the same trend as observed for $O_2^\cdot$ production, except for PMA-activated neutrophils of ASTA-fed group, which showed 25% lower $H_2O_2$ production than control neutrophils (Fig. 4).

On the other hand, NO$\cdot$ production (and probably other RNS that generate nitrite as product) was apparently more affected by ASTA supplementation than by FO per se (Fig. 5). Even though no significant difference was observed in rested neutrophils, LPS stimulus only resulted in 45% lower NO$\cdot$ production in neutrophils isolated from ASTA-fed animals (either alone or combined with FO). Concomitantly, the influx of Ca$^{2+}$ ions in LPS-activated neutrophils

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ASTA</th>
<th>FO</th>
<th>FO + ASTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>156.1 ± 20.7</td>
<td>163.1 ± 5.6²FOA</td>
<td>173.7 ± 47.9</td>
<td>144.6 ± 6.5²A</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>106.4 ± 2.9</td>
<td>164.3 ± 27.2²FOA</td>
<td>92.6 ± 13.3</td>
<td>106.7 ± 13.0²A</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>74.9 ± 4.1</td>
<td>100.8 ± 12.7²FOA</td>
<td>72.5 ± 10.1</td>
<td>78.7 ± 16.3</td>
</tr>
<tr>
<td>GSH</td>
<td>0.081 ± 0.041</td>
<td>0.108 ± 0.058²FOA</td>
<td>0.110 ± 0.046</td>
<td>0.158 ± 0.056²FOA</td>
</tr>
<tr>
<td>GSSG</td>
<td>0.097 ± 0.026</td>
<td>0.093 ± 0.029²FOA</td>
<td>0.087 ± 0.028²FOA</td>
<td>0.029 ± 0.006²A, FO</td>
</tr>
<tr>
<td>Reducing power</td>
<td>0.294 ± 0.169</td>
<td>0.367 ± 0.228²FOA</td>
<td>0.387 ± 0.204</td>
<td>0.731 ± 0.300²FOA</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>6.68 ± 2.17</td>
<td>6.57 ± 1.44²FOA</td>
<td>8.88 ± 1.63</td>
<td>11.12 ± 1.01²FOA</td>
</tr>
<tr>
<td>TBARS</td>
<td>1.34 ± 0.31</td>
<td>0.85 ± 0.39²FOA</td>
<td>1.36 ± 0.52</td>
<td>1.11 ± 0.38</td>
</tr>
</tbody>
</table>

Related to C (Control), A (ASTA), FO (Fish oil), FOA (Fish oil + ASTA).

$\cdot$ $p < 0.05$

$\cdot$ $p < 0.01$

$\cdot$ $p < 0.005.$

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ASTA</th>
<th>FO</th>
<th>FO + ASTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total SOD</td>
<td>27.10 ± 0.92</td>
<td>28.26 ± 1.98</td>
<td>29.97 ± 4.53</td>
<td>32.29 ± 1.44²C</td>
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<tr>
<td>MnSOD</td>
<td>17.66 ± 0.04</td>
<td>17.85 ± 2.09</td>
<td>20.49 ± 3.61</td>
<td>21.68 ± 1.16²C</td>
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<tr>
<td>CAT</td>
<td>4.73 ± 0.54</td>
<td>3.06 ± 0.54²C</td>
<td>2.67 ± 0.77²C</td>
<td>2.60 ± 0.54²C</td>
</tr>
<tr>
<td>GPx</td>
<td>0.07 ± 0.04</td>
<td>1.07 ± 0.09²C, FO</td>
<td>0.43 ± 0.01²C, A, FOA</td>
<td>1.41 ± 0.15²C, FO</td>
</tr>
<tr>
<td>GR</td>
<td>0.102 ± 0.014</td>
<td>0.155 ± 0.018²C, FO</td>
<td>0.035 ± 0.002²C, A, FOA</td>
<td>0.139 ± 0.012²C, FOA</td>
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<tr>
<td>TBARS</td>
<td>3.20 ± 0.37</td>
<td>3.67 ± 0.55</td>
<td>3.65 ± 1.14</td>
<td>2.95 ± 0.51</td>
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<tr>
<td>Protein carbonyls</td>
<td>15.0 ± 1.0</td>
<td>19.9 ± 1.12²C, FO, FOA</td>
<td>14.0 ± 0.8²A</td>
<td>14.4 ± 0.2²A</td>
</tr>
<tr>
<td>GSH</td>
<td>3.67 ± 0.32</td>
<td>1.01 ± 0.07²C, F, FOA</td>
<td>6.58 ± 0.24²C, A, FOA</td>
<td>1.47 ± 0.04²C, FO, A</td>
</tr>
<tr>
<td>GSSG</td>
<td>4.05 ± 0.46</td>
<td>4.33 ± 0.20²C</td>
<td>1.84 ± 0.18²C, A</td>
<td>1.32 ± 0.05²C, A</td>
</tr>
<tr>
<td>Reducing Power</td>
<td>0.312 ± 0.045</td>
<td>0.104 ± 0.019²C, FO, FOA</td>
<td>0.641 ± 0.102²C, A, FOA</td>
<td>0.358 ± 0.020²A, FO</td>
</tr>
</tbody>
</table>

Related to C (Control), A (ASTA), FO (Fish oil), FOA (Fish oil + ASTA).

$\cdot$ $p < 0.05.$

$\cdot$ $p < 0.01.$

$\cdot$ $p < 0.005.$

![Fig. 2. Scores of phagocytic capacity of rat neutrophils. The assay was carried out for 60 min, using opsonized zymosan particles. Results are expressed as mean ± SEM of three different experiments (n = 8 rats) performed in triplicate. $\cdot$$p < 0.01$ or $\cdot$$p < 0.05$; related to C (Control).](image)

![Fig. 3. Superoxide anion production was measured in neutrophils ($5 \times 10^5$ per well) under baseline and PMA-stimulated conditions. Cells were stimulated with PMA (20 ng/well) for 30 min and superoxide anion generation was measured by dihydroethidium (5 μM) assay. Results are expressed as mean ± SEM of three different experiments (n = 8 rats) performed in triplicate. $\cdot$$p < 0.05$; or $\cdot$$p < 0.005$, related to C (Control), A (ASTA), FO (Fish oil), FOA (Fish oil + ASTA).](image)
was unaltered regardless of the supplementation protocol (ASTA and/or FO, data not shown).

The progression of auto-induced lipid peroxidation after PMA-stimulation in neutrophils was monitored by C11-BODIPY fluorescence decay (Fig. 6). Based on a first-order exponential decay function (see Materials and Methods), it is clear that the plasma membrane of neutrophils from all supplemented groups was less exposed to oxidative injury than that from untreated control animals. Taking kinetic constants into account (k1), ASTA supplementation was proven to limit the progression of auto-oxidation in neutrophil membrane after PMA-stimulus, regardless of FO presence (Table 3). All fitting exponential curves were relatively well correlated (R² > 0.600).

4. Discussion

4.1. Plasma parameters

As observed by other authors, ASTA supplementation alone (1 mg/kg herein) resulted in hyperlipidemia and hypercholesterolemia in experimental animals (Table 1) [35]. Similar effects were also observed in rats supplemented with the marine carotenoid canthaxanthin, the immediate precursor of ASTA in the carotenogenesis pathway [35]. The hypercholesterolemic effect of these xanthophylls is not related to reported mechanisms of non-polar carotenoids in mammalians, because beta-carotene does not induce changes in plasma cholesterol.

Higher lipid concentration in plasma of ASTA-fed rats was not followed by hyperglycemic conditions or increased antioxidant capacity, as demonstrated by GSH/GSSG scores and oxidative modifications in plasma lipids (Table 1). Based on previous studies from our group, the ASTA effect on plasma glucose, cholesterol or triacylglycerol concentrations is supposedly dependent on the carotenoid content in the diet, since those changes were not observed when animals were fed with a 20-fold higher dose of ASTA [12]. Interestingly, Aoi et al. (2008) showed that exercising mice fed with 0.02% (w/w) ASTA consumed more lipids than glucose as energy source, resulting in an increased running time to exhaustion [36]. Although FO supplementation alone did not alter physically catastrophic redox parameters in plasma (compared to control), its combination with ASTA substantially augmented GSH content, its redox turnover (conversion of oxidized GSSG back to its reduced form, GSH) and, consequently, thiol-dependent reducing power of plasma (Table 1). Glutathione-based and lipid peroxidation markers in plasma confirm our previous results of the biochemical and putative anxiolytic effects of the ASTA + FO treatment [37]. Important hypolipidemic and hypercholesterolemic effects were also observed in the ASTA + FO group (compared to ASTA-fed animals), suggesting a specific effect of FO supplementation, since similar results were also found in the FO-group. Accordingly, experiments in humans indicate a profound hypolipidemic effect of FO, especially by lowering plasma triacylglycerol [38]. In agreement with Engler et al. [39], we also observed hypolipidemic/hypercholesterolemic effects associated with 65% higher NO₂ concentration in plasma of FO + ASTA supplemented Wistar rats (Table 1), although supplementation with FO alone demonstrated a non-significant tendency for higher values (p = 0.175). Reduction in lipidemia and cholesterolemia coupled to adequate redox rebalance and higher NO₂ bioavailability in plasma has been currently suggested as positive events for preventing the progression of early coronary heart disease in high-risk patients [40].

4.2. Non-activated neutrophils

The long-term FO + ASTA supplementation resulted in substantial antioxidant responses in rat neutrophils, compared to control. Both total and mitochondrial MnSOD activities were proportionally increased by approximately 20%, whereas CAT showed 43% lower activities (Table 2). Higher SOD activities in neutrophils from
Thus, it is desirable to investigate the redox balance and progression of lipid oxidation in neutrophils during the inflammatory process.

Table 3

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>y0</th>
<th>A1</th>
<th>k1</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.946 (±0.005)</td>
<td>0.828 (±0.581)</td>
<td>260.93 (±70.6)</td>
<td>0.918</td>
</tr>
<tr>
<td>ASTA</td>
<td>0.984 (±0.016)</td>
<td>3.10 (±9.33)</td>
<td>1314.1 (±274.4)</td>
<td>0.600</td>
</tr>
<tr>
<td>FO</td>
<td>0.972 (±0.003)</td>
<td>0.839 (±0.968)</td>
<td>2172.2 (±78.6)</td>
<td>0.859</td>
</tr>
<tr>
<td>FO + ASTA</td>
<td>0.984 (±0.002)</td>
<td>1.29 (±2.83)</td>
<td>1574.4 (±76.0)</td>
<td>0.652</td>
</tr>
</tbody>
</table>

* p < 0.05, related to: C (Control); A (ASTA group); FO (Fish oil group); or FOA (Fish oil + ASTA group).

4.3. Activated neutrophils

Fish oil supplementation was related to the increased phagocytic activity of neutrophils (both FO and FO + ASTA groups), since ASTA supplementation alone did not result in higher phagocytic scores (Fig. 2). Moreover, after PMA activation, neutrophils from FO-fed groups (both FO- and FO + ASTA, but not from ASTA-group) showed reduced O₂⁻ production, whereas all supplemented animals had lower H₂O₂ generation (Figs. 3 and 4). These data suggest that phagocytic activity was more closely related to changes in O₂⁻ production of activated neutrophils and/or to pre-acquired positive redox balance during the FO- and FO-ASTA supplementation period. Nevertheless, it is plausible to suggest that the improvement of the immune function (phagocytic activity herein) was a direct effect of FO supplementation in our experimental groups.

The consensus from histological studies and tracking fluorescent-targeted cells is that the majority of inflammatory neutrophils never leave an inflamed site to return to blood stream, since most activated cells die in situ due to cellular processes closely related to oxidative stress: apoptosis or necrosis [45]. It is well conceived that apoptosis is the preferred injury limiting mechanism for the removal of non-functional neutrophils from inflamed sites. Moreover, it is notably related to augmented rates of ROS/RNS production (by intrinsic pathway), Ca²⁺ influx disruption, and progressive oxidative events in several immune cells, including neutrophils [46]. Therefore, it is possible that the antioxidant background in neutrophils (obtained during the supplementation period) could affect: (i) their life-span as active immune cells; (ii) their functionality during inflammatory processes; and (iii) the mechanism by which active neutrophils are degraded (with consequences for remaining cells and tissue). As also observed in lymphocytes [12], ASTA supplementation here offered putative anti-apoptotic effects in neutrophils, based on reduced production of NO⁻ and unaltered intracellular Ca²⁺ release (Fig. 5).

In addition, the powerful antioxidant activity of ASTA [14] was here evidenced in neutrophils by the significantly lower rate constant (k₁; Table 3) of the exponential progression of membrane oxidation after PMA activation in neutrophils from ASTA-fed animals (both ASTA- and FO + ASTA groups; Fig. 6). This sensitive assay by the radical-sensitive probe C11-BODIPY depicts the slower progression of lipid oxidation in neutrophil membrane from both ASTA- and FO + ASTA-fed animals (see Section 2). Hence, it is reasonable to assume that ASTA supplementation offered extended protection to auto-induced oxidation in neutrophils membrane, with possible influence on cell survival and life-span.

Putative anti-apoptotic effects in rat neutrophils are here suggested from combined FO + ASTA supplementation, since lower O₂⁻ production was concomitantly monitored with lower NO⁻ production.
concentrations, and both radicals promptly reacted in intracellular compartments to produce the pro-apoptotic factor, peroxynitrite (ONOO⁻) [47]. Interestingly, low doses of NO• were shown to prevent killing of rat hepatocytes by tert-butylhydroperoxide, whereas further increasing doses resulted in increased killing [48]. This dual pro-/antioxidant role of NO• has been also reported in several other cell types and could represent an important redox switch for triggering apoptosis in neutrophils.

4.4. Mechanistic aspects

A bulk of data has demonstrated that the beneficial action of dietary carotenoids on the immune system is independent of their provitamin A or retinal-derived activities. Most of the immune improving effects provided by nonprovitamin A carotenoids – such as lycopene, canthaxanthin, lutein and ASTA – on humans and animals has been strongly associated with their antioxidant properties, even though recent studies also addressed their role in gene regulation, apoptosis and angiogenesis [49]. The pharmacodynamics of ASTA plays an essential role in determining its plasma concentration, putative direct interaction with circulating neutrophils, and targeting of tissues. Petri & Lundebye (2007) evaluated the tissue distribution of ASTA after oral administration to rats for two weeks, and found that ASTA accumulated predominantly in the spleen, kidneys, adrenals, liver, skin and eyes [50]. Tested as an anti-carcinogenic agent, the serum concentration of ASTA was approximately 1.2 μM following a 0.02% ASTA supplementation protocol for four weeks [51]. Nevertheless, single/multiple oral administrations of ASTA resulted in short-term accumulation in mice liver, in contrast to the fast elimination of unmodified ASTA from plasma [52]. ASTA short-term concentration in mammal liver was shown to proportionally inhibit the activity of hepatic NADPH P450 reductase, leading to detrimental toxicological dysfunctions [53]. Although still obscure, the deactivation of hepatic NADPH P450 reductase could be associated with a hypothetical additional NADPH supply for ASTA-mediated triacylglycerol and cholesterol biosynthesis in the liver. This hypothesis is in agreement with hyperlipidemia and hypercholesterolemia observed in ASTA-fed rats, as shown in Table 1. The specific mechanism of NADPH P450 reductase inhibition by ASTA is dose-dependent and related to inhibition of cytochrome c reduction in microsomes [54]. Therefore, ASTA could directly affect electron transfer on the microsomal membrane of hepatocytes due to its powerful antioxidant properties [14,55]. Furthermore, ASTA alone or combined with FO increased higher activities of the antioxidant enzymes GPx and GR by an unknown mechanism. Although still obscure, the deactivation of hepatic NADPH P450 reductase could be associated with a hypothetical additional NADPH supply for ASTA-mediated triacylglycerol and cholesterol biosynthesis in the liver. This hypothesis is in agreement with hyperlipidemia and hypercholesterolemia observed in ASTA-fed rats, as shown in Table 1. The specific mechanism of NADPH P450 reductase inhibition by ASTA is dose-dependent and related to inhibition of cytochrome c reduction in microsomes [54]. Therefore, ASTA could directly affect electron transfer on the microsomal membrane of hepatocytes due to its powerful antioxidant properties [14,55]. Furthermore, ASTA alone or combined with FO increased higher activities of the antioxidant enzymes GPx and GR belonging to the GSH-recycling system that needs constant supply of NADPH from the pentose phosphate pathway (Table 2).

Although ASTA supplementation alone did not increase GSH content of plasma (p = 0.105; Table 1), the combined ASTA + FO supplementation significantly augmented its thiol-based antioxidant capacity (approximately 2-fold higher), with putative protective effects on circulating neutrophils. Accordingly, both ASTA- and ASTA + FO-fed animals showed lower rates of lipid oxidation, as monitored by the fluorescent probe C17BODIPY581/591 (K1 in Table 3). However, it remains to be investigated whether ASTA and/or FO were properly absorbed by neutrophil membrane or whether this was simply a result of plasma GSH-neutrophil membrane interaction. FO treatment alone resulted in unexpected downregulation of both GPx and GR by an unknown mechanism. On the other hand, the total conversion of arachidonic acid through the lipoxygenase pathway in platelets was shown to be lowered by GSH depletion, which could be a consequence of diminished GR activities [56]. Interestingly, GPx and GR activities in neutrophils of ASTA + FO-fed animals were significantly augmented (62% and 36%, respectively), whereas proper intracellular GSH and GSSG contents both decreased by approximately 60% in neutrophils (Table 2).

The main ASTA + FO effects on neutrophil functions might be associated with the GSH-recycling system (with coadjutant collaboration from total SOD, Table 2), which apparently prioritizes the use of available plasma GSH content to protect neutrophil membrane.

The immune system is known to critically depend on accurate cell–cell communication in order to mount an efficient response [57]. Consequently, it is tempting to suppose that ASTA + FO might help to maintain fluidity and integrity of neutrophil membranes by reducing the oxidative damage during oxidative bursts, with possible positive effects on associated cell receptors, as well as redox-sensitive transcription factors (especially NF-κB), cytokines and prostaglandins [58]. Interestingly, ASTA + FO supplementation also resulted in significantly lower production of O₂• and H₂O₂ during the oxidative burst of neutrophils (Figs. 3 and 4, respectively). Altogether, the redox rebalance in activated neutrophils resulted in significantly higher phagocytic activities, as shown in Fig. 2. Regarding physicochemical properties of biological membranes, it is worth mentioning that rigid, rod-like ASTA molecules, anchored transversally across lipid bilayers, restrict molecular motion of lipids and proteins, thus affecting membrane fluidity and increasing structural stability [59]. On the other hand, n-3/PUFA such as DHA and EPA (present in FO) were shown to induce an opposite effect on biological membranes [60]. Based on the fact that major ROS production during the oxidative burst of neutrophils also occurs in membranes (catalyzed by NADPH oxidases), it is reasonable to assume that the proportion of n-3/PUFA and ASTA incorporated in neutrophil membranes could drastically affect physicochemical properties of lipid bilayers, with crucial consequences in terms of neutrophil function and whole immune responsiveness. Further studies are necessary to better understand such molecular-based – but truly relevant – aspects of ASTA and/or FO supplementation in immune enhancement.

5. Concluding remarks

The present study strongly suggests beneficial effects of FO + ASTA supplementation for immune competence, based on the redox balance in plasma (significant increase in GSH-dependent reducing power), non-activated neutrophils (increased glutathione-one-recycling enzymes GPx and GR) and PMA-activated neutrophils (lower O₂•, H₂O₂, and NO• generation, reduced membrane oxidation, but higher phagocytic activity) of Wistar rats. The effects of ASTA and FO combination were summative rather than synergistic, since FO strongly contributed to hyalopidemic/hycholesterolemic effects on plasma plus increased phagocytic activity in activated neutrophils, whereas ASTA better offered antioxidant/anti-apoptotic effects to PMA-activated neutrophils. The bulk of data reinforces the hypothesis that habitual consumption of marine fish (e.g. salmon, which is a natural source of both astaxanthin and fish oil) is strongly associated with human health, in particular with improvement of immune response, and lower risks for vascular and infectious pathologies [61].

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All authors of the present manuscript declare that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence our work.

References


