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Running head: Astaxanthin on mitochondria function in dogs

Astaxanthin modulates age-associated mitochondrial dysfunction in healthy dogs¹

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ABSTRACT: Young (2.97 ± 0.01 yr; 8.16 ± 0.15 kg BW) and geriatric (10.71 ± 0.01 yr; 9.46 ± 0.18 kg BW) healthy female Beagle dogs ($n = 14/\text{age group}$) were fed 0 or 20 mg astaxanthin daily for 16 wk to examine modulation of mitochondrial function. Fasted blood was sampled on wk 0, 8, and 16. Mitochondria membrane permeability, ATP production, cytochrome c oxidase/reductase, and number were assessed in leukocytes, while astaxanthin uptake, glutathione, superoxide dismutase, nitric oxide, 8-hydroxy-2'-deoxyguanosine, 8-isoprostane and protein carbonyl were measured in plasma. Aging increased ($P < 0.05$) complex III cytochrome c oxidoreductase, but decreased ($P < 0.05$) 8-hydroxy-2'-deoxyguanosine and protein carbonyl. Mitochondrial function improved in both young and geriatric dogs by increasing ($P < 0.05$) ATP production, mitochondria mass, and cytochrome c oxidoreductase activity, especially in geriatric dogs compared with young dogs. Astaxanthin feeding also increased ($P < 0.05$) the reduced glutathione to oxidized glutathione ratio in young dogs, and decreased ($P < 0.05$) nitric oxide in both young and geriatric dogs. Dietary astaxanthin improved mitochondrial function in blood leukocytes, most likely by alleviating oxidative damage to cellular DNA and protein.

Key words: astaxanthin, canine, inflammation, leukocytes, mitochondria

INTRODUCTION

Aging is associated with a decline in immune response and increase in the incidence of cancer, arthritis, cardiovascular, and eye diseases (Grubeck-Loebenstein, 1997). This impairment of immune function results from cell damage by reactive oxygen species (**ROS**) and reactive nitrogen species (**RNS**; Ames et al., 1993). Excessive ROS and RNS can destroy cellular macromolecules such as membrane lipids (Tahara et al., 2001), proteins (Barja, 2002), and mitochondria DNA (Shigenaga et al., 1994). Mitochondria are the most important source of

oxidative products generated during aerobic respiration (Shigenaga et al., 1994). The rate of oxidant production by mitochondria is inversely correlated with maximum life span of a species (Sastre et al., 2002). In fact, mitochondria function declines with age (Lenaz et al., 2002; Nicholls, 2002).

Antioxidants effectively quench harmful oxidative cellular products associated with age-related diseases. The oxycarotenoid astaxanthin is a potent antioxidant (Martin et al., 1999), with an antioxidant activity greater than that of β -carotene, α -carotene, lutein (Naguib, 2000), and α -tocopherol (Di Mascio et al., 1990). Astaxanthin spans the cell membrane bilayer (Shibata et al., 2001) where it decreases membrane fluidity (Barros et al., 2001) and reduces lipid peroxidation (Nakano et al., 1999). Astaxanthin enhanced cell-mediated and humoral immune response in dogs and cats (Chew et al., 2004; Chew et al., 2011), inhibited mammary tumor growth (Chew et al., 1999a), reduced *Helicobacter pylori* bacterial load and gastric inflammation (Bennedsen et al., 1999), and increased immune response (Chew et al., 1999b) in mice. Dogs readily absorb astaxanthin into the blood and concentrate it in leukocyte mitochondria (Park et al., 2010).

Therefore, we hypothesized that dietary astaxanthin will decrease oxidative damage to the mitochondrial lipids, proteins, and DNA of immune cells, and maintain mitochondrial efficiency and activity in not only geriatric, but possibly also, young dogs. A study was conducted to determine the effect of daily administration of astaxanthin on modulation of mitochondrial function in young and geriatric healthy female Beagle dogs.

MATERIALS AND METHODS

All procedures were approved by the Institutional Review Board (IACUC #3374) of Washington State University.

Animals and Study Design

Young (2.97 ± 0.01 yr; 8.16 ± 0.15 kg BW) and geriatric (10.71 ± 0.01 yr; 9.46 ± 0.18 kg BW) female Beagle dogs ($n = 14/\text{age group}$) were randomly assigned to feeding 0 (control) or 20 mg astaxanthin daily for 16 wk. This level of astaxanthin supplementation elicited optimum immune-enhancing response in dogs (Chew et al., 2011), and was adequately taken up in cell organelles (Park et al., 2010). Astaxanthin was incorporated into a commercial diet after kibble extrusion (P&G Pet Care, Lewisburg, OH) to provide 20 mg astaxanthin/200 g food (as-fed); all dogs were fed twice a day (100 g/feeding) throughout the experiment to maintain BW. The diet met or exceeded the requirements for all essential nutrients and contained: 85.3g moisture, 275.8 g CP, 60.7 g ash, 115.0 g fat, 9.9 g Ca, 9.3 g P, 21.3 g crude fiber, and 18,914 kJ GE/kg, and the n-6 to n-3 fatty acid ratio was 7.9. Dogs were housed in 2×3 m pens in a temperature (20 to 22°C)- and light (14 h light)-controlled facility. Starting at 0700 h on sampling days, fasted blood (20 mL) was collected by jugular venipuncture into heparinized vacutainers at wk 0, 8, and 16. Blood tubes were stored on ice during sample collection, followed immediately by processing.

Peripheral Blood Mononuclear Cell Isolation

Peripheral blood mononuclear cells (**PBMC**) were isolated by gradient centrifugation as previously described (Chew and Park, 2009). Contaminating red blood cells were lysed by hypotonic shock. The resulting cell pellet was resuspended in 1 mL hypoosmolar cavitation buffer [100 mmol/L of sucrose, 1 mmol/L of ethylene glycol tetraacetic acid, 220 mmol/L of 3-(N-morpholino) propanesulfonic acid, 4-morpholinepropanesulfonic acid, pH 7.4, 0.1% BSA] containing 10 mmol/L of triethanolamine (Sigma-Aldrich Co., St. Louis, MO), 5% Percoll

(Amersham, Piscataway, NJ), and protease inhibitors (10 μ mmol/L of aprotinin, pepstatin, leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride; Sigma-Aldrich Co.), and kept on ice.

Mitochondria Preparation

The cell suspension was transferred to a pre-chilled chamber (Mini-bomb Cell Disruption Chamber; Kimble-Kontes, Vineland, NJ) that was maintained at 4°C throughout the cavitation procedure (Gottlieb and Adachi, 2000). The chamber was pressurized to 2,896 kPa with nitrogen gas and allowed to equilibrate for 20 min. Sample was collected drop-wise into a glass tube, and cavitation was repeated for 5 min after rinsing with 0.5 mL cavitation buffer. We obtained > 95% cell disruption using this 2-step cavitation procedure. The nuclei and unbroken cells were removed from the suspension by centrifugation at $760 \times g$ for 10 min at 4°C. The supernatant was transferred to a clean microfuge tube and centrifuged at $3,500 \times g$ for 15 min at 4°C; the resulting pellet contained > 90% intact mitochondria, with minimal lysosomal and peroximal contamination, as verified with electron microscopy. The final pellet was resuspended in 50 μ mol/L iso-osmolar storage buffer [20 mmol/L of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 300 mmol/ of L glucose, and 0.1 mmol/L of ethylene glycol tetraacetic acid; Sigma-Aldrich Co.] containing protease inhibitors, and stored in 2 aliquots at -80°C until analysis.

Mitochondria Content Determination by Confocal Microscopy

Isolated PBMC (1×10^6 cells) were stained with the mitochondrion-selective probe (MitoTracker Red CMXRos; Molecular Probes, Carlsbad, CA) at 100 nmol/L in PBS (8 mM Na_2HPO_4 , 2 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, and 150 mM NaCl; pH 7.3), fixed with 3.7% formaldehyde, and the nuclei counterstained (1 μ mol/L SYTOX Green; Molecular Probes, Carlsbad, CA) following the manufacturer's protocol. After washing, the cells were resuspended in saline and

air-dried onto positively-charged slides. Slides were cover slipped with anti-fade agent (ProLong Gold; Molecular Probes), sealed, and stored at -20°C . Three representative fields were evaluated (Zeiss 510 Meta-system CSLM, Zeiss Axiovert 200M, and Apochromat 63X/1.4 DICIII; Carl Zeiss Microimaging Inc., Thornwood, NY). Fluorochromes were excited with Argon/2 (488) and HeNe1 (543) lasers, configuration was multitrack FITC/Rhodamine, and mitochondria content determined by image analysis (Scion Image/NIH Image; <http://www.nist.gov/lispix/imlab/prelim/dnld.html>). Mitochondrial content was expressed as the ratio of fluorescence density (red fluorescence) and the nuclear area.

Membrane Permeability and ATP Production

Isolated PBMC (0.5×10^6 cells) were incubated with 2 nmol/L CMXRos following a pre-incubation in the presence or absence of 400 $\mu\text{mol/L}$ carbonyl cyanide *m*-chlorophenylhydrazone (Sigma-Aldrich Co.) to determine membrane fluidity (Zamzami et al., 2000). Cell populations were then evaluated (FACSCaliber; BD Biosciences, San Jose, CA). The PBMC from blood collected on wk 16 were evaluated for ATP content using a luminescence assay (PerkinElmer, Waltham, MA). Briefly, 1×10^6 cells in PBS were pipetted into black plates (OptiClear Plates; Packard Bioscience, Meriden, CT) and lysis buffer added. Following incubation at 37°C on a rotating-platform, luciferase/luciferin substrate solution was added, plates were incubated, dark-adapted for 10 min, and counted for 5 s.

Cytochrome c Oxidoreductase (Complex III)

Complex III activity was measured by monitoring the reduction of cytochrome c at 550 nm (Birch-Machin et al., 1994; Krahenbuhl et al., 1994). Briefly, decylubiquinol was prepared by reducing decylubiquinone (Sigma-Aldrich Co.) with sodium bromide, followed by organic solvent extraction as previously described (Zheng et al., 1990; Krahenbuhl et al., 1991). The

stock solution concentration was determined by measuring the absorbance difference between 247 and 272 nm, and using an extinction coefficient of $8 \text{ mM}^{-1} \times \text{cm}^{-1}$ for calculation ($8 \text{ mM} = 2.58 \text{ mg/mL}$). Ferricytochrome c ($60 \text{ }\mu\text{mol/L}$; Sigma-Aldrich Co.) was prepared in reaction buffer (50 mmol/L of potassium phosphate, pH 7.4, 0.1 mmol/L of ethylenediaminetetraacetic acid, 0.2% BSA, and 3 mmol/L of sodium azide; Sigma-Aldrich Co.), vortexed, and kept on ice. Non-inhibitable activity was determined by adding 10 mg/L of antimycin A (*Streptomyces sp.*; Sigma-Aldrich Co.) to a reaction tube and subtracting the non-specific activity from the net activities of sample assay tubes. Net activity was determined by measuring the reduction of cytochrome c at 550 nm with a reference wavelength of 580 nm ($\epsilon = 19 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

Cytochrome c Oxidase (Complex IV)

Complex IV activity was determined by measuring oxidation of cytochrome c at 550 nm with a reference wavelength of 580 nm (CYTOC-OX; Sigma-Aldrich Co.). Unit of activity was defined as oxidation of $1.0 \text{ }\mu\text{mol}$ ferrocyanochrome c/min at pH 7.0 at 25°C , and cytochrome c oxidase measured was standardized per mg protein.

Oxidative Stress and Inflammation Biomarkers

Reduced (**GSH**) and oxidized (**GSSG**) glutathione levels in blood reflect oxidative stress, and were measured using a colorimetric assay (BIOXYTECH GSH/GSSG-412; OXIS International, Inc., Beverly Hills, CA) with the following modifications. Samples were prepared as described in the assay protocol, and kept on ice until assay. An ELISA plate reader (BioTek μ Quant, BioTek, Winooski, VT) was used to determine the change in absorbance at 412 nm for 3 min. Sample, standard, chromagen, enzyme, and nicotinamide adenine dinucleotide phosphate-oxidase volumes were adjusted accordingly to utilize a microtiter plate format. The detection

limit for total GSH is 0.54 $\mu\text{mol/L}$, and intra-assay CV were 0.96 and 6.45% in whole blood for GSH and GSSG, respectively.

Plasma superoxide dismutase (**SOD**) is an important component of antioxidant defense mechanisms. Total SOD was measured using a colorimetric assay (Superoxide Dismutase Assay Kit; Cayman Chemical, Ann Arbor, MI). Plasma samples were assayed concurrently with SOD standards, and concentrations were calculated based on linear regression of the standard curve and linearized rate for each sample.

Nitric oxide (**NO**) is an important cellular signaling molecule and reflects inflammatory status. Total NO formation was determined using a commercial assay kit (Nitrate/Nitrite Colorimetric Assay Kit LDH Method; Assay Designs, Ann Arbor, MI). Plasma concentrations were determined using a nitrate standard curve.

Lipid peroxidation was assessed by measuring total plasma 8-isoprostane using a competitive ELISA (8-Isoprostane EIA Kit; Cayman Chemical). Following alkaline hydrolysis of the samples, batch purifications of 8-isoprostane were prepared using affinity sorbent as described in the manufacturer's protocol. Concentrations were determined using an 8-isoprostane standard curve.

Protein carbonyl plasma levels, which are indicative of protein oxidation, were determined using a colorimetric assay (Protein Carbonyl Assay kit; Cayman Chemical). Concentrations were determined using the extinction coefficient of 2,4-dinitrophenylhydrazine at 370 nm.

The DNA oxidative damage was also assessed. Prior to assay, plasma was centrifuged at $13,700 \times g$ for 3 min at 4°C in a microfuge to remove particulates; the supernatant was used to quantify levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) by competitive ELISA

(BIOXYTECH 8-OHdG-EIA Kit; OXIS International, Beverly Hills, CA). Astaxanthin content in plasma was analyzed using reverse-phase HPLC as previously described (Park et al., 1998).

Statistical Analysis

Data were analyzed by the GLM procedure of SAS (SAS Inst. Inc. Cary, NC). Normality was analyzed using Shapiro-Wilkes test. The statistical model included age, treatment, week, BW, age x week, and treatment x week. Final statistical analysis was by ANCOVA, using wk 0 as the covariate. Means were considered significant at $P < 0.05$.

RESULTS

Plasma Astaxanthin

Astaxanthin was not detectable in any baseline plasma samples, and was also not detectable in plasma of young or geriatric dogs fed the control diet throughout the study, as expected. Maximal concentrations of astaxanthin in plasma from dogs receiving the treatment diet were observed by wk 8, reaching 64.0 ± 4.0 and 53.4 ± 6.9 nmol/L in geriatric and young dogs, respectively. These concentrations were maintained through wk 16 (58.3 ± 5.1 and 51.4 ± 5.9 nmol/L in geriatric and young dogs, respectively).

Oxidative Stress and Inflammation Biomarkers

Overall, dietary astaxanthin suppressed ($P < 0.01$) plasma 8-OHdG concentrations in dogs (7.64 ± 0.47 ng/mL) compared with control (9.05 ± 0.30 ng/mL; Table 1). This inhibition was statistically significant on wk 16 in the geriatric dogs but not in young dogs. There was no overall age effect on 8-OHdG concentrations. Plasma protein carbonyl concentrations were elevated at all time points ($P < 0.05$) in geriatric dogs compared with young dogs. On wk 8, astaxanthin supplementation decreased ($P < 0.01$) plasma protein carbonyl concentrations in

geriatric (35%) and young (45%) dogs when compared with unsupplemented dogs. This inhibitory effect of astaxanthin was maintained through wk 16. In contrast, there was no age or astaxanthin effect on plasma concentrations of 8-isoprostane. Astaxanthin increased ($P < 0.05$) blood SOD concentrations in old dogs on wk 16 (Table 1), Young dogs tended to have greater plasma SOD than geriatric dogs on wk 16. Total NO concentration was similar between young and geriatric dogs (Table 1). Astaxanthin inhibited ($P < 0.01$) plasma NO concentration on wk 16 in both age groups.

Blood GSH and GSSG concentrations (overall average, $1,477 \pm 49$ and 69 ± 7 $\mu\text{mol/L}$, respectively) were not affected by astaxanthin supplementation; however, concentrations of GSSH in geriatric dogs (89.2 ± 12.1 $\mu\text{mol/L}$) tended to be greater than in young dogs (49 ± 6.1 $\mu\text{mol/L}$; Figure 1). The ratio of GSH to GSSG increased ($P < 0.05$) in young dogs fed astaxanthin compared with those fed the control diet, although a similar diet effect was not observed in geriatric dogs.

Leukocyte Mitochondrial Content and Membrane Permeability

Although astaxanthin feeding did not influence mitochondrial mass in leukocyte mitochondria in young dogs, it increased ($P < 0.05$) mitochondria mass in geriatric dogs compared with those fed the control diet (Table 2). Age did not affect leukocyte mitochondrial content. There was no age or astaxanthin supplementation effect with mitochondria membrane permeability.

Mitochondria ATP Production

Astaxanthin feeding increased ($P < 0.05$) mitochondria ATP production by 12 to 14% in both young and geriatric dogs compared with those fed the control diet (Figure 2). There was no age effect.

Mitochondria Cytochrome c Oxidoreductase and Oxidase

Although cytochrome c oxidoreductase (complex III) production by the mitochondria was similar in young and old dogs fed the control diet throughout the study, geriatric dogs fed astaxanthin had 570-600% higher production ($P < 0.001$) by wk 8 when compared with baseline values (Table 2). In contrast, young dogs fed astaxanthin did not exhibit similar changes. Neither age nor astaxanthin supplementation influenced complex IV cytochrome c oxidase activity.

DISCUSSION

Mitochondria are particularly vulnerable to oxidative attack (Szeto, 2006), and when excessive ROS and RNS overwhelm the body's antioxidant capacity, exogenous antioxidants such as astaxanthin are needed to remove these harmful products. Results of the present experiment indicate that aging is associated with increased mitochondrial oxidative damage (increased GSSG and protein carbonyl, and decreased GSH to GSSG ratio), astaxanthin supplementation alleviates these unfavorable outcomes by decreasing mitochondrial oxidative and inflammatory damage (decreased GSSG, NO, protein carbonyl, and 8-OHdG; increased GSH to GSSG ratio) and by improving mitochondrial function through increased ATP production, mitochondrial mass, and complex III cytochrome c oxidoreductase.

Results of the present experiment indicated that astaxanthin, when fed to young and geriatric dogs, improved leukocyte mitochondria function. In addition, although ATP normally declines with age, astaxanthin increased ATP production in both young and geriatric dogs. This represents the first evidence for enhanced ATP production induced by dietary astaxanthin. Decline in leukocyte mitochondrial ATP generation (produced through oxidative phosphorylation) clearly threatens the integrity of the immune response and subsequent health.

Complexes I, III, and IV are involved in electron pumping and the subsequent generation of the inwardly-directed proton gradient across the inner mitochondrial membrane. It has been shown that age can inhibit the electron transport chain at complex III, which generates ROS (Barja, 1999). Complex I, II, and III are produced by the mitochondria, while complex IV is produced in the cytosol and subsequently transported into the mitochondria. In this study, astaxanthin increased complex III activity; the response to astaxanthin feeding on wk 16 was 2.2 times greater in geriatric dogs compared with young dogs. There was no effect of age or astaxanthin supplementation on complex IV activity in this study, indicating that the major effect of astaxanthin on the electron transport chain is on complex III and the subsequent reduction of ROS.

In this study, age and dietary astaxanthin had no influence on mitochondria membrane potential. However, mitochondrial mass was increased in astaxanthin supplemented dogs, regardless of age. The average volume per mitochondrion and the number of functionally active mitochondria per cell decreased with age, which is consistent with other studies (Markowska et al., 1994; Ledda et al., 2001). Astaxanthin had no effect on nuclei area, mean volume or area/nuclei. This change in mitochondria may partially explain the increased ATP production observed in geriatric dogs. Similar fluorescent-labeling methodologies have been used to determine cellular mitochondrial content associated with Alzheimer's disease (de la Monte et al., 2000) and with other neurodegenerative disorders (Foster et al., 2006). Besides ATP production, mitochondria also serve a broad range of functions including the regulation of cytosolic Ca^{+2} levels, intracellular pH and apoptosis (Foster et al., 2006). Therefore, our study demonstrated that astaxanthin supplementation increases ATP production by leukocyte mitochondria in dogs, most likely through increased complex III and cellular mitochondrial content. Because the

mitochondria are the major source of ROS, astaxanthin most likely serves to quench excess harmful ROS, thereby alleviating oxidative and/or inflammatory damage and maintaining cellular integrity.

In fact our study supports this contention. Astaxanthin supplementation increased the GSH to GSSG ratio in whole blood of both young and geriatric dogs; the response was greater in young dogs compared with geriatric. Glutathione reductase catalyzes the reduction of the oxidized glutathione GSSG to the glutathione GSH, and thereby functions as an antioxidant; its reducing and nucleophilic properties play an important role in the antioxidant system and metabolic pathways of aerobic cells.

Production of NO was inhibited by astaxanthin to the same degree in both the young and geriatric dogs compared with unsupplemented controls. The NO produced in response to inflammatory or mitogenic stimuli reacts with superoxide anion to produce the highly toxic peroxynitrite. Astaxanthin has been reported to inhibit the production of inflammatory mediators such as NO, prostaglandin E₂, tumor necrosis factor- α , and interleukin-1 β in macrophages, possibly by suppressing upstream I- κ B kinase activity, thereby, blocking NF- κ B activation (Pashkow et al., 2008). As a molecular link in the inflammatory process, NF- κ B is frequently found to be activated at sites of inflammation in diverse diseases.

The inner mitochondrial membrane is highly proteinaceous (Van Remmen and Richardson, 2001). Damage to mitochondrial proteins can be a direct result of ROS attack or a consequence of lipid peroxidation (Barja, 2002). Mitochondrial protein damage increases with age, resulting in decreased mitochondrial efficiency and reduce cellular energy production (Shigenaga et al., 1994). Mitochondrial DNA is more prone to oxidative damage than nuclear DNA (Shigenaga et al., 1994), and mitochondrial DNA damage has also been reported to

increase with age (Mori et al., 1998; Hosokawa et al., 2000). Because mitochondrial DNA is central to the machinery that controls the mitochondria and, therefore, cell metabolism, maintaining mitochondria DNA integrity is paramount for normal cell function. In this study, we demonstrated that dietary astaxanthin inhibited cellular DNA damage (decreased 8-OHdG) and protein damage (decreased protein carbonyl).

On the other hand, astaxanthin did not influence plasma 8-isoprostane concentrations. Fatty acids in the lipid bilayer of the mitochondrial membrane are prone to peroxidation because of their close proximity to the electron transport chain. Damage to these macromolecules tends to reduce membrane fluidity and consequently alter membrane function (Van Remmen and Richardson, 2001). Even though lipid peroxidation in mitochondrial membranes increases with age (Bagchi et al., 1996; Melov, 2002), this age effect in dogs was not apparent in this study. The lack of the astaxanthin effect on lipid peroxidation is in contrast to others who demonstrated a reduced oil-induced oxidative stress, lowered serum lipid peroxides (Nakano et al., 1999) and transaminase activities (Nakano et al., 1995) in fish. In addition, astaxanthin reduced LDL oxidation in humans (Iwamoto et al., 2000) and increased plasma HDL in rats (Murillo, 1992).

In this study, astaxanthin was rapidly absorbed into the blood by both young and old dogs, with a steady-state concentration by 8 wk. This is in general agreement with previous studies in dogs and cats with ready absorption of astaxanthin into the blood, and the highest proportion of total astaxanthin uptake in immune cell mitochondria (Park et al., 2010). Others have also detected the highest concentration of astaxanthin in the mitochondria of human neuroblastoma dopaminergic cells (Liu et al., 2009) and in the human mesangial cell line (Manabe et al., 2008). In addition, the protective characteristics of astaxanthin can lead to improvement of immune function. Improved immune response, both cell-mediated as well as

humoral humoral (Chew et al., 2004; Chew et al., 2011), was observed, as was stimulation of mitogen-induced lymphoblastogenesis and enhanced cytotoxic activity in murine splenocytes (Chew et al., 1999b). Others have reported that astaxanthin is more effective than β -carotene and lutein in enhancing T cell antigen-dependent antibody production in mice (Jyonouchi et al., 1994). Astaxanthin partially restored immunosenescence, an age-associated decline in immune function (Grubeck-Loebenstein, 1997) by increasing antibody production in humans (Jyonouchi et al., 1995). Dietary β -carotene prevented an age-related decline in immune health in dogs (Massimino et al., 2003). In fact, β -carotene improved certain cell-mediated immune response in geriatric dogs to levels observed in young dogs.

Astaxanthin's unique structure allows it to span biological membranes, with the polar end on the outside of the membrane and the lipophilic polyene chain sitting within the bilayer (Miki, 1991). This precise transmembrane alignment allows astaxanthin to preserve the membrane structure (McNulty et al., 2007), decrease membrane fluidity (Barros et al., 2001), and consequently function as an antioxidant in preventing lipid peroxidation (Subczynski et al., 1996). Also, it likely provides proximity to cofactors, such as ascorbate; the latter acts as a reservoir for accepting radical cations, thereby recharging the electron transfer capacity (May, 1999).

Therefore, astaxanthin supplementation reduced oxidative and inflammatory damage, and consequently enhanced mitochondrial function in healthy young and geriatric dogs. The heightened response of certain mitochondrial function from astaxanthin supplementation was greater in geriatric dogs than in young dogs. In conclusion, astaxanthin is a naturally occurring pigment that can potentially be used to alleviate age-related oxidative and inflammatory damage and enhance mitochondria function.

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Table 1. Changes in oxidative stress biomarkers in young and geriatric dogs fed 0 or 20 mg astaxanthin for 16 wk

| Item | Young | | Geriatric | |
|--|-------------|--------------|--------------|--------------|
| | Control | Astaxanthin | Control | Astaxanthin |
| Plasma 8-hydroxy-2'-deoxyguanosine, nmol/L | | | | |
| wk 0 | 8.51 ± 0.92 | 8.83 ± 1.27 | 10.24 ± 1.07 | 9.18 ± 1.28 |
| wk 8 | 9.06 ± 0.88 | 7.49 ± 1.04 | 9.38 ± 1.02 | 7.04 ± 1.12 |
| wk 16 | 8.09 ± 0.77 | 7.12 ± 0.48 | 9.02 ± 0.61 | 6.19 ± 0.79* |
| Plasma protein carbonyl, nmol/L | | | | |
| wk 0 | 17.8 ± 1.3 | 16.8 ± 1.8 | 23.7 ± 3.3 | 20.2 ± 1.6 |
| wk 8 | 15.3 ± 1.3 | 8.6 ± 0.5* | 20.1 ± 1.4 | 13.3 ± 1.9* |
| wk 16 | 14.8 ± 1.0 | 11.5 ± 0.9* | 21.8 ± 1.2 | 16.0 ± 2.1* |
| Plasma 8-isoprostane, pg/mL | | | | |
| wk 0 | 37.5 ± 4.2 | 41.6 ± 2.3 | 43.5 ± 5.1 | 36.9 ± 2.7 |
| wk 8 | 43.5 ± 3.7 | 44.9 ± 3.1 | 42.1 ± 3.7 | 43.6 ± 3.1 |
| wk 16 | 38.9 ± 3.5 | 42.9 ± 4.6 | 36.1 ± 2.5 | 41.3 ± 4.0 |
| Plasma superoxide dismutase, U/mL | | | | |
| wk 0 | 1.81 ± 0.11 | 1.73 ± 0.14 | 1.45 ± 0.11 | 1.43 ± 0.21 |
| wk 8 | 1.77 ± 0.21 | 1.83 ± 0.12 | 1.81 ± 0.20 | 1.99 ± 0.32 |
| wk 16 | 2.01 ± 0.36 | 1.99 ± 0.07 | 1.49 ± 0.12 | 1.64 ± 0.31* |
| Plasma nitric oxide, U/mL | | | | |
| wk 0 | 5.52 ± 0.62 | 6.02 ± 0.63 | 5.27 ± 0.51 | 5.06 ± 0.61 |
| wk 8 | 5.21 ± 0.56 | 4.77 ± 0.60 | 4.91 ± 0.62 | 3.92 ± 0.26 |
| wk 16 | 5.52 ± 0.46 | 3.53 ± 0.35* | 5.10 ± 0.55 | 3.82 ± 0.34* |

*Within age group and week, different from control ($P < 0.05$).

Table 2. Changes in leukocyte membrane permeability, mitochondrial content, Complex III and IV in young and geriatric dogs fed 0 or 20 mg astaxanthin for 16 wk

| Item | Young | | Geriatric | |
|---|-------------|-------------|-------------|--------------|
| | Control | Astaxanthin | Control | Astaxanthin |
| Leukocyte membrane permeability, fluorescence intensity | | | | |
| wk 0 | 1,157 ± 57 | 1,155 ± 51 | 1,342 ± 71 | 1,236 ± 66 |
| wk 8 | 1,376 ± 103 | 1,313 ± 101 | 1,269 ± 145 | 1,323 ± 85 |
| wk16 | 1,781 ± 104 | 1,776 ± 98 | 1,829 ± 47 | 1,883 ± 124 |
| Leukocyte mitochondrial content, mitochondrial density:nuclear area | | | | |
| wk 0 | 130 ± 5 | 134 ± 5 | 134 ± 5 | 136 ± 5 |
| wk 16 | 167 ± 10 | 185 ± 12 | 143 ± 14 | 204 ± 11* |
| Leukocyte mitochondria Complex III, U/g protein | | | | |
| wk 0 | 2.06 ± 0.84 | 3.63 ± 1.74 | 0.77 ± 0.29 | 0.99 ± 0.19 |
| wk 8 | 2.00 ± 0.50 | 6.53 ± 1.10 | 1.52 ± 0.28 | 5.61 ± 1.93* |
| wk 16 | 1.37 ± 0.22 | 2.67 ± 0.45 | 0.96 ± 0.10 | 5.93 ± 2.65* |
| Leukocyte mitochondria Complex IV, U/g protein | | | | |
| wk 0 | 6.49 ± 2.68 | 7.15 ± 3.32 | 7.84 ± 3.09 | 8.62 ± 4.01 |
| wk 8 | 5.61 ± 1.91 | 4.42 ± 2.32 | 5.49 ± 2.48 | 5.08 ± 2.04 |
| wk 16 | 5.56 ± 1.89 | 6.77 ± 2.00 | 6.91 ± 2.27 | 4.45 ± 1.93 |

*Within age group and week, different from control ($P < 0.05$).

Figure 1. Reduced (GSH) to oxidized (GSSG) glutathione in whole blood of young and geriatric dogs fed 0 or 20 mg astaxanthin for 16 wk. Values are means \pm SEM. Data were analyzed by ANCOVA (n = 14). *Within age group and week, different from control ($P < 0.05$).

Figure 2. Leukocyte mitochondrial ATP production on wk 16 in young and geriatric dogs fed 0 or 20 mg astaxanthin for 16 wk. Values are means \pm SEM. Data were analyzed by ANCOVA (n = 14). *Within age group and week, different from control ($P < 0.05$).

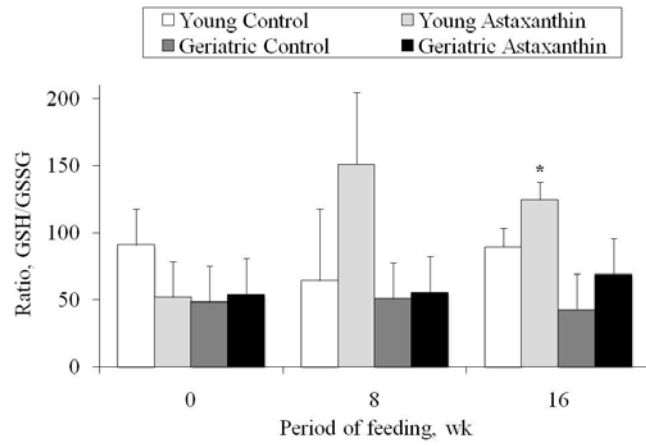


Figure 1.

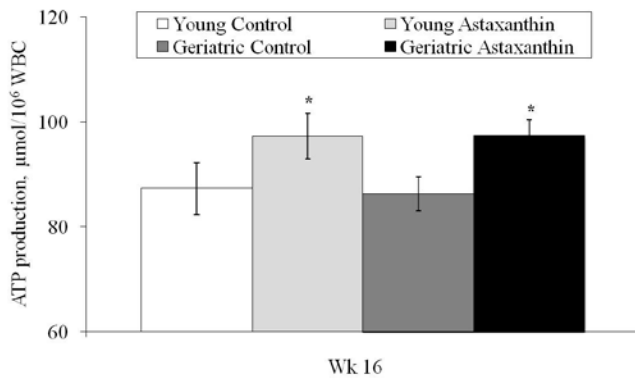


Figure 2.