

## Effect of Astaxanthin Supplementation on Inflammation and Cardiac Function in BALB/c Mice

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**Abstract.** Astaxanthin is an antioxidant with immunomodulatory, anti-inflammatory and anticancer properties. This study evaluated the use of dietary astaxanthin to decrease oxidative stress and improve cardiac function, thereby providing a potential cardioprotective supplement. Female BALB/c mice (8 weeks of age) were fed a semi-synthetic diet containing 0, 0.02 or 0.08% astaxanthin for 8 weeks. Cardiac function was assessed by echocardiography bi-weekly, and blood and tissue samples were collected at 8 weeks. Plasma astaxanthin concentrations increased ( $p < 0.05$ ) dose-dependently to 0.5 and 4  $\mu\text{mol/l}$  in the astaxanthin-supplemented mice. Blood glutathione concentrations and lymphocyte mitochondrial membrane potential were not significantly affected by astaxanthin treatment. However, mice fed 0.08% astaxanthin had higher ( $p < 0.05$ ) heart mitochondrial membrane potential and contractility index compared to the control group. These results support the possible use of dietary astaxanthin for cardiac protection.

Astaxanthin is an oxycarotenoid and has been shown to be an effective inhibitor of oxidative damage (1, 2); its immunomodulatory and anti-inflammatory action have also been clearly demonstrated (3). Astaxanthin increased immunoglobulin production in human cells *in vitro* (4), enhanced mouse splenic lymphocyte response and function, elevated mitogen-induced blastogenesis and cytotoxic activity (5), and increased production of IL-1 $\alpha$  and TNF- $\alpha$  (6). In fact, astaxanthin pre-treatment prior to drug administration showed a protective effect by alleviating lipid oxidation during naproxen-induced gastric ulceration in rats (7). Cellular enzymes involved in defense against oxidative stress were also increased; these include superoxide dismutase, catalase and glutathione peroxidase.

Hypertension can trigger oxidative stress in cardiac tissues (8). However, this can be counteracted with astaxanthin supplementation as demonstrated in a nitric oxide-induced vasorelaxation rat model (9, 10). Astaxanthin was also effective as a protective pre-treatment in rats (11) and dogs (12) following coronary artery occlusion. Not only was infarct size dose-dependently decreased, but myocardial salvage increased. Extending the period of pre-treatment in the rat further increased the beneficial effects (13). A mouse peritoneal inflammation model was used to show reduced lipid peroxidation with administration of disodium disuccinate astaxanthin, indicating that it may be useful therapeutically in response to multiple triggers of oxidative stress in cardiac tissue (14). In addition to the normal causes of cardiac oxidative stress such as hypertension, ischemia, obesity and dyslipidemia which can lead to endothelial dysfunction, damage can occur in tissues due to exercise-induced stress in the heart through mitochondrial respiration and xanthine oxidase activation (15). Dietary astaxanthin supplementation reduced DNA damage and lipid peroxidation in the mouse heart following strenuous exercise. In addition, high amounts of radical formation in cardiac cells can lead to contractile impairment resulting from lipid peroxidation, protein oxidation and DNA damage to the mitochondria, sarcolemma, and sarcoplasmic reticulum (16). Disruption of mitochondrial membrane potential (MMP) prior to severe cell injury (17) and increased inflammatory cytokine production (18) in cardiac cells are associated with apoptosis (19) and contractile failure in chemotherapy-induced cardiotoxicity (18). Therefore, the objective of this study was to investigate the feasibility of dietary astaxanthin, a potent antioxidant, to improve cardiac function in BALB/c mice.

### Materials and Methods

**Animals and diet.** Female BALB/c mice (8 weeks old, 19.7 $\pm$ 0.02 g, n=63) were randomly assigned to be fed a semi-synthetic diet (AIN-93M diet, Dyets, Bethlehem, PA, USA) containing 0, 0.02, or 0.08% astaxanthin (Carophyll Pink 8% beadlet; Hoffmann-La Roche, Nutley, NJ, USA) for 8 weeks. The diet was prepared as previously described (20). Daily food intakes and weekly body weights were measured.

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**Key Words:** Astaxanthin, cardioprotection, immunomodulation.

Mice were housed in an environment-controlled room (23°C, 12-hour dark-light cycle) and had free access to water and food. All experimental procedures were approved by the Institutional Animal Care and Use Committee, Washington State University.

**Echocardiography.** Echocardiographic examination was performed every two weeks (21) by a trained cardiologist using two-dimensional and M-mode ultrasound parameters to monitor cardiac size and function (ATL Ultrasound, HDI 3000, Bothell, WA, USA). Mice were anesthetized with isoflurane gas and positioned on a plexiglass platform designed for veterinary echocardiography. The images were captured using a digital echocardiography software program (MPACS, LLC, Echolink and Archman™, Madison, WI, USA). The following measurements were taken to assess cardiac function: ratio of left atrial diameter to the root of aortic diameter (LA/Ao) to measure the size of left atrium, left ventricular diastolic (LVIDd) and systolic inner diameter (LVIDs) to evaluate left ventricular structural changes, and percentile fractional shortening (FS) to measure the contractility of the left ventricle. Calculation of FS was by the formula:  $(LVIDd-LVIDs)/LVIDd \times 100$ . Four consecutive measurements were taken for each evaluation and the average value was used for analysis.

**Blood and tissue collection.** At the end of the feeding period, blood was collected by heart puncture into a heparinized syringe. Aliquots of whole blood were prepared for analysis of total glutathione (GSHt) and oxidized glutathione (GSSG) (described later). Plasma was obtained from the remaining blood and stored at -80°C. Hearts and livers were weighed and heart mitochondria immediately isolated.

**Mitochondria purification.** Heart mitochondria were purified at 4°C using a discontinuous Percoll gradient method (22). Purity of isolated mitochondria was confirmed by electron microscopy. The purified mitochondria pellet was resuspended in freshly prepared cell-free buffer (220 mmol/l mannitol, 68 mmol/l sucrose, 2 mmol/l NaCl, 2.5 mmol/l  $KH_2PO_4$ , 0.5 mmol/l EGTA, 2 mmol/l  $MgCl_2$ , 5 mmol/l pyruvate, 10 mmol/l HEPES, 100  $\mu$ mol/l PMSF, 2  $\mu$ mol/l leupeptin, 1.4 nmol/l pepstatin, 1 mmol/l DTT) at 4°C, and immediately used for MMP measurement.

**Heart and lymphocyte MMP.** Accumulated chloromethyl-X-rosamine (MitoTracker®Red CMXRos, Molecular Probes Inc., OR, USA; CMXRos), which stains mitochondria in live cells (23), were used to determine MMP in heart and leukocytes. Negative control samples were incubated in 400  $\mu$ mol/l carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma, St. Louis, MO, USA) for 30 min at 37°C before CMXRos staining. Mitochondria were stained with 2  $\mu$ mol/l CMXRos for 23 min at room temperature in the dark. All samples were immediately analyzed by flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA, USA) using CellQuest Pro software, version 5.1.1, (BD Bioscience).

**Plasma analyses.** Concentrations of GSHt and GSSG were measured in whole blood using the Biotech GSH/GSSG-412 assay kit (OxisResearch, Portland, OR, USA). Changes in absorbance at 412 nm were measured for 3 min by spectrophotometry (Beckman Coulter Inc., Fullerton, CA, USA). GSHt and GSSG concentrations and their ratio were calculated based on generated standard curves using the reaction rate where slope of the regression equation is equal to rate.

Plasma was analyzed for IL-1 $\alpha$ , IL-6, and TNF- $\alpha$  by ELISA (BD Bioscience). The detection limit was 31.3, 15.6, and 15.6 pg/ml for IL-1 $\alpha$ , IL-6 and TNF- $\alpha$ , respectively. Plasma SAA concentration was measured by sandwich ELISA (PHASE™ RANGE Murine Serum Amyloid A, TriDelta plc, Co. Wicklow, Ireland). The assay detection limit was 0.59  $\mu$ g/ml.

Plasma astaxanthin was extracted with a 1:1 (v/v) mixture of petroleum ether:anhydrous ether and analysed by HPLC (Waters Alliance 2690, 996 photo-diode array detector, Waters, Milford, MA, USA). Astaxanthin and samples eluted on a 3  $\mu$ m silica column (Luna, 100A; 150x4.6 mm; Phenomenex, Torrance, CA, USA) using hexane:acetone (82:18, v/v) as the mobile phase and a flow rate of 1.2 ml/min (24).

**Statistical analysis.** Data were first tested for normality with the Shapiro-Wilk test. Diet intake and body weight were analyzed by analysis of covariance (ANCOVA; SAS Institute, Cary, NC, USA). Other variables were analyzed by one-way analysis of variance using the General Linear Model procedure, and treatment means were compared by the Student's *t*-test. A probability value of  $p < 0.05$  was considered statistically significant.

## Results

Final body weight (mean  $\pm$  standard error of the mean [SEM]: 22.1  $\pm$  0.1 g), heart weight (0.118  $\pm$  0.001 g), liver weight (1.17  $\pm$  0.02 g) and daily food intake (2.6  $\pm$  0.1 g/day overall average) were not significantly influenced by dietary astaxanthin (Table I). While astaxanthin was not detectable in mice fed the control diet, concentrations increased ( $p < 0.05$ ) dose-dependently at week 8 with astaxanthin supplementation (Table I). The 13-*cis* astaxanthin was the major geometrical isomer (77%) in plasma, with all-*trans* (22%) and 9-*cis* (1%) also being present.

**Echocardiography.** Baseline for echocardiographic measurements were: 1.02  $\pm$  0.02, 2.61  $\pm$  0.03 and 31.9  $\pm$  0.8 mm for LA/Ao, LVIDd and FS, respectively (data not shown). After 8 wk of supplementation, mice fed 0.08% astaxanthin had increased ( $p < 0.05$ ) FS (41.51  $\pm$  1.82 compared to control (35.27  $\pm$  2.14) (Figure 1). Dietary astaxanthin had no significant effect on LA/Ao ratio and LVIDd.

**Cardiac and lymphocyte MMP.** Heart MMP increased with dietary astaxanthin supplementation (Figure 2) in a dose-dependent manner. Although MMP tended to be higher in the 0.02% supplemented mice, MMP was 50% higher ( $p < 0.05$ ) in mice fed 0.08% astaxanthin. In contrast, dietary astaxanthin had no significant influence on lymphocyte MMP.

**Plasma analyses.** Dietary astaxanthin did not significantly influence concentrations of GSHt, GSSG, and GSHt/GSSG ratio in whole blood (Table II), although there was a tendency for an increased GSHt/GSSG ratio and decreased GSSG in mice supplemented with astaxanthin.

Table I. Daily food intake, body and organ weights and plasma astaxanthin concentrations in mice fed 0, 0.02 or 0.08% astaxanthin for 8 weeks.

	Dietary astaxanthin (%)		
	0	0.02	0.08
Food intake (g/day)	2.7±0.1	2.4±0.1	2.5±0.1
Final body weight (g)	22.3±0.4	21.9±0.4	22.0±0.2
Heart weight (g)	0.112±0.004	0.113±0.003	0.119±0.003
Liver weight (g)	1.11±0.04	1.13±0.05	1.18±0.05
Plasma astaxanthin (µmol/l)	0 <sup>c</sup>	0.57±0.10 <sup>b</sup>	4.97±0.77 <sup>a</sup>

Values are means±standard error of the mean, n=21/group; Different letters within rows indicate statistical significance,  $p<0.05$ .

Astaxanthin feeding inhibited TNF- $\alpha$  production but had no significant effect on plasma IL-1 $\alpha$ , IL-6 and SAA (Table II). Concentrations of these cytokines tended to be highly variable.

## Discussion

Dietary astaxanthin increased plasma astaxanthin concentrations in a dose-dependent manner. While plasma astaxanthin in the 0.02% group reached 0.5 µmol/l, concentrations in mice fed 0.08% astaxanthin were 9 times higher (4.9 µmol/l). After a single oral dose of astaxanthin (all-*trans*:9-*cis*:13-*cis*=74:9:17 ratio), Osterlie *et al.* (25) reported the presence of all three isomers (all-*trans*:9-*cis*:13-*cis*=50:13:37 ratio) in human plasma. A similar study with rainbow trout showed all-*trans* astaxanthin to be the major isomer present in the plasma (92%), with much lower percentage of 9-*cis* (3%) and 13-*cis* (5%) astaxanthin (26). Using the same source of astaxanthin in the current study, it was demonstrated that all three astaxanthin isomers were detectable in the plasma; however, the major astaxanthin isomer in mice was the 13-*cis* (>70%) isomer. Liu and Osawa (27) showed differential antioxidant activity between the three isomers of astaxanthin, with 9-*cis* showing the highest activity and 3-*cis* being more active when compared to all-*trans*. These data demonstrate differences in all-*trans* astaxanthin isomerization by different species, which may have implications in the bioactivity of astaxanthin composition used for supplementation.

Excess ROS has been implicated in compromising cardiac health by leading to cardiac damage; on the other hand, antioxidants can not only alleviate oxidative damage but also provide cardioprotection (9, 11). A number of agents have been shown to have chemoprotective properties; these include vitamin E, lycopene, melatonin, garlic extract and panax ginseng extract (20, 28, 29). In this study, mice had significantly higher FS and heart MMP when 0.08% astaxanthin for 8 weeks, suggesting a possible role of

Table II. Blood GSH and GSSG concentrations and plasma IL-1 $\alpha$ , IL-6, TNF- $\alpha$  and SAA in mice fed 0, 0.02 or 0.08% astaxanthin for 8 weeks.

	Dietary astaxanthin (%)		
	0	0.02	0.08
GSHt (µmol/l)	1291±58	12178±111	1232±35
GSSG (µmol/l)	180±9	164±16	1734±12
GSHt/GSSG ratio	5.35±0.60	5.89±0.95	5.41±0.61
IL-1 $\alpha$	5.95±1.86	4.58±1.10	11.11±2.61
IL-6	25.97±7.30	30.32±7.90	31.05±11.74
TNF- $\alpha$	19.51±7.71 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>
SAA	5.56±0.79	5.08±0.67	5.29±0.66

Values are mean±standard error of the mean, n=21/group; Different letters within rows indicate statistical significance,  $p<0.05$ .

astaxanthin in cardiac function. The initial blockage of harmful ROS is an important factor for protection, as was demonstrated in human neuroblastoma cells (30); the action is most likely through the blocking of p38MAPK apoptotic signalling. Reduced MMP leads to the release of cytochrome *c* from the damaged mitochondria, thereby activating caspase 3 followed by apoptosis. Accumulation of astaxanthin in the mitochondria of human mesangial cells decreased release of ROS from the mitochondria and effectively reduced NF $\kappa$ B gene expression (31). Possible mechanisms of astaxanthin decrease of MMP include: (a) myocardial sensitization to intracellular calcium and/or adrenergic stimulation to enhance excitation-contraction coupling, (b) reduction of arterial pressure and the consequent decrease of afterload, and (c) increased arterial wall resistance leading to increased preload and cardiac concentric hypertrophy (32, 33).

During oxidative stress, GSH concentrations may rise due to decreased GPx activity (34). However, astaxanthin had no significant effect on these variables, suggesting that the action of astaxanthin is not mediated through GSHt/GSSG.

Elevated inflammatory cytokines in plasma have also been linked to cardiovascular diseases. In cardiac tissue, IL-1 $\alpha$  induces collagen degradation and tissue remodeling, and the blocking of IL-1 receptors in toll-like receptor-2-knockout mice prevented cardiotoxicity (18). In this study, astaxanthin had no effect on plasma concentrations of IL-1 $\alpha$  and IL-6. Conversely, dietary astaxanthin decreased plasma TNF- $\alpha$ . TNF- $\alpha$  is linked to the pathophysiology of cardiovascular diseases, and increases expression during cardiotoxicity in rodents (34, 18). Antioxidants in garlic extract normalized cardiac TNF- $\alpha$  expression and prevented histological changes. The suppression of plasma TNF- $\alpha$  concentrations in this study suggests cardioprotective role of astaxanthin.

Acute phase protein, SAA, produced in the liver in response to inflammatory stimuli such as IL-1 $\alpha$ , IL-6 and TNF- $\alpha$ , is

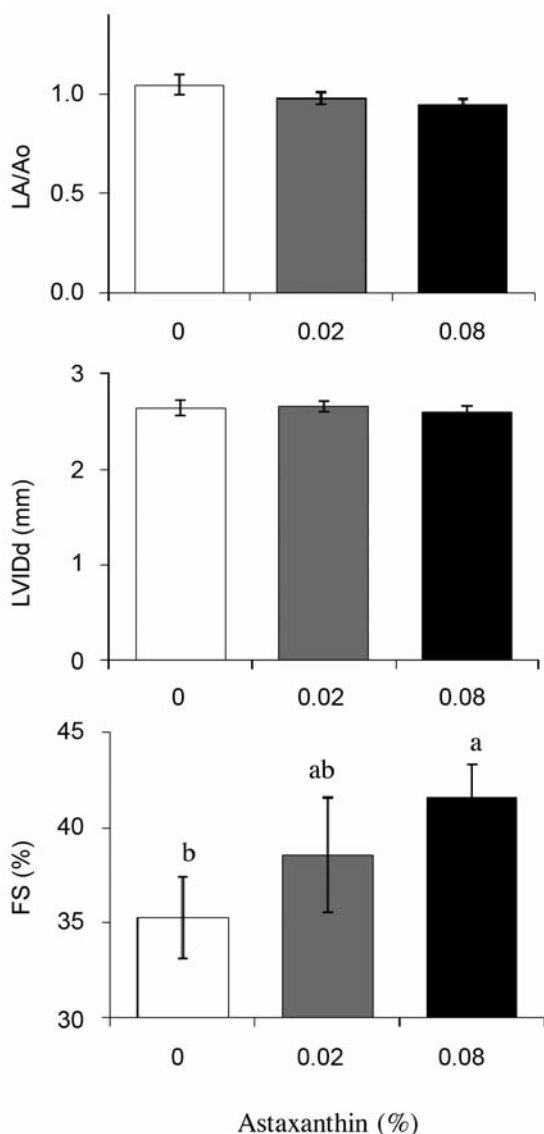


Figure 1. Echocardiographic measures of cardiac function, LA/Ao ratio, LVIDd and FS in mice fed 0, 0.02 or 0.08% astaxanthin for 8 weeks. Values are mean±standard error of the mean, n=14/group. Different letters within a cardiac measurement denote statistical difference, p<0.05.

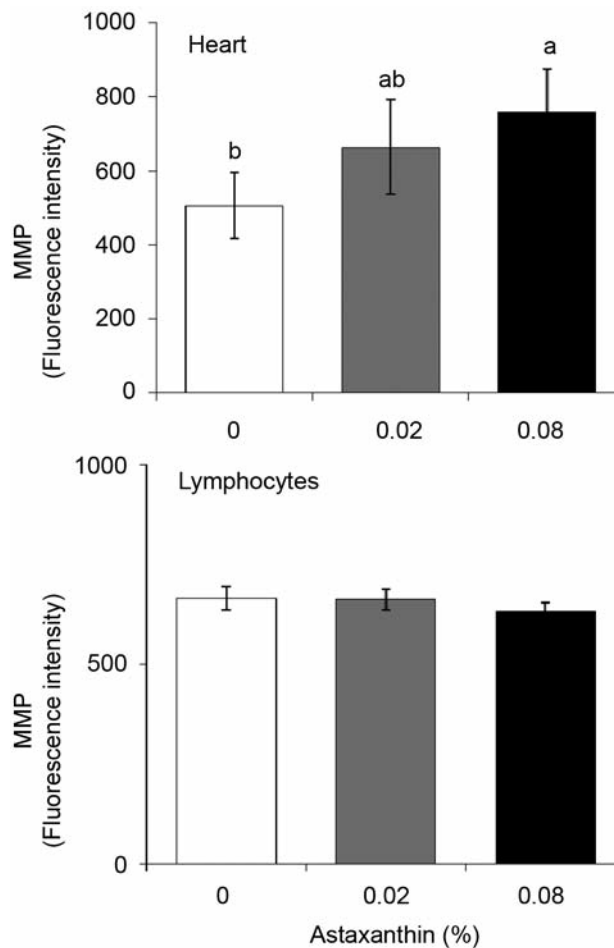


Figure 2. Heart and lymphocyte MMP in mice fed 0, 0.02 or 0.08% astaxanthin for 8 weeks. Values are mean±standard error of the mean, n=14/group. Different letters within a panel denote statistical difference, p<0.05.

linked to the pathogenesis of cardiovascular diseases (35). In the current study, plasma SAA concentrations tended to decrease with astaxanthin supplementation. Although IL-6 levels were increased, there was no ensuing increase in SAA levels.

In summary, dietary astaxanthin increased heart MMP and FS dose-dependently and tended to decrease plasma IL-1 $\alpha$ , TNF- $\alpha$  and SAA concentrations. Taken together, these results suggest the possible role dietary astaxanthin could play during chemotherapy, not only for its ability to counteract induced oxidative stress, but also for its cardioprotective efficacy.

### Acknowledgements

Funding for this project was received from the Agriculture Research Center, Washington State University, Pullman, WA, U.S.A.

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Received March 4, 2010

Revised May 7, 2010

Accepted May 12, 2010