

Contents lists available at [ScienceDirect](http://ScienceDirect)

# Toxicology in Vitro

journal homepage: [www.elsevier.com/locate/toxinvit](http://www.elsevier.com/locate/toxinvit)

## Oxidative stress in human lymphocytes treated with fatty acid mixture: Role of carotenoid astaxanthin

T.R. Campoio, F.A. Oliveira, R. Otton \*

Postgraduate Program – Health Sciences – CBS, Cruzeiro do Sul University, 03342000 Sao Paulo, SP, Brazil

### ARTICLE INFO

#### Article history:

Received 21 February 2011

Accepted 19 April 2011

Available online 27 April 2011

#### Keywords:

Antioxidant

Astaxanthin

Leukocytes

Lymphocytes

Oxidative stress

Fatty acids

### ABSTRACT

Fatty acids (FA) have been shown to alter leukocyte function, and depending on concentration and type, they can modulate both inflammatory and immune responses. Astaxanthin (ASTA) is a carotenoid that shows notable antioxidant properties. In the present study we propose to evaluate the oxidative stress in human lymphocytes induced by a FA mixture and the possible protective role of ASTA. The present study showed that the FA mixture at 0.3 mM caused a marked increase in the production of superoxide anion, hydrogen peroxide and nitric oxide, which was accompanied by an increase in total-SOD activity, in TBARS levels and a reduction of catalase activity and content of GSH and free thiol groups. The FA mixture also promoted an increase in intracellular  $Ca^{2+}$  mobilization and in the proliferative capacity of B-lymphocytes. The addition of ASTA (2  $\mu$ M) partially decreased the ROS production and TBARS levels and increased the levels of free thiol groups. ASTA decreased the proliferative capacity of cells treated with FA but not by reducing intracellular calcium concentration. Based on these results we can conclude that ASTA can partially prevent oxidative stress in human lymphocytes induced by a fatty acid mixture, probably by bleaching/quenching free radical production.

© 2011 Elsevier Ltd. Open access under the [Elsevier OA license](http://www.elsevier.com/locate/elsevier).

### 1. Introduction

Fatty acids (FA) have been shown to alter leukocyte function, and depending on concentration and type, they can modulate both inflammatory and immune responses. These metabolites are important components of the diet and act as both intracellular and extracellular mediators, positively or negatively regulating physiological and pathological conditions (Pompeia et al., 2000). Polyunsaturated fatty acids (PUFAs) of the omega-3 family have overall suppressive effects on lymphocyte by modulating cell-membrane fluidity and composition of lipid rafts, inhibiting lymphocyte proliferation, antibody and cytokine production, adhesion molecule expression, natural killer cell activity and triggering cell death (Costabile et al., 2005; Fan et al., 2003; Larbi et al., 2005; Stulnig et al., 2000). The omega-6 PUFAs have both inhibitory and stimulatory effects on lymphocyte function. In addition to lymphocytes, FA has also been found to modulate phagocytosis of macrophages and neutrophils, reactive oxygen species production, cytokine production and leukocyte migration, also interfering with antigen presentation by macrophages (Calder et al., 1990; Endres et al., 1993; Meydani et al., 1991). The importance of FA

has been corroborated by many clinical trials in which patients present enhancement or impairment of immune function depending on which FA is provided in supplementation. Several mechanisms have been proposed to explain fatty acid modulation of immune response, such as changes in membrane fluidity and signal transduction pathways, regulation of gene transcription, protein acylation, and calcium release (Pompeia et al., 2000).

Cell and plasma levels of FA are significantly increased under fasting conditions, hypoxia, obesity, strenuous exercise and type 1 and 2 diabetes. In these situations, we also observed a significant immune suppression (Bazan, 1970; Delarue et al., 2004; Gardiner et al., 1981; Itani et al., 2002; Otton et al., 2004). Indeed, diabetic individuals present a high occurrence of infections associated with complications such as heart disease, atherosclerosis, cataract formation, peripheral nerve damage, retinopathy, and others which contribute to decrease quality of life of the patients (Valko et al., 2007).

In our previous study (Otton et al., 2004) we showed that blood peripheral lymphocytes obtained from poorly controlled diabetic patients presented increased DNA fragmentation as compared with cells obtained from healthy patients. Lymphocytes from alloxan-induced diabetic rats also showed increased DNA fragmentation when compared with cells from controls. Concomitantly, there was also high occurrence of chromatin condensation and blebbing formation. These observations strongly support the proposition that uncontrolled diabetes leads to impaired immune function

\* Corresponding author. Address: Av. Regente Feijó, 1295, 03342000 São Paulo, SP, Brazil. Tel./fax: +55 11 26726200.

E-mail addresses: [rosemary.otton@cruzeirodosul.edu.br](mailto:rosemary.otton@cruzeirodosul.edu.br), [rosemaryotton@hotmail.com](mailto:rosemaryotton@hotmail.com) (R. Otton).

due to higher number of lymphocyte death. More recently our group showed that lymphocytes from healthy human subjects as well as leukemia cell lines (Raji and Jurkat cells) after treatment with a fatty acid mixture that mimics the proportion and concentration found in plasma from diabetic patients, raises the proportion of cells in apoptosis (Otton and Curi, 2005), by a mechanism involving the release of cytochrome c from mitochondria, activation of caspases, increase in the production of NO and superoxide, and induction of calcium release (Otton et al., 2007).

The production of free radicals is increased in diabetic patients, generating an oxidative stress condition as showed by many authors. According to these authors, many different pathways may contribute to increased oxidative stress in diabetes, including increased plasma levels of FA (Newsholme et al., 2007). The increase in fatty acid levels may alter reactive oxygen species (ROS) production via activation of NADPH-oxidase, by induction of mitochondrial uncoupling, by inducing calcium mobilization as well as the activation of the transcription factor NF- $\kappa$ B via Toll like receptor 4 (TLR-4) signaling (Atli et al., 2004; Baynes, 1991; Catherwood et al., 2002; Green et al., 2004; Inoguchi et al., 2000; Otton et al., 2007; Rolo and Palmeira, 2006; Sano et al., 1998). Based on these effects, many authors have suggested the use of antioxidants in the treatment of diabetic complications, especially those involving excessive production of free radicals.

Carotenoids act as antioxidants by quenching singlet oxygen and free radicals (Palozza and Krinsky, 1992; Tsuchiya et al., 1992). These compounds are colored pigments widely distributed in vegetables, fruits and seafood and are implicated in the prevention of degenerative diseases including coronary heart disease and cancer (Gerster, 1993; Morris et al., 1994). The xanthophyll carotenoid astaxanthin (3,3'-dihydroxy- $\beta,\beta'$ -rotene-4,4'-dione; ASTA), a reddish-colored C-40 compound, is a powerful broad-ranging antioxidant that occurs naturally in a wide variety of living organisms, such as microalgae, fungi, complex plants, and crustaceans (Hussein et al., 2006). It is a quencher of ROS and reactive nitrogen species (RNS) single- and 2-electron oxidants as well as a chain-breaking scavenger of free radicals. ASTA, unlike other carotenoids, contains two additional oxygenated groups on each ring structure, resulting in enhanced antioxidant properties (Guerin et al., 2003). It has been reported that ASTA has a high antioxidant activity: 10 times higher than other carotenoids such as lutein, canthaxanthin, and  $\beta$ -carotene and 100 times higher than  $\alpha$ -tocopherol (Goto et al., 2001; Naguib, 2000). This potent antioxidant activity has been observed to modulate biological functions ranging from lipid peroxidation to tissue protection against light damage (McNulty et al., 2007; Santocono et al., 2006).

At the same time, ASTA displays interesting anti-inflammatory effects by preserving redox-sensitive (and essential) structures of human lymphocytes, although the applied dose apparently hinders lymphocyte proliferation (Bolin et al., 2010). As fatty acids are potent inducers of oxidative stress and as reported by many authors that ASTA has an important and prominent antioxidant activity, we propose to evaluate the oxidative stress caused by a mixture of fatty acids previously used by our group, and the possible ASTA protective role of oxidative stress induced by the FA mixture.

## 2. Materials and methods

### 2.1. Reagents

Astaxanthin (ASTA) and most of other chemicals were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA), excepting the RPMI-1640 culture medium, pluronic acid, Vybrant MTT Cell Proliferation kit and acetoxymethylester (Fura-2 AM) which were from Life Technologies (California, USA). Common

reagents for buffers (e.g. PBS) and regular laboratory solutions were obtained from Labsynth (Diadema, SP, Brazil).

### 2.2. Subjects

The Ethical Committee of the Universidade Cruzeiro do Sul (protocol number 030/07) approved the experimental procedure of this study. Around 30 healthy adult women and men (mean age  $27.0 \pm 9.0$ ) were included in the present study. All subjects did not present systemic or topical therapeutic regimen at least for the last 2 months. Subjects with a smoking history, alcohol habits, obesity or any other systemic diseases were excluded of the study (based on an anamnesis protocol).

### 2.3. Cell isolation and culture condition

Lymphocytes were obtained through the collection of human peripheral blood by venipuncture procedure in vacuum/siliconized tubes containing 0.1 mM EDTA. Peripheral blood lymphocytes were isolated under sterile conditions by using a density gradient present in the reagent Histopaque 1077 (Sigma–Aldrich) according to the manufacturer's instructions. After centrifugation, lymphocytes were counted in a Neubauer chamber using Trypan blue (1%). Lymphocytes ( $1 \times 10^6$ /mL) were cultured in 5 mL of RPMI 1640 supplemented as described above. The cells were treated with 0.3 mM of the fatty acid mixture added or not of 2  $\mu$ M of ASTA solubilized in DMSO and cultured at 5% CO<sub>2</sub> for up to 24 h at 37 °C. After this period, the cells were collected, centrifuged and stored at  $-80$  °C. To perform the assays of enzymes activities and oxidative damages in biomolecules, cells were defrosted and immediately used. For acute effects of FA on cell ROS production and intracellular calcium mobilization, after isolation lymphocytes were resuspended in Tyrode's solution (137 mM NaCl, 2.68 mM KCl, 0.49 mM MgCl<sub>2</sub>, 12 mM NaHCO<sub>3</sub>, 0.36 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.6 mM D-glucose, and 5 mM acid HEPES, pH 7.4) and freshly used.

The fatty acid mixture used in the present study was previously described (Otton and Curi, 2005). Briefly, the proportion of fatty acids was as follows: 1.74% lauric (C12:0), 5.2% myristic (C14:0), 31% palmitic (C16:0), 1.1% palmitoleic (C16:1), 41% stearic (C18:0), 4.6% oleic (C18:1), 9.6% linoleic (C18:2), 1.3% linolenic (C18:3), 3.2% arachidonic (C20:4), 0.45% eicosapentaenoic (C20:5), and 1.8% docosahexaenoic (C20:6) acids. In this study, the 0.3 mM FA concentration used is frequently found in plasma from diabetic patients (Bajaj et al., 2002; Woerle et al., 2002). The percentage of ethanol used to prepare the FA mixture, was always lower than 0.05% of the total volume of culture medium. This concentration of ethanol has shown not to be toxic for the cells (Siddiqui et al., 2001). All experiments were performed with cells left untreated (control) or treated with ethanol (vehicle). Bovine serum albumin (BSA) was added at 0.2% as an extracellular fatty acid chelator. There was no difference between untreated and ethanol-treated cells in all cases.

### 2.4. Determination of lymphocyte proliferation capacity

The proliferation response of lymphocytes was determined using the Vybrant MTT Cell proliferation (Life Technologies) 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 determined by optical density at 570 nm. The cells ( $5 \times 10^5$  cell/well) were treated for 48 h with 0.3 mM of the fatty acid mixture added or not of 2  $\mu$ M of ASTA and stimulated with concavalin A (Con A) (20  $\mu$ g/mL) or lipopolysaccharide (LPS) (100  $\mu$ g LPS/mL) to stimulate T and B cell proliferation, respec-

tively. Absorbance was measured in 570 nm and the results were expressed as optical density (OD).

### 2.5. Intracellular $\text{Ca}^{2+}$ release

Changes in cytosolic  $\text{Ca}^{2+}$  levels were monitored by fluorescence using the calcium-sensitive probe Fura 2-AM (Otton et al., 2010). Briefly, cells ( $1 \times 10^6/300 \mu\text{L}$ ) were acutely treated with 0.3 mM of the FA mixture added or not by 2  $\mu\text{M}$  of ASTA. The loading period for 5  $\mu\text{M}$  Fura 2-AM was 1 h at 37 °C in  $1 \times 10^6$  cells/well in Tyrode's solution. Afterwards, cells were washed and intracellular  $[\text{Ca}^{2+}]_i$  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  $[\text{Ca}^{2+}]_i$  was performed by calibration with ionomycin (100  $\mu\text{M}$ , maximum concentration) followed by EGTA addition (60  $\mu\text{M}$ , minimum concentration) according to the Grynkiewicz equation, using the  $K_{\text{diss}}$  of 224 nM (Grynkiewicz et al., 1985).

### 2.6. Measurement of ROS production by lymphocytes

#### 2.6.1. Phenol red assay

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) production was measured according to Pick and Mizel (1981) which is based on the horseradish peroxidase catalysis of the phenol red oxidation by  $\text{H}_2\text{O}_2$ . Lymphocytes ( $5 \times 10^5/\text{well}$ ) were acutely treated (3 h) with 0.3 mM of the fatty acid mixture added or not by 2  $\mu\text{M}$  of ASTA in the absence and presence of phorbol myristate acetate (PMA; 20 ng/well) used as a ROS inducer. After 3 h the absorbance was measured at 620 nm to evaluate  $\text{H}_2\text{O}_2$  concentration (compared to a standard curve).

#### 2.6.2. Dihydroethidium assay

Dihydroethidium (DHE) is a fluorescence probe and was used to measure the intracellular superoxide anion production. Once inside the cell, DHE is rapidly oxidized to ethidium (a red fluorescent compound) by superoxide with minor collaboration of other ROS. Lymphocytes ( $5 \times 10^5/\text{well}$ ) were incubated with 5  $\mu\text{M}$  DHE for 15 min at room temperature in the dark. At the beginning of the assay control cells were stimulated with PMA (20 ng/well) and 0.3 mM of the FA mixture added or not by 2  $\mu\text{M}$  of ASTA. Cells were incubated in the dark at room temperature for additional 30 min. DPI (diphenylene iodonium 10  $\mu\text{M}$ ), an inhibitor of NADPH oxidase (Chen et al., 2007), was used to investigate if superoxide anion production occurred through NADPH-oxidase activation. Sodium azide (SA – 400  $\mu\text{M}$ ) was used as a mitochondrial inhibitor. Afterwards, fluorescence was analyzed in a microplate reader (Tecan, Salzburg, Austria) (wavelengths of excitation and emission were 396 and 590 nm, respectively).

#### 2.6.3. DCFH-DA assay

The probe DCFH-DA was primarily used as an indicator of the production of  $\text{H}_2\text{O}_2$  (Keston and Brandt, 1965) but is also described as being oxidized by other ROS such as  $\text{HO}^\cdot$ ,  $\text{ROO}^\cdot$ , NO and peroxynitrite (Crow, 1997; Wang and Joseph, 1999). The cells ( $5 \times 10^5/\text{well}$ ) were preloaded with DCFH-DA (5  $\mu\text{M}$ ) by incubation in culture medium for 30 min. DCFH-DA is cleaved intracellularly by non specific esterase and turns into high fluorescent 2,7-dichlorofluorescein (DCF) upon oxidation by ROS. After the loading period, cells were treated with FA with or without ASTA at 2  $\mu\text{M}$  and cultured for 18 h. The experiments were conducted in the presence and absence of PMA (20 ng/well). After the culture period, cells were centrifuged and resuspended in 300  $\mu\text{L}$  of Tyrode's buffer and the fluorescence was monitored in spectrofluorimeter Tecan (Salzburg, Austria) with excitation at 485 nm and emission at

530 nm. The results of this experiment were expressed as relative units of fluorescence.

### 2.7. Nitric oxide production

Nitric oxide production was performed according to Ding et al. (1988) through nitrite ( $\text{NO}_2^-$ ) determination. Nitric oxide ( $\text{NO}^\cdot$ ) is rapidly converted into  $\text{NO}_2^-$  in aqueous solutions and, therefore, the total  $\text{NO}_2^-$  concentration can be used as a stoichiometric indicator of  $\text{NO}^\cdot$  production in culture. Lymphocytes ( $5 \times 10^5/\text{well}$ ) were cultured with 0.3 mM of the FA mixture with or without 2  $\mu\text{M}$  of ASTA and LPS (10  $\mu\text{g}/\text{well}$ ) for 4 h. EGTA (ethylene glycol tetraacetic acid, 500  $\mu\text{M}$ ) was used as a calcium quelator and therefore to discard NO production by constitutive calcium-dependent NOS. N-Acetyl-Cysteine (NAC – 500  $\mu\text{M}$ ) was added as an antioxidant to discard ROS induction on iNOS activation. The absorbance was measured in 550 nm to estimate  $\text{NO}_2^-$  concentrations based on a standard  $\text{NaNO}_2$  solution.

### 2.8. Preparation of homogenates

For the enzymatic activities, oxidative lesions in biomolecules and glutathione content cells were pelletized ( $5 \times 10^6$ ) after 24 h culture and mixed with 0.6 mL of the assay-specific extraction buffer and ruptured by ultrasonication in a Vibra Cell apparatus (Connecticut, USA), then centrifuged for 10 min, 10,000g at 4 °C. The supernatant was used for further analysis.

### 2.9. Lymphocyte antioxidant enzyme activities

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were determined in lymphocytes using a microplate reader (Tecan, Salzburg, Austria). CAT activity was measured as described by Aebi (1984) based on the direct decomposition of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). SOD activity was measured using the method described by Ewing and Janero (1995), which involves the reduction of  $\text{O}_2^-$  radicals by nitroblue tetrazolium (NBT) following a linear first order kinetic during 3 min. Glutathione peroxidase (Mannervik, 1985) and glutathione reductase (Carlberg and Mannervik, 1985; Rahman et al., 2006) were measured based on the oxidation of  $\beta$ -NADPH in the presence of *tert*-butyl hydroperoxide used as substrate.

### 2.10. GSH/GSSG ratio

Lymphocytes ( $5 \times 10^6$ ) were used for determination of glutathione status, using the method described by Rahman et al. (2006). Both total GSH and GSSG were analyzed using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to combine 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  $\mu\text{M}$  of GSH and GSSG.

#### 2.6.8. Oxidative damages

The lipid peroxidation in lymphocytes was performed by measuring the concentration of thiobarbituric acid-reactive substances in cell homogenates as described previously by Fraga and colleagues (Fraga et al., 1988). The assay evaluated 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 reached room temperature and the TBARS content was estimated by a standard curve of 10  $\mu\text{M}$  1,1,3,3-tetraethoxypropane.

Thiol and carbonyl groups were evaluated as biomarkers of aminoacid oxidation in total protein fractions, which were isolated from crude homogenate of cells ( $5 \times 10^6$ ) by precipitation with 20% trichloroacetic 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 (Biteau et al., 2003; Murphy and Kehrer, 1989). 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  $\epsilon = 2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (Murphy and Kehrer, 1989).

### 2.11. Protein determination

The total protein content of lymphocytes was measured by the method of Bradford (Bradford, 1976), using BSA as standard.

### 2.12. Statistical analyses

All data are expressed as mean values and standard errors of at least three independent experiments. Data were analyzed by one-way ANOVA followed by the Tukey's post hoc test. The software employed for statistical analyses was GraphPad Prism (version 4; GraphPad Software, San Diego, CA, USA).

## 3. Results

### 3.1. Lymphocyte proliferation

The functional activity of lymphocytes was assayed by their capacity to proliferate in response to a specific stimulation. Fig. 1 shows the MTT assay results after stimulation with Con A (a T lymphocytes mitogen) or LPS (a B lymphocytes mitogen) for 48 h. FA at 0.3 mM increased both basal (without stimulation) and LPS-stimulated proliferative capacity of human lymphocytes by 38% and 30%, respectively as compared with non stimulated control group. The addition of astaxanthin to cells treated with FA caused a decrease in the proliferation of lymphocytes in basal, Con A and LPS-stimulated conditions by 43%, 26% and 30%, respectively as compared with 0.3 mM of FA mixture.

### 3.2. Intracellular calcium concentration $[Ca^{2+}]_i$

Intracellular  $Ca^{2+}$  mobilization was significantly enhanced by the mixture of FA in human lymphocytes (about 31-fold) when

compared to the control group (Fig. 2). The increase in  $Ca^{2+}$  levels was sustained during 20 min of kinetic monitoring. Treatment with ASTA was unable to prevent the calcium increase induced by FA. BSA (0.2%) addition was able to partially decrease calcium mobilization probably by chelating free FA.

### 3.3. ROS and RNS production

To measure intracellular superoxide anion, hydrogen peroxide and nitric oxide production, cells were acutely treated with the FA mixture with or without ASTA as indicated in the material and methods section. As shown in Fig. 3A, the treatment of human lymphocytes with the FA mixture increased the intracellular superoxide anion levels by 135% as compared with the PMA-control group and as assessed by using DHE probe. The addition of ASTA to FA-treated cells promoted a reduction of 20% in superoxide production. Treatment of PMA-control cells with DPI, a NADPH-oxidase inhibitor, totally inhibited superoxide anion production, whereas sodium azide (SA) partially inhibited superoxide anion production. DPI addition in cells treated with fatty acid mixture partially decreased (20%) the superoxide anion production (Fig. 3A).

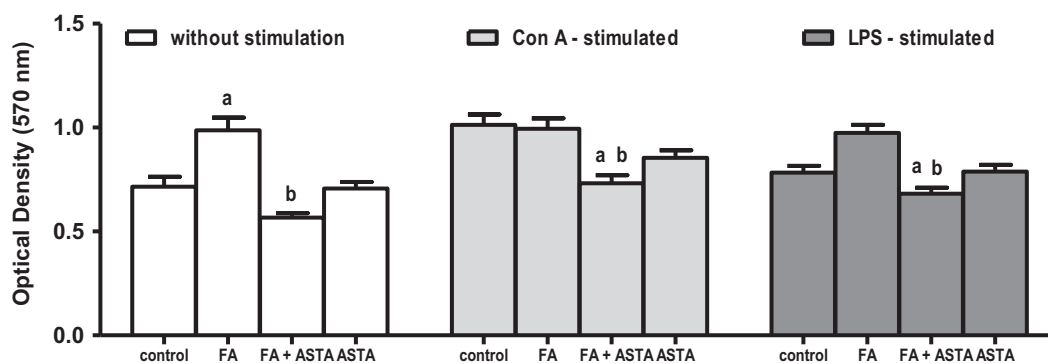
A similar pattern was observed when DCFH-DA probe was used as a general ROS probe (Fig. 3B). An increase of threefold in total ROS production was observed in lymphocytes treated with the FA mixture as compared with PMA-control group. ASTA-treatment decreased the ROS production induced by FA in 20%. Addition of BSA, used as a FA chelating agent, reduced the ROS production in about 32%.

We observed an increase of up to twofold in the production of hydrogen peroxide induced by the FA mixture as compared with control without stimulation. ASTA-treatment partially reduced (23%) the  $H_2O_2$  production observed in FA group as compared with PMA-control group (Fig. 3C).

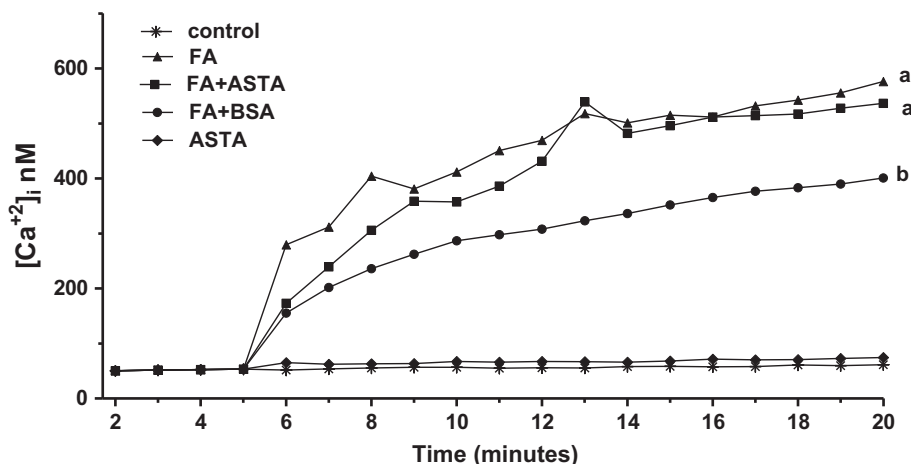
There was an increase of 97% in NO production after treatment with 0.3 mM of FA as compared with control group without LPS. Treatment of cells with ASTA in the FA group did not prevent the increase caused by the presence of FA. ASTA *per se*, raises nitric oxide production by 99% as compared with control group without LPS (Fig. 3D). N-acetylcysteine (NAC) and BSA partially reduced the NO production induced by the FA mixture.

### 3.4. Antioxidant enzyme activities and oxidative damage

To determine whether the increased levels of ROS induced by the FA mixture can modulate antioxidant status of cells, we evaluated the antioxidant enzyme activities after 24 h of treatment (Table 1). The FA mixture decreased the activity of CAT by 42% and increased the total-SOD activity by 27% as compared to the control



**Fig. 1.** Proliferation response of human lymphocytes to Con A and LPS-stimulation. Cells ( $5 \times 10^5$ /well) were cultured for 48 h with 0.3 mM of the fatty acid mixture (FA) added or not by 2  $\mu$ M of ASTA. The results are presented as mean  $\pm$  SEM (four different experiments). <sup>a</sup> $p < 0.05$  compared to control group. <sup>b</sup> $p < 0.05$  as compared with the cells treated with the fatty acid mixture 0.3 mM.



**Fig. 2.** Total intracellular  $\text{Ca}^{2+}$  mobilization (nM) in human lymphocytes. Cells ( $1 \times 10^6$ /well) were previously loaded with  $5 \mu\text{M}$  Fura 2-AM during 1 h and then incubated with  $0.3 \text{ mM}$  of the fatty acid mixture added or not of  $2 \mu\text{M}$  of ASTA for 20 min. The results are presented as mean  $\pm$  SEM. <sup>a</sup> $p < 0.05$  compared to control group. <sup>b</sup> $p < 0.05$  as compared with the cells treated with the fatty acid mixture  $0.3 \text{ mM}$ .

group. The FA group with ASTA restored total-SOD activity to those of the control group, whereas CAT activity decreased by 71% and GR activity increased by 80% as compared with the control group. Among all front line antioxidant enzymes tested, total-SOD was increased by 52% and GR activity was decreased by 28% due to ASTA treatment.

Oxidative damages in biomolecules were also modulated by the FA mixture. TBARS levels were dramatically increased by treatment of cells with a FA mixture (210%) and ASTA-treatment partially restored TBARS levels (112%) as compared with the control cells. Free protein SH-group was decreased in 69% in cells treated with FA. After treatment with  $2 \mu\text{M}$  of ASTA a partial restoring of 41% was observed in thiol content groups. Carbonyl groups were not modulated by the treatment of cells with FA and ASTA (data not shown).

### 3.5. Glutathione redox status

A significant reduction in the content of GSH and GSSG of 73% and 35%, respectively was observed in lymphocytes treated with  $0.3 \text{ mM}$  of the FA mixture when compared to the control group. This reduction was not prevented by ASTA addition (Fig 4).

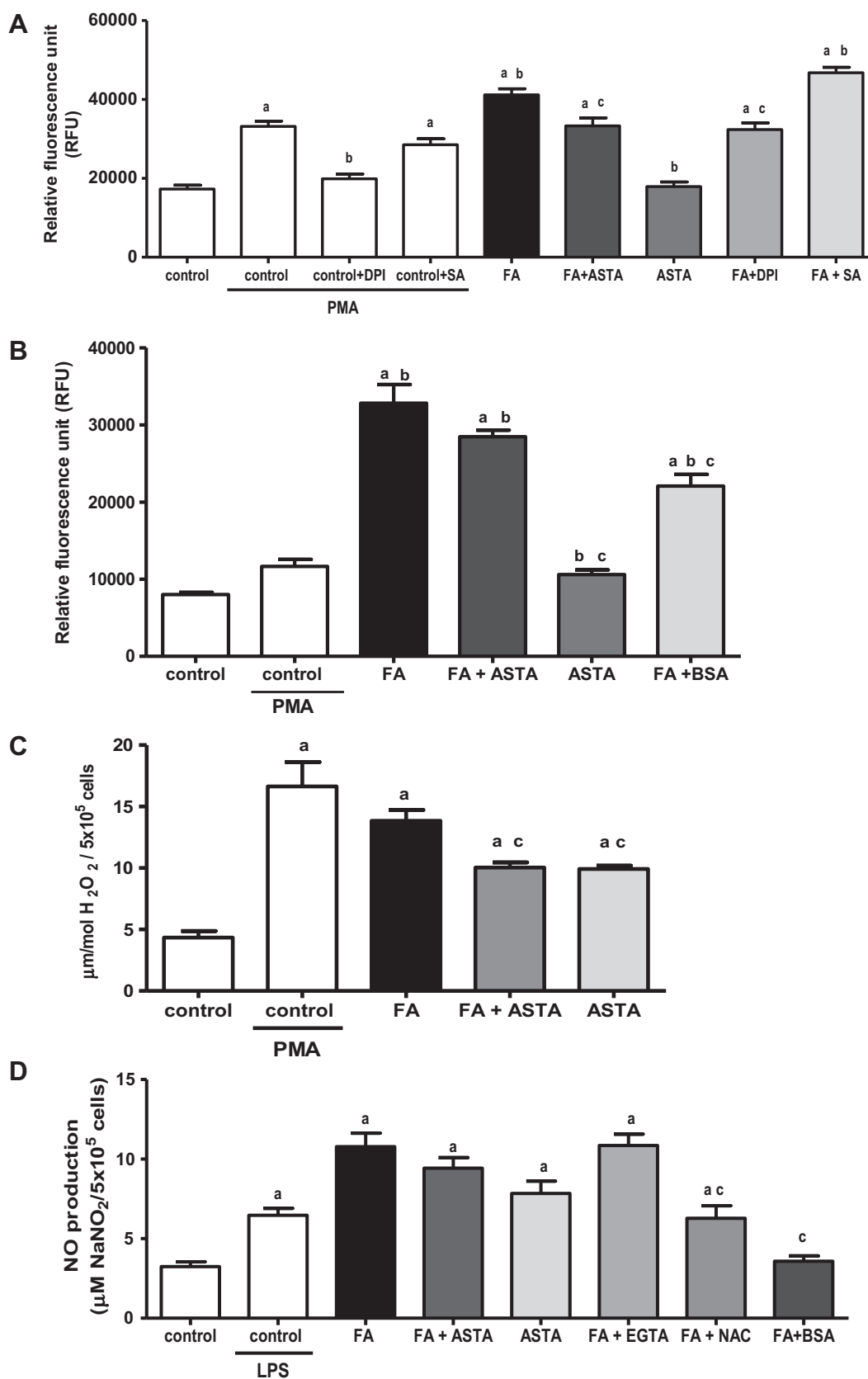
## 4. Discussion

It has been postulated that FA may influence cells of the immune system, including lymphocytes by modifying cell-membrane composition (Fan et al., 2004; Li et al., 2006), altering intracellular signaling pathways (Gorjao et al., 2007; Lee et al., 2004; Madani et al., 2001; Mizota et al., 2009), proliferation capacity, interleukins release (Nunes et al., 2008; Sacerdote et al., 2005; Verlengia et al., 2004), ROS production (Cury-Boaventura and Curi, 2005; Stentz and Kitabchi, 2006; Otton et al., 2007), gene expression (Verlengia et al., 2004) and calcium mobilization (Otton et al., 2007).

Overall, the mixture of FA used in the present study caused a marked increase in the production of superoxide anion, hydrogen peroxide and nitric oxide, which was accompanied by an increase in total-SOD activity and in levels of TBARS as well as a reduction of catalase, levels of free thiol groups and GSH content. These parameters could be understood as an increase in oxidative stress. The FA mixture also promoted an increase in intracellular  $\text{Ca}^{2+}$  mobilization and in the proliferative capacity of B-lymphocytes. Treatment of cells with the antioxidant ASTA partially decreased the oxidative stress imposed by the FA mixture.

$\text{Ca}^{2+}$  signaling is essential for diverse biological processes.  $\text{Ca}^{2+}$  ions are especially suited as intracellular second messengers because of the strong homeostatic mechanisms that maintain intracellular free  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) in resting cells at  $100 \text{ nM}$  or less. In the face of extracellular  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_o$ ) that are four orders of magnitude higher ( $1\text{--}2 \text{ mM}$ ). Cytoplasmic  $\text{Ca}^{2+}$  concentrations are maintained at low levels primarily through the action of plasma membrane  $\text{Ca}^{2+}$ -ATPases (PMCA) that pump  $\text{Ca}^{2+}$  out of the cell across the plasma membrane. Additionally, the sarco-endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPases (SERCA) pumps  $\text{Ca}^{2+}$  into the lumen of the endoplasmic reticulum (ER). In the longer term (hours), sustained  $\text{Ca}^{2+}$  entry is critical for essentially all responses initiated through T cell, B cell, and Fc receptors, including proliferation and cytokine production by T cells, cytokine production by mast cells and natural killer (NK) cells, differentiation of B cells into plasma cells, and the differentiation of naive T cells into Th1, Th2, and Th17 effectors subtypes (Hogan et al., 2010). As showed in our work, intracellular calcium concentration was exceptionally enhanced and sustained during 20 min of monitoring in cells treated with FA mixture (Fig. 2) and addition of ASTA to FA-treated cells was unable to restore calcium to basal levels. At the same time, proliferative capacity of lymphocytes was increased by the presence of FA mixture, and ASTA addition restored proliferative capacity of lymphocytes to control values (Fig. 1). Based on this data we are able to suggest that proliferative response of lymphocytes, which is a well-known calcium-dependent process is not the only mechanism involved in this process since ASTA decreased proliferative capacity of cells treated with FA but did not reduce intracellular calcium concentration. It has been shown that ASTA is a potent inhibitor of tyrosine kinases, inhibiting the MAPK pathway, decreasing the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK and MEK pathway, down regulating the NF- $\kappa\text{B}$  activation and ERK1/2 and pMSK-1 pathway (Lee et al., 2003; Kim et al., 2010). Whether ASTA is reducing lymphocyte proliferation by inhibiting the phosphorylation of key proteins implicated in the process of lymphocyte proliferation remains to be elucidated.

Concerning NO, calcium also seems not to be involved in both ASTA and FA-induction of its production (Fig 3D) since the treatment of cells with EGTA (used as a calcium chelating agent) was not able to reduce NO levels, which demonstrates that NO production induced by FA and ASTA occurs by activation of iNOS gene expression, which is a calcium-independent isoenzyme. In fact, it has been demonstrated that the saturated FA are potent inducers



**Fig. 3.** ROS and RNS production by using DHE (A), DCFH-DA (B), phenol red (C) and Griess reagent (D) in human lymphocytes treated with 0.3 mM of the fatty acid mixture added or not by 2 µM of ASTA, NAC (N-acetylcysteine), BSA (bovine serum albumin) and EGTA (ethylene glycol tetraacetic acid). Cells ( $5 \times 10^5$ /well) were stimulated with PMA (20 ng/well) or LPS (10 µg/well) when indicated. The results are presented as mean  $\pm$  SEM from at least four different experiments. (a)  $p < 0.05$  compared to group control without stimulation; (b)  $p < 0.05$  compared to stimulated-control group; (c)  $p < 0.05$  as compared with the cells treated with the fatty acid mixture 0.3 mM.

**Table 1**  
Activities of the antioxidant enzymes and oxidative damages in biomolecules.

	Control	FA	FA + ASTA	ASTA
Total/SOD <sup>a</sup>	9 ± 0.5	12 ± 0.3*	9 ± 0.8**	14 ± 0.9*
Mn/SOD <sup>b</sup>	9 ± 0.7	8 ± 0.6	6 ± 0.8	8 ± 1.2
CAT <sup>c</sup>	10 ± 0.6	5 ± 0.4*	1 ± 0.1**	12 ± 0.6
GPx <sup>d</sup>	40 ± 2.1	38 ± 1.5	25 ± 2.3**	20 ± 2.1**
GR <sup>e</sup>	33 ± 0.9	47 ± 4.5	60 ± 5.2*	24 ± 0.8**
TBARS <sup>f</sup>	75 ± 3.6	233 ± 35.4*	160 ± 12.9*	131 ± 1.3
Protein-SH <sup>g</sup>	287 ± 19	89 ± 4.4*	167 ± 18.6**	291 ± 19.4

<sup>a</sup> Total/SOD expressed in (unit mg protein<sup>-1</sup>).

<sup>b</sup> MnSOD expressed in (unit mg protein<sup>-1</sup>).

<sup>c</sup> CAT expressed in (μmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> L<sup>-1</sup> mg protein<sup>-1</sup>).

<sup>d</sup> GPx expressed in (mU mg protein<sup>-1</sup>).

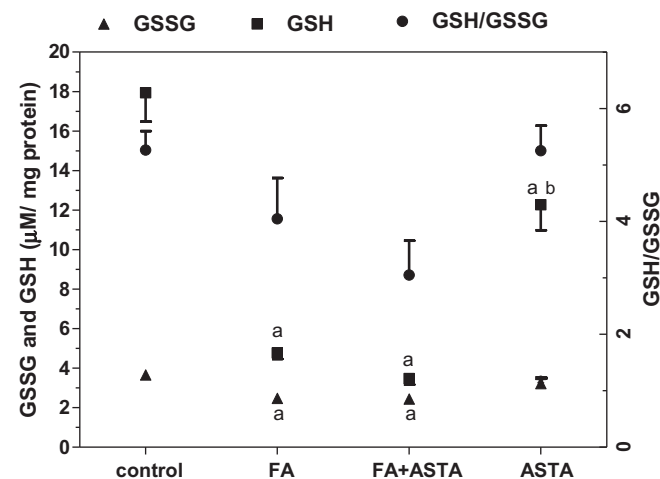
<sup>e</sup> GR expressed in (unit mg protein<sup>-1</sup>).

<sup>f</sup> TBARS expressed in (nmol MDA mg protein<sup>-1</sup>).

<sup>g</sup> Protein-SH expressed in (μmol-SH mg protein<sup>-1</sup>).

\* *p* < 0.05 compared to group control.

\*\* *p* < 0.05 as compared with the cells treated with the fatty acid mixture 0.3 mM.



**Fig. 4.** GSH, GSSG concentration and GSH/GSSG ratio in human lymphocytes after 24 h of treatment with 0.3 mM of the fatty acid mixture added or not by 2 μM of ASTA. (a) *p* < 0.05 compared to group control. (b) *p* < 0.05 as compared with the cells treated with the fatty acid mixture 0.3 mM.

of activation of the transcription factor NF-κB, through its connection with the *Toll like receptor 4* (TLR4) (Lee et al., 2004). When the FA binds to the receptor TLR4, there is an immediate activation of intracellular pathway leading to NF-κB activation and increased gene transcription of iNOS with subsequent increase in NO<sup>•</sup> production. In FA-treated cells with BSA, there was a total inhibition of NO<sup>•</sup> production. Therefore, we can assume that the increase of NO<sup>•</sup> production induced by the mixture of FA could be due to activation of NF-κB and increased iNOS expression by direct activation of TLR4. It was recently shown by our group that ASTA also increases the production of NO<sup>•</sup> in human lymphocytes and neutrophils (Bolin et al., 2010; Macedo et al., 2010). As previously shown, ASTA was able to reduce the arterial blood pressure mediated by increase of NO<sup>•</sup> production (Hussein et al., 2005). However, ASTA reduced the activation the transcription factor NF-κB and decreased the IL-6 production in microglial cells (Kim et al., 2010). In the current study, ASTA led to an increase in NO<sup>•</sup> production and association of ASTA and FA-treated cells was not able to restore the NO<sup>•</sup> production (Fig 3D). Therefore, we can suggest the ROS participation on NO<sup>•</sup> induction, since a slight reduction on ROS production promoted by ASTA also promoted a small reduction in NO<sup>•</sup> levels on FA + ASTA group. In fact, NAC treatment partially reduced the production of NO<sup>•</sup> induced by FA, indicating a partial contribu-

tion of ROS in the NO<sup>•</sup> production by FA. Contrasting results were obtained by Choi et al. (2008) which showed astaxanthin inhibiting the production of inflammatory mediators by blocking iNOS and COX-2 activation or by the suppression of iNOS and COX-2 degradation. Then, as in our FA mixture there is a great content of saturated FA and this FA can induce both the activation of TLR4 pathway which in turn activates nuclear transcription factor NFκB by different ways as previously described by other authors (Lee et al., 2004), we can assume there is the activation of TLR4-pathway, with a consequent induction of NFκB, followed by iNOS activation, which culminates in increased NO levels. ASTA was unable to abrogate the NO<sup>•</sup> producing induced by the FA mixture.

Excessive levels of reactive oxygen species not only directly damage cells by oxidizing DNA, protein and lipids, but indirectly damage cells by activating a variety of stress-sensitive intracellular signaling pathways such as NF-κB, p38 MAPK, JNK/SAPK, hexosamine and others. Activation of these pathways results in the increased expression of numerous gene products that may cause cellular damage and play a major role in the etiology of the late complications of diabetes (Newsholme et al., 2007). In our study we showed that the FA mixture caused a large increase in superoxide anion and hydrogen peroxide production as showed by dihydroethidium, DCFH-DA and phenol red assays (Fig 3). This increase in ROS production was accompanied by an increase of damage in lipids and proteins (Table 1), whereas catalase activity and GSH content were decreased. In an attempt to reduce the ROS production induced by the mixture of FA we added ASTA which resulted in a partial reduction of 20% (on average) in ROS production. Many antioxidants are particularly known to provide protection from ROS-mediated cellular damage. This effect is considered to be a defense mechanism against the attack of ROS. In addition, antioxidants have been linked to regulatory functions in cell growth, survival, cytotoxicity, and transformation possibly involving redox regulation and chemical toxicity (Larcombe et al., 2010).

One mechanism to explain the increase in ROS production induced by FA could be by the interaction of polyunsaturated, saturated and monounsaturated FA, which are present in our FA mixture, with components of the respiratory chain, thereby inhibiting the electron transport chain, when electrons are directly delivered to Complex III, e.g. from succinate. FA strongly enhance complex III-associated superoxide anion generation (Schonfeld and Reiser, 2006; Schonfeld and Wojtczak, 2007). Also, an elevation of intracellular Ca<sup>2+</sup> induced by increased Ca<sup>2+</sup> influx through voltage-gated Ca<sup>2+</sup> channels caused by the FA mixture can stimulate mitochondrial generation of ROS. Moreover, Ca<sup>2+</sup> via protein kinase C (PKC) activation enhances NADPH oxidase-dependent generation of ROS, and thus induces oxidative stress (Kruman et al., 1998; Morgan et al., 2007; Yu et al., 2006). Interestingly, the high levels of ROS induced by FA were not totally inhibited by DPI (Fig. 3A), whereas in PMA-control group there was a reduction on ROS production to basal levels. This phenomenon indicates that not only NADPH-oxidase is involved in ROS production of lymphocytes treated with FA. Furthermore, when SA was used as an electron transport chain inhibitor there was no reduction in ROS production induced by FA (Fig 3A).

In summary, our data suggest that FA induces oxidative stress through increased production of superoxide anion, hydrogen peroxide and NO production, decreasing enzymatic activity of catalase and GSH content and increasing intracellular calcium concentration, which can be involved in increasing B-lymphocyte proliferation. Moreover, the increase in ROS and NO production explains the increase in lipid peroxidation and damage to cell proteins. Our data also show that ASTA can decrease the exacerbated production of ROS induced by FA, but only partially. Based on these results we can conclude that ASTA can partially prevent oxidative

stress in human lymphocytes induced by a fatty acid mixture, probably by bleaching/quenching free radical production.

### Disclosure statement

All authors of the present manuscript disclose no actual potential conflict of interest including any financial, personal or other relationships with people or organizations.

### Acknowledgements

The authors are grateful to the technical assistance of Fineto Jr., C., Guerra, B.A., Marin, D.P. and Bolin, P.A. This research is supported by Fundação de Amparo a Pesquisa do Estado de São Paulo – FAPESP (2008/0888-6 and 2007/03334-6), Cruzeiro do Sul University and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

### References

- Aebi, H., 1984. Catalase in vitro. *Methods Enzymol.* 105, 121–126.
- Atli, T., Keven, K., Avci, A., Kutlay, S., Turkcapar, N., Varli, M., Aras, S., Ertug, E., Canbolat, O., 2004. Oxidative stress and antioxidant status in elderly diabetes mellitus and glucose intolerance patients. *Arch. Gerontol. Geriatr.* 39, 269–275.
- Bajaj, M., Berria, R., Pratipanawatr, T., Kashyap, S., Pratipanawatr, W., Belfort, R., Cusi, K., Mandarino, L., DeFronzo, R.A., 2002. Free fatty acid-induced peripheral insulin resistance augments splanchnic glucose uptake in healthy humans. *Am. J. Physiol. Endocrinol. Metab.* 283, E346–E352.
- Baynes, J.W., 1991. Role of oxidative stress in development of complications in diabetes. *Diabetes* 40, 405–412.
- Bazan Jr., N.G., 1970. Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. *Biochim. Biophys. Acta* 218, 1–10.
- Biteau, B., Labarre, J., Toledano, M.B., 2003. ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* 425, 980–984.
- Bolin, A.P., Macedo, R.C., Marin, D.P., Barros, M.P., Otton, R., 2010. Astaxanthin prevents in vitro auto-oxidative injury in human lymphocytes. *Cell Biol. Toxicol.* 26, 457–467.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Calder, P.C., Bond, J.A., Harvey, D.J., Gordon, S., Newsholme, E.A., 1990. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochem. J.* 269, 807–814.
- Carlberg, I., Mannervik, B., 1985. Glutathione reductase. *Methods Enzymol.* 113, 484–490.
- Catherwood, M.A., Powell, L.A., Anderson, P., McMaster, D., Sharpe, P.C., Trimble, E.R., 2002. Glucose-induced oxidative stress in mesangial cells. *Kidney Int.* 61, 599–608.
- Chen, C.Y., Liu, T.Z., Chen, C.H., Wu, C.C., Cheng, J.T., Yiin, S.J., Shih, M.K., Wu, M.J., Chern, C.L., 2007. Isoobtusilactone A-induced apoptosis in human hepatoma Hep G2 cells is mediated via increased NADPH oxidase-derived reactive oxygen species (ROS) production and the mitochondria-associated apoptotic mechanisms. *Food Chem. Toxicol.* 45, 1268–1276.
- Choi, S.K., Park, Y.S., Choi, D.K., Chang, H.I., 2008. Effects of astaxanthin on the production of NO and the expression of COX-2 and iNOS in LPS-stimulated BV2 microglial cells. *J. Microbiol. Biotechnol.* 18, 1990–1996.
- Costabile, M., Hii, C.S., Melino, M., Easton, C., Ferrante, A., 2005. The immunomodulatory effects of novel beta-oxa, beta-thia, and gamma-thia polyunsaturated fatty acids on human T lymphocyte proliferation, cytokine production, and activation of protein kinase C and MAPKs. *J. Immunol.* 174, 233–243.
- Crow, J.P., 1997. Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite in vitro: implications for intracellular measurement of reactive nitrogen and oxygen species. *Nitric Oxide* 1, 145–157.
- Cury-Boaventura, M.F., Curi, R., 2005. Regulation of reactive oxygen species (ROS) production by C18 fatty acids in Jurkat and Raji cells. *Clin. Sci. (Lond.)* 108, 245–253.
- Delarue, J., LeFoll, C., Corporeau, C., Lucas, D., 2004. N-3 long chain polyunsaturated fatty acids: a nutritional tool to prevent insulin resistance associated to type 2 diabetes and obesity? *Reprod. Nutr. Dev.* 44, 289–299.
- Ding, A.H., Nathan, C.F., Stuehr, D.J., 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141, 2407–2412.
- Endres, S., Meydani, S.N., Ghorbani, R., Schindler, R., Dinarello, C.A., 1993. Dietary supplementation with n-3 fatty acids suppresses interleukin-2 production and mononuclear cell proliferation. *J. Leukoc. Biol.* 54, 599–603.
- Ewing, J.F., Janero, D.R., 1995. Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. *Anal. Biochem.* 232, 243–248.
- Fan, Y.Y., McMurray, D.N., Ly, L.H., Chapkin, R.S., 2003. Dietary (n-3) polyunsaturated fatty acids remodel mouse T-cell lipid rafts. *J. Nutr.* 133, 1913–1920.
- Fan, Y.Y., Ly, L.H., Barhoumi, R., McMurray, D.N., Chapkin, R.S., 2004. Dietary docosahexaenoic acid suppresses T cell protein kinase C theta lipid raft recruitment and IL-2 production. *J. Immunol.* 173, 6151–6160.
- Fraga, C.G., Leibovitz, B.E., Tappel, A.L., 1988. Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes. *Free Radic. Biol. Med.* 4, 155–161.
- Gardiner, M., Nilsson, B., Rehnrcrona, S., Siesjo, B.K., 1981. Free fatty acids in the rat brain in moderate and severe hypoxia. *J. Neurochem.* 36, 1500–1505.
- Gerster, H., 1993. Anticarcinogenic effect of common carotenoids. *Int. J. Vitam. Nutr. Res.* 63, 93–121.
- Gorjao, R., Hirabara, S.M., de Lima, T.M., Cury-Boaventura, M.F., Curi, R., 2007. Regulation of interleukin-2 signaling by fatty acids in human lymphocytes. *J. Lipid Res.* 48, 2009–2019.
- Goto, S., Kogure, K., Abe, K., Kimata, Y., Kitahama, K., Yamashita, E., Terada, H., 2001. Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. *Biochim. Biophys. Acta* 1512, 251–258.
- Green, K., Brand, M.D., Murphy, M.P., 2004. Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes. *Diabetes* 53 (Suppl. 1), S110–118.
- Gryniewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3450.
- Guerin, M., Huntley, M.E., Olaizola, M., 2003. Haematococcus astaxanthin: applications for human health and nutrition. *Trends Biotechnol.* 21, 210–216.
- Hogan, P.G., Lewis, R.S., Rao, A., 2010. Molecular basis of calcium signaling in lymphocytes: STIM and ORAI. *Annu. Rev. Immunol.* 28, 491–533.
- Hussein, G., Goto, H., Oda, S., Iguchi, T., Sankawa, U., Matsumoto, K., Watanabe, H., 2005. Antihypertensive potential and mechanism of action of astaxanthin: II. Vascular reactivity and hemorheology in spontaneously hypertensive rats. *Biol. Pharm. Bull.* 28, 967–971.
- Hussein, G., Sankawa, U., Goto, H., Matsumoto, K., Watanabe, H., 2006. Astaxanthin, a carotenoid with potential in human health and nutrition. *J. Nat. Prod.* 69, 443–449.
- Inoguchi, T., Li, P., Umeda, F., Yu, H.Y., Kakimoto, M., Imamura, M., Aoki, T., Etoh, T., Hashimoto, T., Naruse, M., Sano, H., Utsumi, H., Nawata, H., 2000. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 49, 1939–1945.
- Itani, S.I., Ruderman, N.B., Schmieder, F., Boden, G., 2002. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and I $\kappa$ B- $\alpha$ . *Diabetes* 51, 2005–2011.
- Keston, A.S., Brandt, R., 1965. The fluorometric analysis of ultramicro quantities of hydrogen peroxide. *Anal. Biochem.* 11, 1–5.
- Kim, Y.H., Koh, H.K., Kim, D.S., 2010. Down-regulation of IL-6 production by astaxanthin via ERK-, MSK-, and NF- $\kappa$ B-mediated signals in activated microglia. *Int. Immunopharmacol.* 10, 1560–1572.
- Kruman, I., Guo, Q., Mattson, M.P., 1998. Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells. *J. Neurosci. Res.* 51, 293–308.
- Larbi, A., Grenier, A., Frisch, F., Douziche, N., Fortin, C., Carpentier, A.C., Fulop, T., 2005. Acute in vivo elevation of intravascular triacylglycerol lipolysis impairs peripheral T cell activation in humans. *Am. J. Clin. Nutr.* 82, 949–956.
- Larcombe, S.D., Coffey, J.S., Bann, D., Alexander, L., Arnold, K.E., 2010. Impacts of dietary antioxidants and flight training on post-exercise oxidative damage in adult parrots. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* 155, 49–53.
- Lee, S.J., Bai, S.K., Lee, K.S., Namkoong, S., Na, H.J., Ha, K.S., Han, J.A., Yim, S.V., Chang, K., Kwon, Y.G., Lee, S.K., Kim, Y.M., 2003. Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing I $\kappa$ B kinase-dependent NF- $\kappa$ B activation. *Mol. Cells* 16, 97–105.
- Lee, J.Y., Zhao, L., Youn, H.S., Weatherill, A.R., Tapping, R., Feng, L., Lee, W.H., Fitzgerald, K.A., Hwang, D.H., 2004. Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. *J. Biol. Chem.* 279, 16971–16979.
- Li, Q., Tan, L., Wang, C., Li, N., Li, Y., Xu, G., Li, J., 2006. Polyunsaturated eicosapentaenoic acid changes lipid composition in lipid rafts. *Eur. J. Nutr.* 45, 144–151.
- Macedo, R.C., Bolin, A.P., Marin, D.P., Otton, R., 2010. Astaxanthin addition improves human neutrophils function: in vitro study. *Eur. J. Nutr.* 49, 447–457.
- Madani, S., Hichami, A., Legrand, A., Belleville, J., Khan, N.A., 2001. Implication of acyl chain of diacylglycerols in activation of different isoforms of protein kinase C. *FASEB J.* 15, 2595–2601.
- Mannervik, B., 1985. Glutathione peroxidase. *Methods Enzymol.* 113, 490–495.
- McNulty, H.P., Byun, J., Lockwood, S.F., Jacob, R.F., Mason, R.P., 2007. Differential effects of carotenoids on lipid peroxidation due to membrane interactions: X-ray diffraction analysis. *Biochim. Biophys. Acta* 1768, 167–174.
- Meydani, S.N., Endres, S., Woods, M.M., Goldin, B.R., Soo, C., Morrill-Labrode, A., Dinarello, C.A., Gorbach, S.L., 1991. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. *J. Nutr.* 121, 547–555.
- Mizota, T., Fujita-Kambara, C., Matsuya, N., Hamasaki, S., Fukudome, T., Goto, H., Nakane, S., Kondo, T., Matsuo, H., 2009. Effect of dietary fatty acid composition on Th1/Th2 polarization in lymphocytes. *JPN J. Parenter Enteral Nutr.* 33, 390–396.



- Morgan, M.J., Kim, Y.S., Liu, Z., 2007. Lipid rafts and oxidative stress-induced cell death. *Antioxid. Redox Signal.* 9, 1471–1483.
- Morris, D.L., Kritchevsky, S.B., Davis, C.E., 1994. Serum carotenoids and coronary heart disease. The lipid research clinics coronary primary prevention trial and follow-up study. *JAMA* 272, 1439–1441.
- Murphy, M.E., Kehrer, J.P., 1989. Oxidation state of tissue thiol groups and content of protein carbonyl groups in chickens with inherited muscular dystrophy. *Biochem. J.* 260, 359–364.
- Naguib, Y.M., 2000. Antioxidant activities of astaxanthin and related carotenoids. *J. Agric. Food Chem.* 48, 1150–1154.
- Newsholme, P., Haber, E.P., Hirabara, S.M., Rebelato, E.L., Procopio, J., Morgan, D., Oliveira-Emilio, H.C., Carpinelli, A.R., Curi, R., 2007. Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. *J. Physiol.* 583, 9–24.
- Nunes, E.A., Bonatto, S.J., de Oliveira, H.H., Rivera, N.L., Maiorka, A., Krabbe, E.L., Tanhoffer, R.A., Fernandes, L.C., 2008. The effect of dietary supplementation with 9-cis:12-trans and 10-trans:12-cis conjugated linoleic acid (CLA) for nine months on serum cholesterol, lymphocyte proliferation and polymorphonuclear cells function in Beagle dogs. *Res. Vet. Sci.* 84, 62–67.
- Otton, R., Curi, R., 2005. Toxicity of a mixture of fatty acids on human blood lymphocytes and leukaemia cell lines. *Toxicol. In Vitro* 19, 749–755.
- Otton, R., Soriano, F.G., Verlengia, R., Curi, R., 2004. Diabetes induces apoptosis in lymphocytes. *J. Endocrinol.* 182, 145–156.
- Otton, R., da Silva, D.O., Campoio, T.R., Silveira, L.R., de Souza, M.O., Hatanaka, E., Curi, R., 2007. Non-esterified fatty acids and human lymphocyte death: a mechanism that involves calcium release and oxidative stress. *J. Endocrinol.* 195, 133–143.
- Otton, R., Marin, D.P., Bolin, A.P., Santos Rde, C., Polotow, T.G., Sampaio, S.C., de Barros, M.P., 2010. Astaxanthin ameliorates the redox imbalance in lymphocytes of experimental diabetic rats. *Chem. Biol. Interact.* 186, 306–315.
- Palozza, P., Krinsky, N.L., 1992. Antioxidant effects of carotenoids in vivo and in vitro: an overview. *Methods Enzymol.* 213, 403–420.
- Pick, E., Mizel, D., 1981. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J. Immunol. Methods* 46, 211–226.
- Pompeia, C., Lopes, L.R., Miyasaka, C.K., Procopio, J., Sannomiya, P., Curi, R., 2000. Effect of fatty acids on leukocyte function. *Braz. J. Med. Biol. Res.* 33, 1255–1268.
- Rahman, I., Kode, A., Biswas, S.K., 2006. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat. Protoc.* 1, 3159–3165.
- Rolo, A.P., Palmeira, C.M., 2006. Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. *Toxicol. Appl. Pharmacol.* 212, 167–178.
- Sacerdote, P., Martucci, C., Vaccani, A., Bariselli, F., Panerai, A.E., Colombo, A., Parolaro, D., Massi, P., 2005. The nonpsychoactive component of marijuana cannabidiol modulates chemotaxis and IL-10 and IL-12 production of murine macrophages both in vivo and in vitro. *J. Neuroimmunol.* 159, 97–105.
- Sano, T., Umeda, F., Hashimoto, T., Nawata, H., Utsumi, H., 1998. Oxidative stress measurement by in vivo electron spin resonance spectroscopy in rats with streptozotocin-induced diabetes. *Diabetologia* 41, 1355–1360.
- Santocono, M., Zurria, M., Berrettini, M., Fedeli, D., Falcioni, G., 2006. Influence of astaxanthin, zeaxanthin and lutein on DNA damage and repair in UVA-irradiated cells. *J. Photochem. Photobiol. B* 85, 205–215.
- Schonfeld, P., Reiser, G., 2006. Rotenone-like action of the branched-chain phytanic acid induces oxidative stress in mitochondria. *J. Biol. Chem.* 281, 7136–7142.
- Schonfeld, P., Wojtczak, L., 2007. Fatty acids decrease mitochondrial generation of reactive oxygen species at the reverse electron transport but increase it at the forward transport. *Biochim. Biophys. Acta* 1767, 1032–1040.
- Siddiqui, R.A., Jensi, L.J., Neff, K., Harvey, K., Kovacs, R.J., Stillwell, W., 2001. Docosahexaenoic acid induces apoptosis in Jurkat cells by a protein phosphatase-mediated process. *Biochim. Biophys. Acta* 1499, 265–275.
- Stentz, F.B., Kitabchi, A.E., 2006. Palmitic acid-induced activation of human T-lymphocytes and aortic endothelial cells with production of insulin receptors, reactive oxygen species, cytokines, and lipid peroxidation. *Biochem. Biophys. Res. Commun.* 346, 721–726.
- Stulnig, T.M., Berger, M., Roden, M., Stingl, H., Raederstorff, D., Waldhausl, W., 2000. Elevated serum free fatty acid concentrations inhibit T lymphocyte signaling. *FASEB J.* 14, 939–947.
- Tsuchiya, M., Scita, G., Freisleben, H.J., Kagan, V.E., Packer, L., 1992. Antioxidant radical-scavenging activity of carotenoids and retinoids compared to alpha-tocopherol. *Methods Enzymol.* 213, 460–472.
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* 39, 44–84.
- Verlengia, R., Gorjao, R., Kanunfre, C.C., Bordin, S., de Lima, T.M., Martins, E.F., Newsholme, P., Curi, R., 2004. Effects of EPA and DHA on proliferation, cytokine production, and gene expression in Raji cells. *Lipids* 39, 857–864.
- Wang, H., Joseph, J.A., 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic. Biol. Med.* 27, 612–616.
- Woerle, H.J., Popa, E., Dostou, J., Welle, S., Gerich, J., Meyer, C., 2002. Exogenous insulin replacement in type 2 diabetes reverses excessive hepatic glucose release, but not excessive renal glucose release and impaired free fatty acid clearance. *Metabolism* 51, 1494–1500.
- Yu, J.H., Kim, K.H., Kim, H., 2006. Role of NADPH oxidase and calcium in cerulein-induced apoptosis: involvement of apoptosis-inducing factor. *Ann. NY Acad. Sci.* 1090, 292–297.