Effect of Dietary Astaxanthin at Different Stages of Mammary Tumor Initiation in BALB/c Mice

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Abstract. *The effects of astaxanthin on tumor growth, cardiac function and immune response in mice were studied. Female BALB/c mice were fed a control diet (diet C) for 8 weeks, 0.005% astaxathin for 8 weeks (diet A), or diet C for weeks 1-5 followed by diet A thereafter (diet CA). Mice were injected with a mammary tumor cell line on day 7 and tumor growth was measured daily. Mice fed diet A had extended tumor latency and lower tumor volume ($p<0.05$). Interestingly, those fed diet CA showed the fastest tumor growth. Astaxanthin feeding elevated plasma astaxanthin concentrations; there was no difference in plasma astaxanthin between mice fed CA and those fed A. Mice fed diet A, but not CA, had a higher ($p<0.05$) natural killer cell subpopulation and plasma interferon-γ concentration compared to those fed diet C. Astaxanthin delayed tumor growth and modulated immune response, but only when astaxanthin was given before tumor initiation. This suggests that an adequate blood astaxanthin status is needed to protect against tumor initiation; conversely, astaxanthin supplementation after tumor initiation may be contraindicated.*

Astaxanthin has potent antioxidant and anti-inflammatory properties, and is antihypertensive and neuroprotective (1, 2). It reduces the risk of cardiovascular disease (3) and cancer, and modulates immune function (4). The anticancer activity of astaxanthin is mediated through reduced lipid peroxidation and DNA damage, enhanced immune function and down-regulation of pro-inflammatory cytokines. The inhibitory activity of astaxanthin on mammary tumor growth in BALB/c mice was higher than that of β-carotene and canthaxanthin (2). Similarly, Jyonouchi *et al*. (4) reported that astaxanthin prevented fibrosarcoma growth through the activation of cytotoxic T lymphocytes and increased interferon-γ (IFN-γ) production by tumor-draining lymph nodes and spleen cells in mice. Astaxanthin improved phytohaemagglutinin-induced splenocyte proliferation and splenocyte cytotoxic activity in mice (5). During stress-induced immune suppression, astaxanthin restored the natural killer (NK) cell population (6). We studied the efficacy of astaxanthin supplementation at two stages of tumor growth and its effect on immunomodulation and cardiac function in BALB/c mice.

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Key Words: Antioxidant, astaxanthin, mouse, inflammation, cardiac function.

Materials and Methods

Animals and diet. Female BALB/c mice (8 weeks old, 19.0±0.32 g; Jackson Laboratory, Bar Harbor, ME, USA) were either fed the base control diet (diet C) for 8 weeks, a diet containing 0.005% astaxathin (Zanthin®; US Nutra, Eustis, FL, USA) for 8 weeks (diet A), or the control diet for weeks 1-5 followed by 0.005% astaxanthin for the final 3 weeks (diet CA) (n=21/diet). The base diet was a semi-synthetic diet (AIN-93M; Dyets, Inc., Bethlehem, PA, USA) that meets or exceeds all essential nutrients. Daily food intake and weekly body weight were measured. 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.

Tumor challenge. Mice were fed their respective diets for 7 days before being inoculated with WAZ-2T (-SA) mammary tumor cells (generously provided by Dr. Howard Hosick, Washington State University, Pullman, WA, USA) as previously described (7, 8). The tumor cells (7×10^5 cells/injection) were inoculated into the right inguinal mammary fat pad. Tumor growth was monitored daily starting 10 days after inoculation. Tumor latency, defined by the day when tumor was first palpable, was recorded. Tumor diameter was measured using a pair of digital calipers (Mitsutoyo, Tokyo) as previously described (8). Tumor volume was calculated as a sphere.

Echocardiography. Cardiac function was measured in all mice using ultrasound transthoracic echocardiography (ATL Ultrasound, HDI 3000; Bothell, WA, USA) at the end of the study (9). Two-dimensional and M-mode evaluations were recorded, and images captured using a digital echocardiography software program (MPACS, LLC, Echolink and Archman™ , Madison, WI, USA). The following

Table I. *Tumor latency, tumor weight and organ weight.*

	Tumor		Organs		
Treatment	Latency (days)	Weight (g)	Heart (g)	Spleen (g)	Liver (g)
C	30.75±0.93[ab]	5.5±0.3	0.124±0.004	0.218±0.019	1.074±0.051
CA	30.32±0.9[3b]	5.7±0.3	0.125±0.003	0.203±0.021	0.936±0.053
A	33.67±1.04[a]	5.1±0.4	0.120±0.004	0.218±0.009	1.069±0.050

Values are means±SEM, n=21/treatment. Different letters within a column denote statistical significance of differences, $p<0.05$. C, Control diet throughout the study; CA, control diet for weeks 1-5, astaxanthin diet weeks 6-8; and A, astaxanthin diet throughout the study.

measurements were taken: the ratio of the left atrial diameter to the root of the aortic diameter (LA/Ao) to measure the size of the left atrium, the left ventricular diastolic (LVIDd) and systolic (LVIDs) inner diameter to evaluate the left ventricular structural changes, and the percentile fractional shortening (FS) to measure the contractility of the left ventricle. The FS was calculated using the formula: (LVIDd-LVIDs)/LVIDd ×100. Four consecutive measurements were taken for each evaluation and the average value was used for analysis.

Blood and tissue collection. After echocardiographic examination, mice were anesthetized and blood was collected by heart puncture with a heparinized syringe. Aliquots of whole blood were prepared for analysis of total glutathione (GSHt) and oxidized glutathione (GSSG) and stored at –80˚C until assay. Following centrifugation, plasma was collected and aliquoted, overlayed with nitrogen and stored at –80˚C until analysis of cardiac troponin-I (cTn I), cytokines, serum amyloid-α (SAA), and astaxanthin. Leukocytes collected from the plasma/RBC interface were used for lymphocyte phenotyping and measurement of mitochondrial membrane potential (MMP). The heart, liver, and spleen were weighed, and heart mitochondria immediately isolated.

Mitochondria purification and MMP measurement. Mitochondria were isolated and purified as described (10) and immediately used for MMP measurement. Lymphocyte and cardiac mitochondria were labeled with chloromethyl-x-rosamine (CMXRos; MitoTracker Red, Molecular Probes, Inc. OR, USA) (11), and MMP measured by flow cytometry (FACSCalibur; BD Bioscience, San Jose, CA, USA) using the CellQuest Pro software (version 5.1.1.).

Blood assays. Plasma cTn I (Life Diagnostics, West Chester, PA, USA) and serum SAA (TriDelta plc, Co. Wicklow, Ireland) were measured by sandwich ELISA. The assay detection limit was 0.078 ng/ml and 0.059 μg/ml, respectively. Concentrations of GSHt and GSSG in whole blood were measured using a commercial kit (Biotech GSH/GSSG-412; OxisResearch, Portland, OR, USA). The reaction rate, which is proportional to the GSH and GSSG quantity, was measured at 412 nm. The lower limit of detection for the assay was 0.54 μmol/l.

Plasma interleukin (IL)-1α, IL-6, tumor necrosis factor (TNF)-α, IL-2, and IFN-γ were quantified by ELISA (BD Bioscience, San Jose, CA, USA). The assay detection limits were 31.3, 15.6, 15.6, 3.1 and 3.1 pg/ml, respectively.

Lymphocyte phenotyping. Leukocyte subpopulations were measured with two-color (CD25α/CD19) or three-color labeled antibodies (CD3/CD4/CD8α and CD3/CD19/PanNK-CD49b) using flow cytometric analysis (FACSCalibur; CellQuest program version 5.1.1). Anti-mouse CD3-FITC (BD Biosciences), CD4-APC, CD8α-PE, PanNK/CD49b-PE, CD19-APC, and CD25α-PE (Invitrogen, Carlsbad, CA, USA) antibodies were used.

Astaxanthin extraction and HPLC analysis. Astaxanthin was extracted from plasma as previously described (12), and analyzed by HPLC (Waters Alliance 2690 equipped with a 996 photo-diode array detector; Waters, Milford, MA, USA). Astaxanthin was eluted isocratically on a 3 μ silica column (Luna, 100A; 150×4.6 mm; Phenomenex, Torrance, CA, USA) with a hexane:acetone (82:18, v/v) mobile phase set at a flow-rate of 1.2 ml/min (13).

Statistical analysis. Data were first tested for normality by the Shapiro-Wilk test (SAS Institute, Cary, NC, USA). Body weight and food intake were evaluated by analysis of covariance (ANCOVA). Treatment means were compared using Student's *t*-test. The frequency of tumor incidence, IL-1α, IL-6 and TNF-α expression in plasma was analyzed by Chi-square. A probability value of $p<0.05$ was considered statistically significant.

Results

General. Body weight was similar among treatment groups and averaged 24.6±0.5 g at the end of the study. Diet intake, and final heart, spleen and liver weights also were similar among treatments (Table I). Astaxanthin was not detectable in the plasma of mice fed the control diet. Astaxanthin concentrations in the plasma of both CA and A groups at week 8 were similar and averaged 0.57±0.10 μmol/l.

Mammary tumor growth. The average tumor latency was significantly longer ($p<0.05$) with diet A (33.7±1.0 day) compared to diet C (30.7±1.0 day) (Table I). Tumor latency in the CA group (30.3±1.0 day) was not different from that of the C group. Treatment effects on the incidence of detectable tumor followed a similar trend, with significantly ($p<0.05$) lower tumor incidence in mice fed diet A 23-32 days post-tumor challenge compared to those fed C and CA diets (Figure 1). Overall, mammary tumor growth was rapid after day 35 post-tumor inoculation in all treatment groups (Figure 2). However, mice fed diet A had smaller ($p<0.05$) tumors throughout the study compared to those fed diet C.

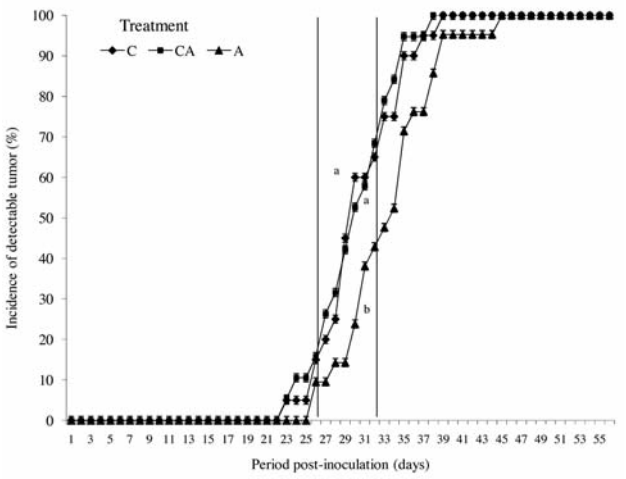

Figure 1. *The percentage of mice with detectable tumor after –SA mammary tumor cell injection at week 0. Mice were fed a control or astaxanthin diet. C, Control diet throughout the study; CA, control diet weeks 1-5; astaxanthin, diet weeks 6-8; and A, astaxanthin diet throughout the study. Values are means, n=21/treatment. Treatment A is significantly lower (p<0.05; Chi-square) than C between days 17 and 32 (as denoted by the area between the two vertical lines).*

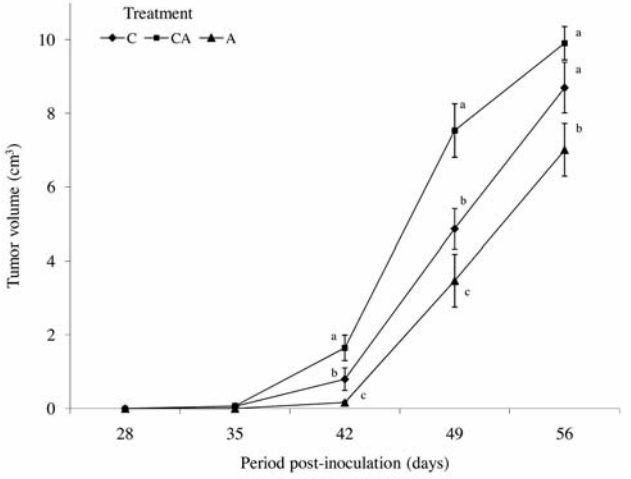

Figure 2. *Final tumor weight on day 56 post-tumor inoculation in mice fed a control or astaxanthin diet. C, Control diet throughout the study; CA, control diet weeks 1-5; astaxanthin, diet weeks 6-8; and A, astaxanthin diet throughout the study. Values are means±SEM, n=21/treatment. Different letters denote a significant difference (p<0.05).*

Supplementing astaxanthin during tumor development in diet CA did not reduce tumor growth; in fact, tumors were larger in mice fed the CA diet than in those fed the C diet 42 and 49 days post-tumor cell inoculation (Figure 2). Final tumor weight was statistically similar in all treatments, with those in diet A being the smallest (Table I).

Cardiac function. The LA/Ao ratio was higher ($p<0.05$) in mice on diet A than those on diet C (Table II), and all were significantly above normal values (ratio <1.1). Astaxanthin (diets A and CA) feeding did not significantly affect LVIDd, LVIDs and FS, although mice on diets A and CA had numerically higher FS% than those on diet C, indicating better contractility. Plasma cTn I concentration was also not significantly different between treatments (Table II).

Astaxanthin feeding increased ($p<0.05$) cardiac MMP compared to mice fed the control diet (Table III). Unlike cardiac MMP, astaxanthin did not change lymphocyte MMP, although values tended to be higher in mice fed diet A.

Oxidative and inflammatory status. The concentration of blood GSHt was significantly lower ($p<0.05$) in mice fed CA and A diet compared to those on diet C (Table III), and values are comparable to reported concentrations (273.5±33.5 μmol/l). Changes in GSSG tended to be similar to GSHt but there was no significant treatment difference. Dietary astaxanthin had no effect on the GSH/GSSG ratio.

Table II. *Echocardiographic measurements and cardiac troponin I concentrations*.

Treatment	LA/Ao	Echocardiography		Plasma	
		LVIDd (mm)	LVIDs (mm)	FS (%)	cTn I (ng/ml)
C	1.60±0.08^{b}	3.12±0.06	1.71±0.08	44.9±2.6	4.09±1.15
CA	1.77±0.09^{ab}	3.17±0.08	1.55±0.12	51.5±2.6	3.31±1.12
A	1.91±0.12^{a}	3.16±0.04	1.68±0.07	46.6±2.1	5.51±1.16

Values are means±SEM, n=21/treatment. Different letters within a column denote statistical significance of differences, $p<0.05$. C, Control diet throughout the study; CA, control diet for weeks 1-5, astaxanthin diet weeks 6-8; and A, astaxanthin diet throughout the study.

Plasma SAA, an acute phase protein, was 2.5- to 3- fold lower ($p<0.05$) in mice supplemented with astaxanthin (diets CA and A) compared to unsupplemented controls (Table III).

Astaxanthin supplementation reduced plasma IL-1α compared to controls; concentrations were significantly lower ($p<0.05$) with diet A while that with diet CA also tended to be lower (Table III). In contrast, mice fed diet CA but not those fed A had significantly higher ($p<0.05$) concentrations of plasma IL-2, IL-6 and TNF-α.

Lymphocyte subsets. Compared to mice fed diet C, mice fed diet CA had higher ($p<0.05$) subpopulations of T-helper (Th) cells, whereas those fed diet A had higher ($p<0.05$) populations of NK cells (Table IV). No significant treatment

Table III. *MMP, antioxidant status and cytokines values.*

	Treatment		
	C	CA	A
Cardiac MMP	227±11[b]	248±7[a]	248±10[a]
Lymphocyte MMP	508±9	486±18	522±15
GSHt (μmol/l)	464±50[a]	274±39[b]	335±38[b]
GSSG (μmol/l)	62.4±10.3	33.6±6.0	47.4±10.5
GSHt/GSSG (μmol/l)	21.0±11.6	9.2±2.2	11.4±2.8
SAA (μg/ml)	98.94±42.8[a]	32.91±15.14[b]	38.42±16.1[b]
IL-1α (pg/ml)	22.8±19.7[a]	6.0±4.6a	0.47±0.48[b]
IL-2 (pg/ml)	0.011±0.001[a]	0.017±0.002[b]	0.006±0.002[a]
IL-6 (pg/ml)	149.7±65.6[b]	389.3±89.9[a]	205.7±60.6[ab]
TNF-α (pg/ml)	32.6±12.5[b]	191.0±81.8[a]	30.7±8.7[ab]
IFN-γ (pg/ml)	1.02±0.15[a]	1.22±0.23[a]	2.03±0.39[b]

Values are means±SEM, n=21. Data were analyzed by the Chi-square test. Different letters within a row denote statistical significance of differences, $p<0.05$. C, Control diet throughout the study; CA, control diet for weeks 1-5, astaxanthin diet weeks 6-8; and A, astaxanthin diet throughout the study.

Table IV. *Percentage of lymphocyte subsets in mice.*

	Treatment		
Lymphocyte subset	C	CA	A
Total T-cells (%)	32.6±4.0[ab]	42.4±5.4[a]	29.3±4.5[b]
Th cells (%)	13.8±2.4[b]	23.4±4.2[a]	15.2±3.4[ab]
Tc cells (%)	8.6±1.2	9.4±1.1	7.8±0.97
Th:Tc ratio	1.64±0.22	2.40±0.34	1.88±0.38
Total B-cells (%)	63.4±3.1	53.6±5.6	62.9±3.7
Activated B-cells (%)	1.94±0.12	1.69±0.13	2.14±0.14
NK cells (%)	12.6±1.5[b]	13.1±1.3[ab]	16.7±1.7[a]

Values are means±SEM, n=15/treatment. Different letters within a row denote statistical significance of differences, $p<0.05$. C, Control diet throughout the study; CA, control diet for weeks 1-5, astaxanthin diet weeks 6-8; and A, astaxanthin diet throughout the study.

differences were observed for populations of total T-, cytotoxic T- (Tc) and B-cells.

Discussion

Astaxanthin has been shown to inhibit breast, bladder, colon and oral cancer and sarcoma in rodents (2). In the current study, astaxanthin was introduced into the diet at two different stages: prior to tumor cell challenge (diet A), or 5 weeks after tumor cell inoculation (diet CA). Astaxanthin fed before tumor injection (diet A) significantly polonged tumor latency and suppressed tumor growth compared to unsupplemented mice or those given astaxanthin later (diet CA). This suggests the importance of adequate astaxanthin or antioxidant status prior to tumor cell challenge. This is supported by Prabhu *et al*. (14) who studied the beneficial effects of astaxanthin on DMH-induced colon cancer in rats. They reported that supplementation with astaxanthin at initiation of cancer significantly inhibited cancer progression, and to a lesser degree with astaxanthin supplementation after cancer induction.

Generally, tumors suppress certain immune cell populations, especially those of T- and NK cells which are key players in tumor immunity (15). The Th cells process tumor cell antigenic information, while the Tc cells work as effectors against tumor cells in an antigen-dependent manner. In this study, feeding astaxanthin after tumor challenge (diet CA) increased not only the percentage of Th cells, but also plasma concentrations of IL-2; such an immune response was not observed in mice fed astaxanthin throughout the study (diet A). Mice on diet A had significantly higher NK cell populations and higher IFN-γ than those on diet C. NK cells play a major role in immune surveillance and the cytotoxic activity of NK cells is enhanced by IFN-γ produced by NK and T-cells. Jyonouchi *et al*. (4) demonstrated that the anticancer action of astaxanthin was mediated through the activation of Tc cells and IFN-γ production in mice *in vivo* while Kurihara *et al*. (6) reported that astaxanthin restored NK cell populations during stress-induced immune suppression.

The persistent expression of inflammatory cytokines such as IL-1α, IL-6 and TNF-α increases cancer progression and cachexia (16, 17). Although plasma IL-1α concentrations were significantly reduced with astaxanthin supplementation, plasma IL-6 and TNF-α concentrations increased only in mice fed astaxanthin after tumor challenge. IL-1α is involved in local tumor invasiveness and angiogenesis in breast cancer (18), while IL-6 and TNF are associated with systemic inflammation that fuels cancer cell growth (16). High plasma IL-6 and TNF-α concentrations in the CA group were consistent with rapid tumor growth; in addition, mice in the CA group also had low activated B-cell populations. Plasma SAA, the acute phase protein produced in the liver in response to inflammatory stimuli such as IL-6, was low in the CA group even though plasma IL-6 was high, possibly due to liver dysfunction.

Dietary astaxanthin significantly increased heart MMP in astaxanthin-supplemented mice, suggesting that astaxanthin may prevent mitochondrial dysfunction and reduce oxidative stress. The protective effect of astaxanthin in improving impaired mitochondrial function in a human neuroblastoma cell line has also been demonstrated (19).

The ratio of LA/Ao, an index of the size of the left atrium, is normally <1.1 in rodents (20). All tumor-bearing mice in this study had higher LA/Ao and LVIDd (>3.0 mm) and FS (>44%), compared to healthy mice (LVIDd: 2.6 mm, FS:

35%). The increased chamber sizes and cardiac contractility are likely due to increased blood volume resulting from tumor growth. Left atrial enlargement was apparent in all the mice, especially those fed diet A, suggesting the presence of additional left ventricular diastolic dysfunction. Left ventricular stiffness through fibrosis, fat, other cellular deposition, or concentric hypertrophy may cause this left ventricular diastolic dysfunction (21).

In summary, astaxanthin fed prior to tumor initiation suppressed tumor growth, increased NK cell populations, and increased plasma IFN-γ concentration in mice. In contrast, astaxanthin fed after tumor initiation resulted in more rapid tumor growth and elevated palsma inflammatory cytokines IL-6 and TNF-α, thus emphasizing the importance of antioxidant status prior to disease initiation.

Acknowledgements

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

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Received December 17, 2009
Revised April 16, 2010
Accepted April 23, 2010