

# The Combined Anti-Inflammatory Effect of Astaxanthin, Lyc-O-Mato and Carnosic Acid *In Vitro* and *In Vivo* in a Mouse Model of Peritonitis

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## Abstract

Inflammation has an important role in the pathogenesis of many diseases. The aims of the present research were to assess the effectiveness of a combination of Astaxanthin, Lycopene rich Tomato extract (Lyc-O-Mato®) and Carnosic acid, at low concentrations, to prevent the release of the inflammatory mediators from LPS stimulated macrophages *in vitro* and *in vivo* using a mouse model of peritonitis. Addition of low concentrations of Astaxanthin Lyc-O-Mato® and Carnosic acid to peritoneal mouse macrophages 1 h before addition of LPS for 24 h caused a synergistic inhibition of NO, PGE<sub>2</sub> and TNF $\alpha$  secretion. The supplementation of a nutrient combination in drinking water resulted in attenuation of basal, but not stimulated, superoxide production by recruited neutrophils to the peritoneal cavity and in inhibition of inflammatory mediators production by peritoneal macrophages. Our results indicate a potential use of these combinations in many inflammatory states.

**Keywords:** Astaxanthin; Proinflammatory mediators; Carotenoids; Polyphenols; Macrophages

## Introduction

Peripheral neutrophils and macrophages have an important role in host defense but also participate in the pathogenesis of a variety of diseases, including autoimmune diseases, and inflammatory disorders [1]. Inflammatory stimuli such as lipopolysaccharide (LPS) and interferon- $\gamma$  activate macrophages to produce a variety of pro-inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and other inflammatory mediators including prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and nitric oxide (NO), which are generated by the activity of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), respectively. These inflammatory mediators are involved in the pathogenesis of many human diseases [2-4]. Consequently, a common strategy for reducing the initiation and progression of inflammation-associated diseases is based on suppressing the production of inflammatory mediators [4,5].

Carotenoids and phenolics are associated with beneficial health effects, which are attributed to the consumption of fruits and vegetables and are related at least in part to their antioxidant activity [6-8]. Astaxanthin (3,3-dihydroxy-beta, beta-carotene-4,4-dione) belongs to the xanthophyll subclass of carotenoids. Several studies have demonstrated that Astaxanthin possesses powerful antioxidant properties, both *in vitro* and *in vivo*, especially as an inhibitor of LDL oxidation [9]. Lycopene is a naturally occurring non pro-vitamin A carotenoid found mainly in tomato and tomato products [10] with potent antioxidant properties [6]. Phenolics compose the major class of plant-derived antioxidants. The commonly used spice and flavoring agent rosemary, derived from the leaves of the plant *Rosmarinus officinalis* L, possesses potent antioxidant properties [8,11].

Several studies have reported the anti-inflammatory effect of different carotenoids, including Astaxanthin, lycopene, and of phenolic Carnosic acid [12-14]. These phytonutrients have been shown to inhibit the production of pro-inflammatory mediators, such as NO, interleukins, TNF- $\alpha$ , COX-2, and the transcription nuclear factor- $\kappa$ B (NF- $\kappa$ B), by stimulated macrophages [12,13,15-17]. However, high concentrations of these compounds were needed to achieve a significant inhibition. Considering that phytonutrients exist in nature and in our diets prevalently in combinations, the present study was originated

to study whether given together at low concentrations, will result in synergistic amplification of their efficacy to inhibit the production of inflammatory mediators by stimulated phagocytes *in vitro*. In addition, the present study aimed to determine whether their putative anti-inflammatory effects are also exerted *in vivo* using a mouse model of sterile peritonitis.

## Materials and Methods

### Isolation and culture of macrophage

Peritoneal macrophages were collected from the peritoneal cavity of 6-8 week old male ICR mice (Harlan, Israel) after an intraperitoneal injection of 1.5 ml of thioglycolate broth (4%) 4 days before harvest, as previously described [18]. Peritoneal macrophages were washed three times with PBS followed by hypotonic lysis of erythrocytes, yielding a highly enriched (90-95%) macrophage cell population. Macrophages were identified by FACS analysis using FITC-conjugated rat anti-mouse F4/80 (MCA497F) (Serotec, Oxford, England) by flow microfluorimetry on FACS (Becton Dickinson, Mountain View, CA). For each sample, 10,000 light scatter-gated viable cells were analyzed. Peritoneal macrophages (1 x 10<sup>6</sup> cells/well) were cultured in 96-well plates at 37°C in 5% CO<sub>2</sub> atmosphere in RPMI 1640 medium containing 10% FCS, 2 mm L-glutamine; 100 U/ml penicillin; 100  $\mu$ g/ml streptomycin (Beit-Haemek, Israel). Cells were stimulated with 100 ng/ml LPS from *Salmonella enterica serotype typhimurium* in the absence or presence of Astaxanthin, Lyc-O-Mato®, or carnosic acid and their combinations. LPS was purchased from Sigma, Israel. Astaxanthin and Lyc-O-Mato® were

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supplied by LycoRed Natural Products Industries Ltd. (Beer-Sheva, Israel). Two preparations of Natural Astaxanthin were used throughout the study and acted very similarly. One preparation of Astaxanthin was a 20% oleoresin, and the other preparation of Astaxanthin was 13.5% oleoresin. Lyc-O-Mato<sup>®</sup>, tomato oleoresin, (consists of lycopene 6% and contains additional tomato phytonutrients such the carotenoids as carotene, phytoene, phytoene as well as natural tomato tocopherols Carnosic acid (98.5% pure) was purchased from Alexis Biochemicals (Lousen, Switzerland). The phytonutrients were dissolved in DMSO (in a final concentration of 5 mM). The mixture was vortexed and incubated in a water bath at 37°C (with shaking) for 10 min, sonicated in a sonicator bath for 15 sec X 3 times. For final concentrations of phytonutrients appropriate volumes from the stock solution were added to warmed culture medium. To the control tubes appropriate volumes of DMSO (0.1-0.2%) were added. The % inhibition in each tube test was calculated in relation to its control.

### NO production assay

NO levels in the supernatant of cell cultures were determined by assaying nitrite concentration using Griess reagent and sodium nitrite as a standard [19].

### PGE<sub>2</sub> measurement

Supernatants were collected from resting and LPS-stimulated cells and immediately stored at -70°C. PGE<sub>2</sub> levels were in cell culture supernatants were determined by Elisa Kits (R7D Systems, Minneapolis, MN, USA).

### TNF $\alpha$ production assay

Concentrations of TNF $\alpha$  in cell culture supernatants were quantified by ELISA kits (Biolegend Inc., San Diego, CA, USA).

### Cell survival

Cell viability was assessed by cell count using trypan blue exclusion or by the colorimetric MTT ([3-(4, 5 -dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium] metabolic activity assay as done before [20]. For MTT measurement cells were cultured in 96 well plates. MTT was dissolved in medium (5 mg/ml) and added to each sample in an amount equal to 10% of the culture medium volume. After incubation for 4 h, the formazan crystals were dissolved in 100 mM HCl, 10% Triton X-100 all in isopropanol in equal volume to culture medium, the medium only served as a background. Absorbance intensity measured by a Thermomax Microplate Reader (Molecular Devices, Melno Park, CA, USA) at 570 nm with a reference wavelength of 690 nm.

### Mouse model of sterile peritonitis

Six to eight weeks old male ICR mice (Harlan Laboratories, Israel), were fed *ad libitum* rodent chow (#19520 Kofolk, Pethach Tikva, Israel) and free reverse osmosis filtered water. This study was performed after approval by Ben-Gurion University of the Negev committee for ethical care and use of animals in experiments, Authorization No. IL-4.05.2009. Mice (average weight 30 gr) were housed in static microisolator cages in 12:12 light dark cycles 18-26°C degrees and 30-70% relative humidity. Mice received in their drinking water a supplementation of Astaxanthin, Lyc-O-Mato<sup>®</sup>, Carnosic acid at the ratio of 0.5:1:1 (36, 81.2, 50  $\mu$ g/ml respectively) during 7 days before induction of peritonitis. The molar ratio was determined from the *in vitro* studies. The supplementation was prepared in micro-emulsion containing: 0.3% ascorbyl palmitate, 0.3% alpha tocopherol, 9.34% medium chain triglycerides 13% polysorbat 80 and 77.6%. The micro-

emulsion without any nutrients served as placebo. The animals drank 4 ml/water per day; thus, the phytonutrient intake of Astaxanthin, Lyc-O-Mato<sup>®</sup>, Carnosic acid was 4.9:10: 6.6 mg/Kg/day, respectively.

Sterile peritonitis was induced by injection of a sterile thioglycollate solution (4% w/v in PBS) to the peritoneal cavity. Neutrophils are recruited to the peritoneal cavity during the first 24 h and then replaced by monocyte-macrophages. Peritoneal cells were harvested after 24 h to isolate neutrophils or after 4 days to isolate macrophages using three washes of the peritoneal cavity with 8 ml RPMI medium. Peritoneal cells were washed three times with PBS followed hypotonic lysis of erythrocytes, yielding a high homogenous (90%) neutrophil cell population harvested 24 h after the induction of peritonitis. Homogenous (90%) macrophage cell population collected 4 days later. Peritoneal cell populations were identified by flow microfluorimetry on FACS (Becton Dickinson, Mountain View, CA) using FITC-conjugated rat anti-mouse neutrophils (MCA771F), FITC-conjugated rat anti-mouse F4/80 (MCA497F), and FITC-conjugated rat anti-mouse CD3 (MCA500F) (Serotec, Oxford, England) for the characterization of neutrophils, monocyte-macrophages and lymphocytes, respectively.

### Superoxides production by neutrophils

Superoxide anion (O<sub>2</sub><sup>-</sup>) release was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome c by the microtiter plate technique as described before [21]. Neutrophils (5  $\times$  10<sup>5</sup> cells/well) suspended in 100  $\mu$ l HBSS were stimulated with PMA (50 ng/ml). The reduction of ferricytochrome c was followed by a change of absorbance at 550 nm at 2 min intervals for 30 min on a Thermomax Microplate Reader (Molecular Devices, Melno Park, Calif., USA). The maximal rates of superoxide generation were determined and expressed as nanomoles O<sub>2</sub><sup>-</sup>/10<sup>6</sup> cells/ 10 min using the extinction coefficient E<sub>550</sub> = 21 mM<sup>-1</sup> cm<sup>-1</sup>.

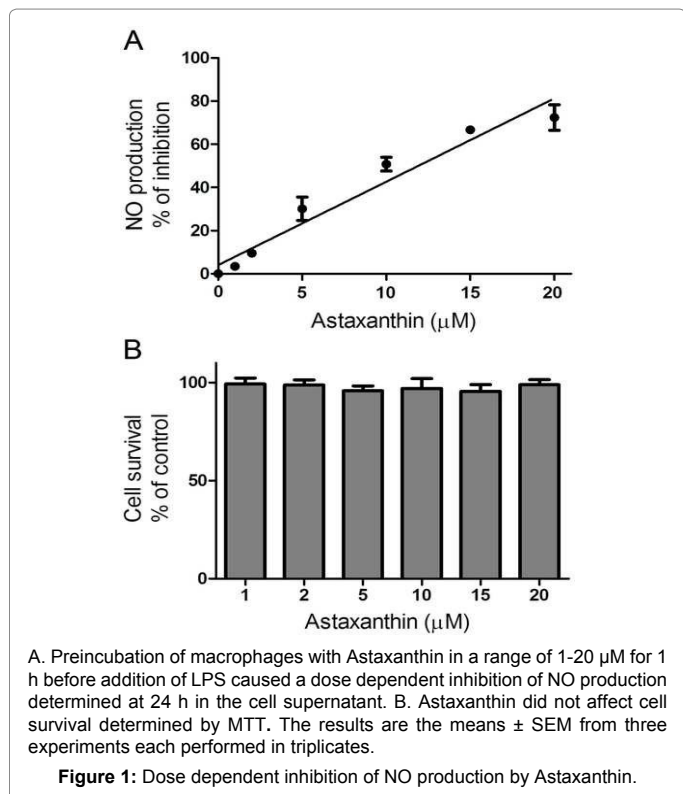
### Statistical analysis

Data are presented as the mean  $\pm$  SEM. Significant differences from control conditions were determined using either one- or two-way analysis of variance (ANOVA) followed by a posteriori Bonferroni's test for multiple comparisons provided by GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

## Results

### Synergistic inhibition of proinflammatory mediators by addition of combinations of Astaxanthin, Lyc-O-Mato<sup>®</sup> and carnosic acid to LPS-stimulated macrophage

The dose response effect of Astaxanthin on NO secretion in culture supernatants of peritoneal macrophages was studied. Astaxanthin was added at different concentrations 1 h before addition of 100 ng/ml LPS to peritoneal macrophages cultured for 24 h at 37°C. The secreted NO was determined in the cell culture supernatant. As shown in Figure 1A, Astaxanthin in the range of 0.1-20  $\mu$ M caused a dose dependent inhibition of NO production. Astaxanthin in this range did not affect cell survival of LPS stimulated macrophages (Figure 1B). We next studied whether combinations of Astaxanthin with another carotenoid (Lyc-O-Mato<sup>®</sup>) and a polyphenol (Carnosic acid) exert a synergistic inhibition of NO production. Addition of 1  $\mu$ M Lyc-O-Mato<sup>®</sup> to Astaxanthin in a range of 0.1-2  $\mu$ M caused a dose dependent synergistic inhibition of NO production (Figure 2A and B). Similarly, addition of 1  $\mu$ M Carnosic acid to Astaxanthin caused a dose dependent synergistic inhibition of NO production (Figure 2C). Addition of both 1  $\mu$ M Lyc-O-Mato<sup>®</sup> and 1  $\mu$ M Carnosic acid to Astaxanthin in a range of 0.1-2  $\mu$ M

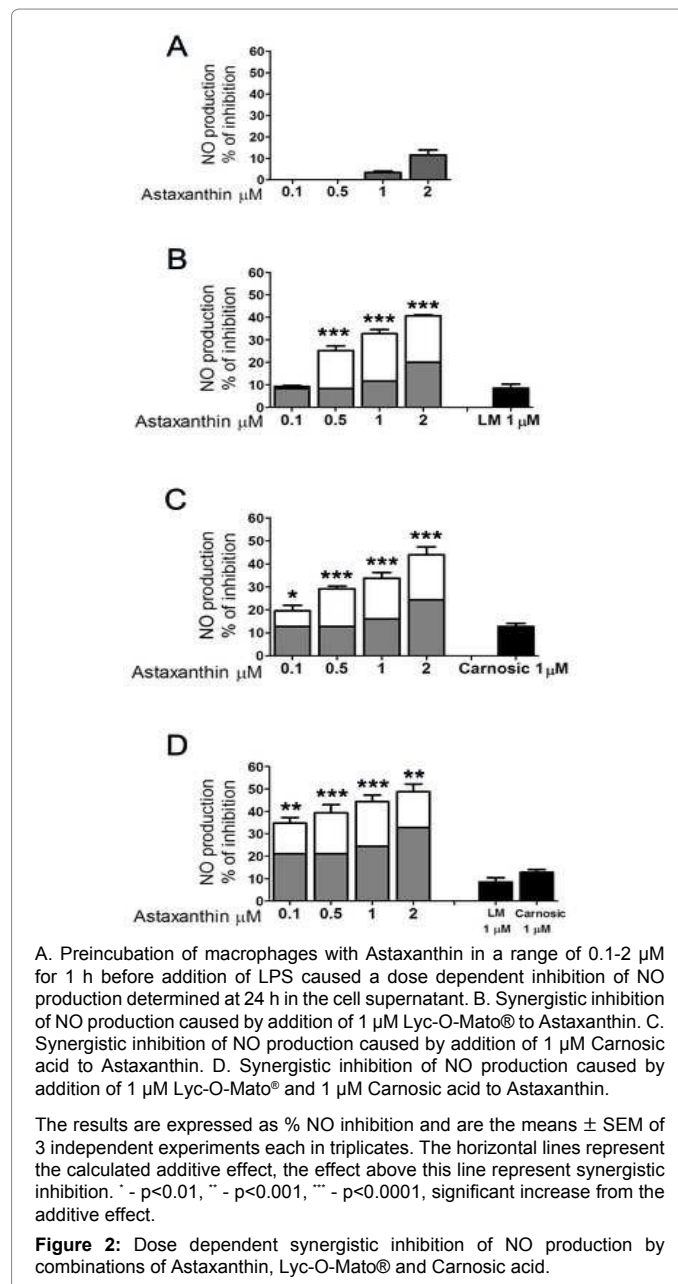


resulted with a higher synergistic inhibition of NO production (Figure 2D).

Similar effects were obtained with respect to TNF $\alpha$  (Figure 3A-D) and PGE $_2$  (Figure 4A-D).

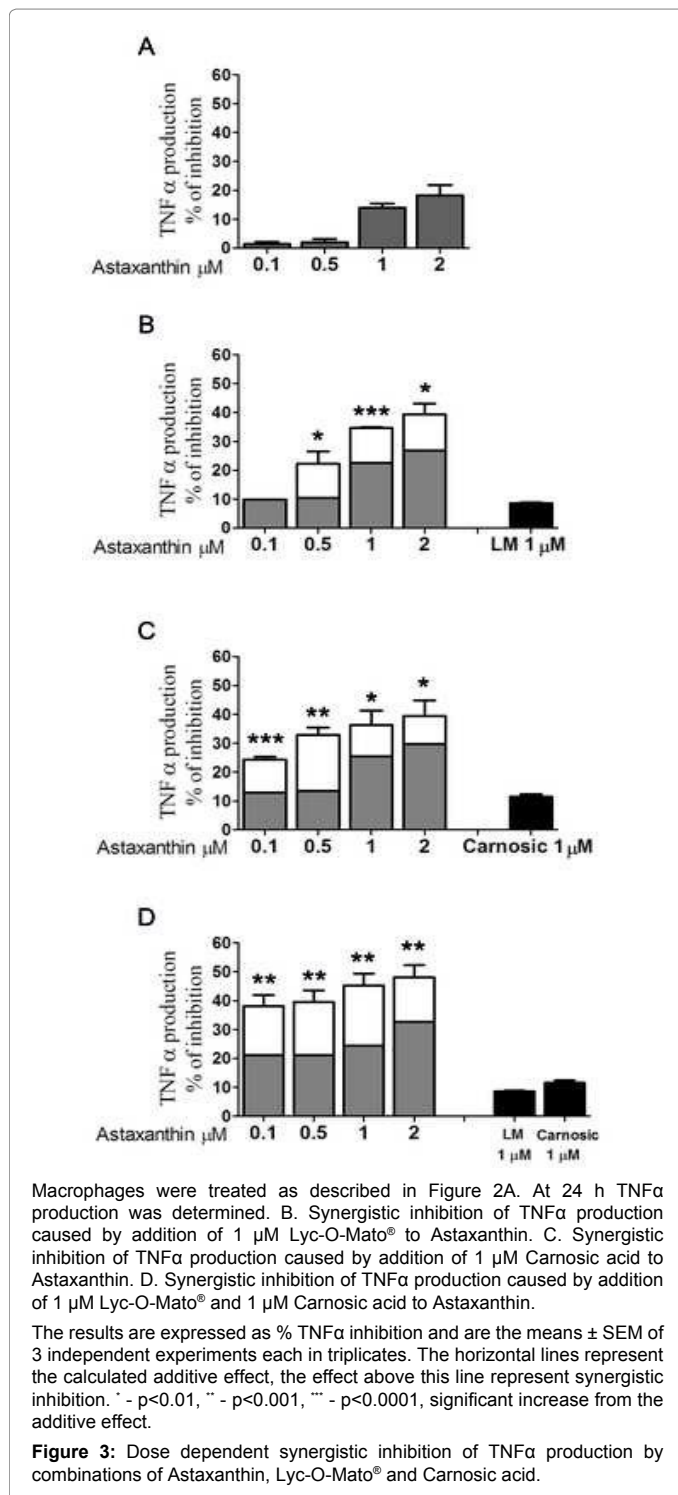
### The phytonutrients combination interfere with the inflammatory process *in vivo*

The next question addressed was whether the phytonutrients combination interfere with the inflammatory process *in vivo* as well. To this end, we used a mouse model of sterile peritonitis. As described in detail in the materials and method section, mice were given phytonutrient mixture or placebo in drinkable water before induction of sterile peritonitis with thioglycollate for 7 days. The molar ratio was 0.5:1:1 for Astaxanthin, Lyc-O-Mato $^{\circledR}$  and Carnosic acid, determined from the *in vitro* studies. This ratio resulted in a significant inhibition of NO production that was not significantly different from using a higher concentration of Astaxanthin (2 M). The quantity of Lyc-O-Mato $^{\circledR}$  used in the present study was 10 mg/kg as done in the previous *in vivo* study [22]. At twenty-four hour, the number of neutrophils recruited to the peritoneal cavity (the site of inflammation) and their activation status were determined. As presented in the Figure 5A, there were no differences in the number of recruited neutrophils to the site of inflammation in mice receiving the nutrient mixture (mix) compared with mice receiving placebo. The means  $\pm$  SEM in mice receiving nutrient for 7 days before peritonitis induction was  $2.35 \pm 0.45 \times 10^7$  neutrophils compared with  $2.15 \pm 0.23 \times 10^7$  neutrophils in mice receiving placebo. Figure 5B presents superoxide production by peritoneal neutrophils. These cells are primed due to the thioglycollate peritoneal injection and reflect the situation of the cells in a site of inflammation. As shown in Figure 5B, there is a significant ( $p < 0.001$ ) reduction (49%) in the release of superoxide by neutrophils from

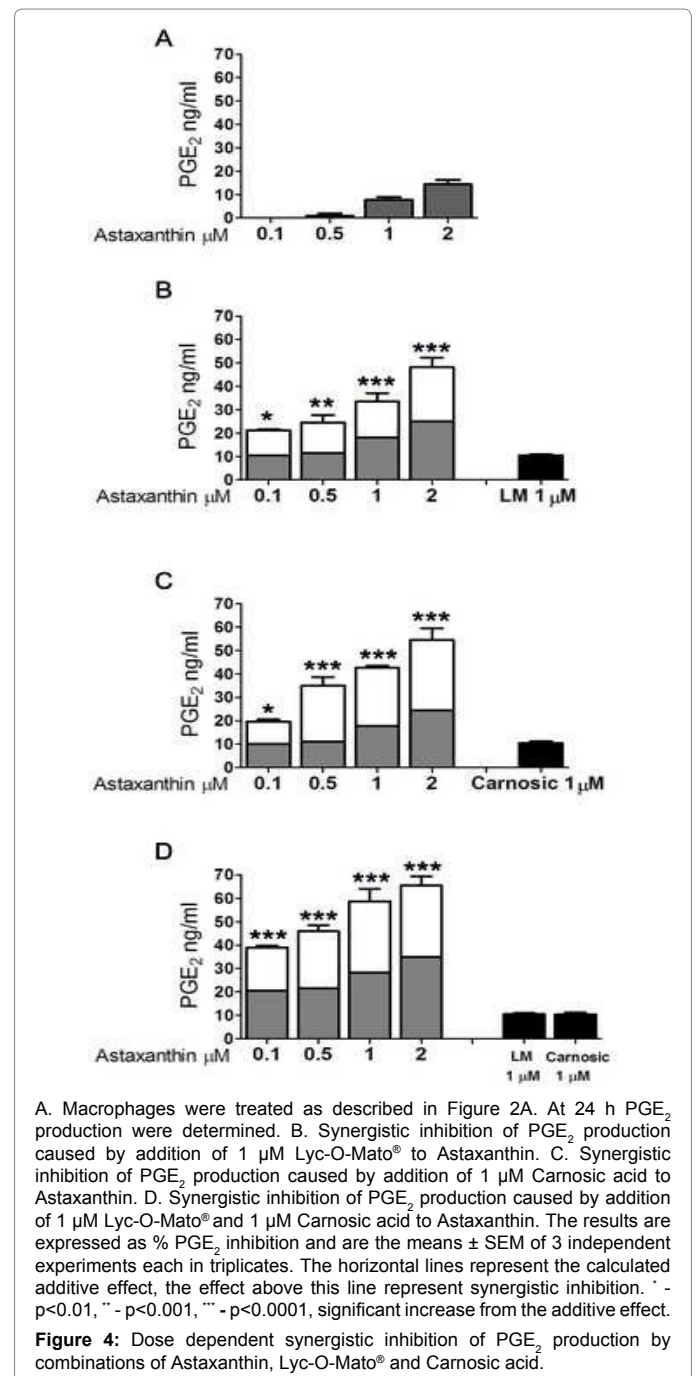


mice receiving the nutrient mixture compared with neutrophils from control mice receiving the placebo ( $3.82 \pm 1.52$  and  $7.48 \pm 1.53$  nmoles O $_2$ /10 $^6$  cells/min, respectively). However, stimulation of the neutrophils (as in the case of infections) resulted with an effective release of superoxides, 7.5-fold higher than without stimulation (Figure 5C). Superoxide production by peritoneal neutrophils of mice on nutrient supply showed a 17% reduction ( $p = n.s.$ ) compared with cells of mice on placebo ( $28.96 \pm 2.01$  and  $34.84 \pm 3.39$  nmoles O $_2$ /10 $^6$  cells/min, respectively).

The anti-inflammatory effect of the nutrient treatment on pro-inflammatory agents release by macrophages at the site of inflammation was also determined. Peritoneal macrophages were isolated and cultured in the absence or presence of 100 ng/ml LPS for 24 h. The supernatant of the cultured peritoneal cells was analyzed for



NO production, PGE<sub>2</sub> production and TNF $\alpha$  production. As shown in Figure 6A, there is a reduction (significance  $p < 0.01$ ) in NO production by peritoneal cells harvested from mice who drank the nutrient compared with placebo;  $2.35 \pm 0.36$  compared with  $3.72 \pm 0.48$   $\mu$ M, respectively after 1 week of treatment. Similarly, there was a reduction (significance  $p < 0.001$ ) in PGE<sub>2</sub> production by peritoneal cells harvested from mice undergoing nutrient drinking compared with placebo,  $1.75 \pm 0.26$  compared with  $2.72 \pm 0.33$  ng/ml (Figure 6B) TNF production

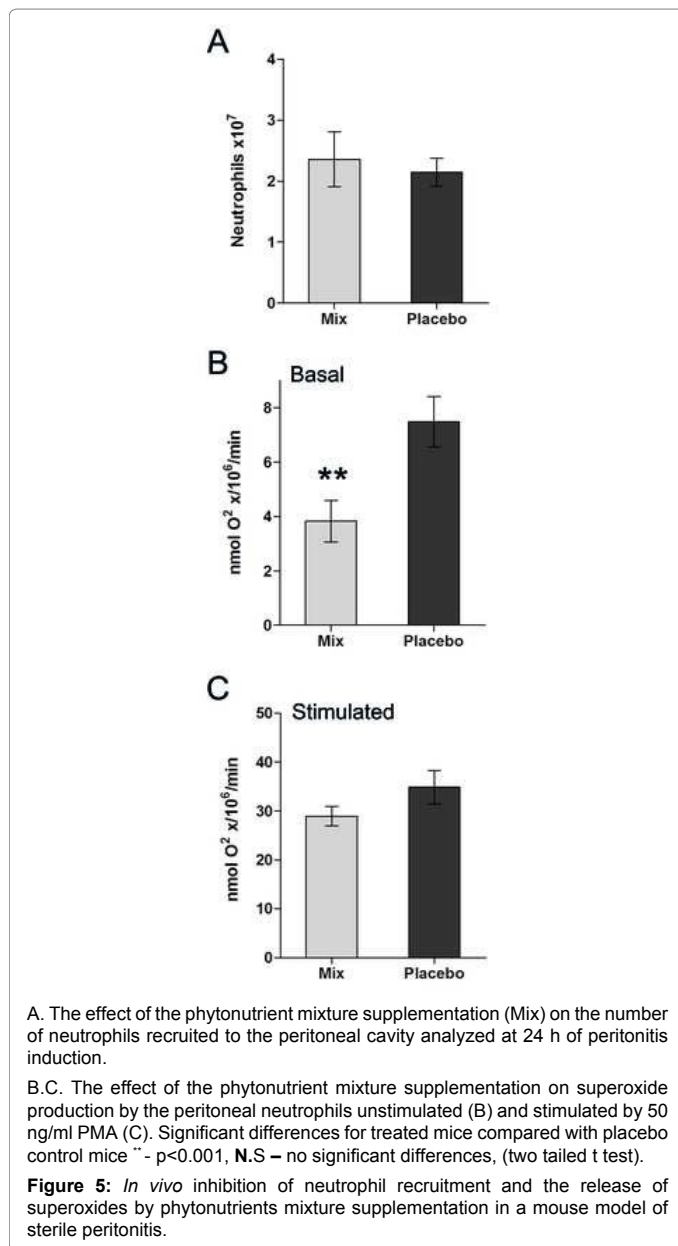


by peritoneal cells harvested from mice undergoing nutrient drinking was significantly lower ( $p < 0.01$ ) compared with placebo,  $64.1 \pm 11.9$  compared with  $110.5 \pm 17.7$  pg/ml (Figure 6C).

The body weights of each mouse on the first day of the of nutrient or placebo supply and at termination were measured. There was no difference in weight gain between the groups studied ( $35.2 \pm 0.8$  gr and  $34.2 \pm 1.0$  gr for the mix treated mice and the control mice, respectively).

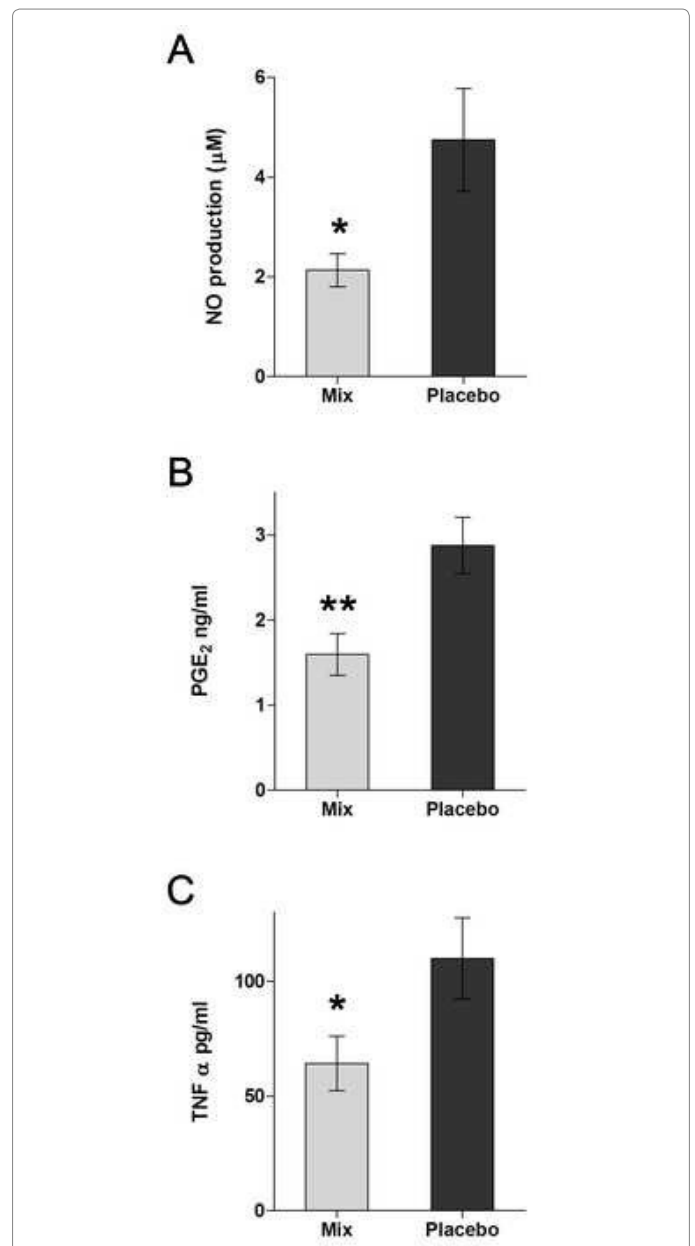
## Discussion

The present study demonstrate that addition of Astaxanthin, Lyc-O-Mato<sup>®</sup> and Carnosic acid at low levels, caused a significant



synergistic inhibition of the production of proinflammatory mediators from LPS stimulated macrophages *in vitro*. Moreover, this nutrient combination supplemented to drinking water, resulted in a significant anti-inflammatory effect in a mouse model of sterile peritonitis *in vivo*, as shown by the reduced release of spontaneous superoxides by neutrophils arriving at the site of inflammation; at the peritoneal cavity, and the reduced production of proinflammatory mediators by macrophages recruited to the peritoneal cavity. The nutrient combination supplementation, however, did not affect the rate of superoxide production from stimulated peritoneal neutrophils. The results suggest that the nutrient feeding was effective in preventing the delirious effect of neutrophils in the site of inflammation, but did not affect their potential to release superoxides when attacked by infection.

Previous studies [12,15,23-26] have reported the inhibition of pro-inflammatory mediators secretion by LPS stimulated macrophages



The effect of the phytonutrient mixture supplementation (Mix) on the release of NO (A) PGE<sub>2</sub> (B) and TNF $\alpha$  (C) by LPS stimulated peritoneal macrophages analyzed at 4 days of peritonitis induction. Significant differences for treated mice compared with placebo control mice are marked with \* -  $p < 0.01$ , \*\* -  $p < 0.001$ , (two tailed t test).

**Figure 6:** *In vivo* inhibition peritoneal macrophages proinflammatory mediators release by phytonutrients mixture supplementation in a mouse model of sterile peritonitis.

using high doses of each of the ingredients (Astaxanthin, Lyc-O-Mato® or Carnosic acid). Similar to those studies we show here that addition of Astaxanthin to LPS stimulated macrophages caused a significant dose dependent inhibition of NO production. Moreover, oral supplementation with Astaxanthin demonstrated a significant reduction in oxidative stress, hyperlipidemia and biomarkers of inflammation [9,27], in LDL oxidation [9], in plasma levels of 12- and 15-hydroxy fatty acids [27], in DNA damage and in CRP plasma levels [28]. Therefore, Astaxanthin appears safe, bioavailable when

given orally and is suitable for further investigation in humans. The results of the present study demonstrate for the first time a significant synergistic inhibitory effect in the production of pro-inflammatory mediators and of cytokines from LPS stimulated macrophages by addition of Lyc-O-Mato® or Carnosic acid at low concentrations, in the range of their human plasma levels after dietary supplementation [29-32]. Addition of 2 µM Astaxanthin with 1 µM Lyc-O-Mato® and 1 µM Carnosic acid (addition of 4 µM nutrients) caused  $48.8 \pm 3.4\%$  inhibition of NO production similar to the inhibition caused by addition of 10 µM of Astaxanthin ( $50.8 \pm 3.2\%$ ). The mechanism behind the synergistic action between the different compounds may be attributed to different physico-chemical properties and/or their preferential location in the membranes that may enable a better effect. The synergistic effect of the phytonutrients may also be due to the differences in the subcellular compartment of the polyphenols and the carotenoids that may enable a better and cooperative antioxidant effect resulting in the synergistic effect. Xanthopylls, such as Astaxanthin, span the membranes with the polar end groups anchored at polar sites of the membranes, while lycopene lacking hydrophilic substituents, remains entirely within the inner part of the membrane and increases motional freedom of lipid polar head groups [33]. Polyphenols and their derivatives are localized in the lipid bilayer membranes or on the bilayer-water interface among the polar head groups of the lipids [34,35]. Since each compound in the combination was reported to be an antioxidant [6,33,36] their mutual presence in different compartment of the membrane, may synergistically enhance each compound's scavenging of the immediate superoxide production and their effect on cell signaling leading to production of other pro-inflammatory mediators.

As shown convincingly in the present study, using the combination of natural ingredients at appropriate combinations, affords a significant advantage in treating inflammation: *i.* use of natural compounds in low concentrations, *ii.* Inhibiting concomitantly a number of pro-inflammatory mediators, in contrast to the action of drugs usually targeted against a single inflammatory agent. The advantage of this combined anti-inflammatory approach was recently corroborated by a study which substantiated the relevance of treatment of the inflammatory processes with the use of medication capable of combined blockage of iNOS, COX-2 expression [37]. The inhibition of TNF $\alpha$  by this supplementation seems as beneficial as the common treatment with anti TNF $\alpha$  antibodies [38,39].

Taken together, our results demonstrate a significant anti-inflammatory effect of the nutrient supplementation (Astaxanthin, Lyc-O-Mato® and Carnosic acid) that caused a significant lower release of pro-inflammatory mediators by peritoneal macrophages. Furthermore, this the nutrient supplementation caused lower priming of the neutrophils arriving to the site of inflammation, as detected by much lower levels of the release of spontaneous free radicals. It is pertinent to note that similar to *in vitro* results, LPS stimulation of peritoneal macrophages from mice fed with the ingredients supplementation released significantly lower levels of PGE<sub>2</sub>, NO and TNF $\alpha$ . Moreover, superoxide production from stimulated peritoneal neutrophils of mice receiving the supplementation did not differ from neutrophils from the placebo mice. Thus, while nutrient feeding may prevent the harmful effect of neutrophils and macrophages at the site of inflammation, it should not suppress their potential to release sufficient superoxides when challenged by infection. Thus, the results indicate a potential use of these combinations for anti-inflammation involved in many inflammatory diseases and aging processes.

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