

Inhibition of Low-Density Lipoprotein Oxidation by Astaxanthin

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Marine animals produce astaxanthin which is a carotenoid and antioxidant. In this study we determined the *in vitro* and *ex vivo* effects of astaxanthin on LDL oxidation. The oxidation of LDL was measured in a 1 ml reaction system consisting of increasing concentrations of astaxanthin (12.5, 25.0, 50.0 $\mu\text{g/ml}$), 400 μM V-70 (2, 2'-azobis(4-methoxy-2, 4-dimethylvaleronitrile)), and LDL (70 $\mu\text{g/ml}$ protein). Astaxanthin dose, dependently significantly prolonged the oxidation lag time (31.5, 45.4, 65.0 min) compared with the control (19.9 min). For the *ex vivo* study 24 volunteers (mean age 28.2 [SD 7.8] years) consumed astaxanthin at doses of 1.8, 3.6, 14.4 and 21.6 mg per day for 14 days. No other changes were made in the diet. Fasting venous blood samples were taken at days 0, +14. LDL lag time was longer (5.0, 26.2, 42.3 and 30.7% respectively) compared with day 0 after consuming astaxanthin at doses of 1.8, 3.6, 14.4 and 21.6 mg for 14 days compared with day 0, but there was no difference in oxidation of LDL between day 0 (lag time 59.9 ± 7.2 min) and day 14 (57.2 ± 6.0 min) in the control group. Our results provide evidence that consumption of marine animals producing astaxanthin inhibits LDL oxidation and possibly therefore contributes to the prevention of atherosclerosis. *J Atheroscler Thromb*, 2000; 7: 216-222.

Key words : Astaxanthin, LDL oxidation, Lag time, Antioxidant

Introduction

Oxidative modification of low-density lipoprotein (LDL) has been implicated in the pathogenesis of atherosclerosis (1, 2). The rapid uptake of oxidative modified LDL via a scavenger receptor leads to the formation of foam cells, and oxidized LDL also has a number of other atherogenic properties (3-5).

One approach to reduce the atherogenicity associated with modified LDL might be the use of antioxidants to prevent the formation of oxidized LDL. The free radical-

mediated oxidation of LDL proceeds to lipid peroxidation, which is the autooxidation of the polyunsaturated fatty acid chains of lipids by a radical chain reaction (6). The diet itself, however, contains several antioxidants that potentially could inhibit the oxidation of LDL.

Natural antioxidants, which include α -tocopherol, β -tocopherol, lycopene and γ -carotene are preferentially oxidized before the oxidation of polyunsaturated fatty acids (7). The peroxidation of LDL lipids is preceded by initial oxidation of the lipoprotein-associated vitamin E and carotenoids (8, 9). Carotenoid functions such as photoprotection (10), enhancement of gap-junction communication (11), quenching of singlet oxygen or radical-trapping antioxidant properties (12-16) have all been thoroughly described in the literature. The action of carotenoids as antioxidants has recently attracted widespread

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attention following observations both *in vitro* and *ex vivo* (17).

Astaxanthin is a carotenoid pigment found in marine animals (18). Astaxanthin of the xanthophyll group possesses no provitamin A activity in contrast to α -carotene. Amongst the diverse biological functions of astaxanthin are involvement in cancer prevention (19), enhancement of immune responses (20) and free radical quenching (21-23). Miki (22) have also revealed that astaxanthin shows a stronger quenching activity against singlet oxygen, approximately one-hundred times stronger than that of α -tocopherol.

Many studies have shown that astaxanthin exhibits protective effects against lipid peroxidation induced by free radicals or oxygen, in organic solution, liposomes, liver microsomes and biological membranes (24-29). Kurashige *et al.* (29) demonstrated that astaxanthin protects the mitochondria of vitamin E-deficient rats from damage by Fe^{2+} -catalyzed lipid peroxidation both *in vivo* and *in vitro*. The inhibitory effect of astaxanthin on mitochondrial lipid peroxidation was stronger than that of α -tocopherol. Terao (30) reported that canthaxanthin and astaxanthin are more effective antioxidants than β -carotene by stabilizing the trapped radicals.

Most of these studies, however, have not investigated the influence of astaxanthin on LDL oxidation. We therefore determined the *in vitro* susceptibility of LDL to oxidation following exposure to astaxanthin using our established method (9, 31, 32) of measuring conjugated dienes. Moreover, since the *ex vivo* effects of astaxanthin-containing supplements on LDL oxidation have not been examined in human subjects, we recruited volunteers to consume astaxanthin and then measured changes in LDL oxidizability.

Materials and Methods

Astaxanthin was extracted and purified by HPLC in large quantity from krill (husk) (18). Lutein, α -tocopherol and V-70 (2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile)) were from Wako Pure Chemicals, Osaka, Japan. Other reagents and solvents were of analytical grade and used without purification.

In vitro study

After overnight fasting, blood was collected in an EDTA-containing (1g/L) tube from a normolipidemic healthy male volunteer. Plasma (1.1 ml) was adjusted to a density of 1.21 kg/l and then layered under 2.8 ml saline solution ($d=1.006$ kg/l) containing 0.1% EDTA in 3.9 ml quick seal tubes. The tubes were centrifuged at $417,000 \times g$ for 40 min at 4°C (TLA 100.4 rotor) in a Beckman Optima TL ultracentrifuge. The concentration of protein in the isolated LDL was determined using a modified Micro BCA method (Micro BCA protein Assay Reagent Kit: PIERCE, Rockford, USA). The final protein

concentration in human LDL was adjusted to 70 mg/l.

Oxidation of LDL was determined as the production of conjugated dienes (9, 31, 32). Freshly prepared LDL (70 mg protein/l) was incubated with astaxanthin (0-50 $\mu\text{g/ml}$), lutein or α -tocopherol (10 $\mu\text{g/ml}$). Oxidation was initiated by adding freshly prepared V-70 solution (final concentration 400 $\mu\text{M/ml}$) at 37°C . Absorbance at 234 nm was automatically recorded at 5 min intervals in a spectrophotometer (Beckman DU-650). The parameter of oxidation determined from the LDL absorbance profile was lag time, which was determined as the intercept of the baseline and propagation phase of the absorbance curve.

Ex vivo study

Twenty-four healthy volunteers (aged 28.2 ± 7.8 y) consumed 1.8 ($n=5$), 3.6 ($n=5$), 14.4 ($n=3$) or 21.6 ($n=5$) mg of astaxanthin-containing supplements per day for 14 days. Six subjects were examined as controls.

All participants were asked to maintain their habitual diet and lifestyle, and to stop taking vitamin supplements 2 weeks before the study began.

A dietary record was obtained for 3 days before the study, and during the experimental period, subjects were given dietary instructions for 14 days. Nutrient intake was calculated from a database of foods based on the composition of Japanese foods. (33)

The study was approved by the ethical committee of National Institute of Health and Nutrition (Japan) and all subjects provided informed consent.

Fasting venous blood samples were taken at the start and the end of the study. The plasma was separated by centrifugation at $2,000 \times g$ for 15 min at 4°C , and LDL was isolated using a single spin ultracentrifugation procedure (34). The concentration of protein in the isolated LDL was determined using a modified Micro BCA method.

Oxidation of LDL (70 $\mu\text{g/ml}$ protein) was initiated by the addition of V-70 (2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile)) solution (final concentration 200 $\mu\text{M/ml}$) at 37°C and monitored using a spectrophotometer (Beckman DU-650) to follow changes of conjugated dienes at 234 nm absorbance every 4 min for 5 h (31, 32). The lag time was determined as the intercepts with the extrapolations of the parts of the curve representing the lag and propagation phases.

The serum lipid (total cholesterol, triglyceride) levels were assayed using enzymatic methods (Kyowa Medex Co., Ltd., Tokyo, Japan). VLDL-cholesterol and LDL-cholesterol levels were estimated using the Friedewald equation (35). HDL-cholesterol level was determined by a precipitation method (Kyowa Medex Co., Ltd., Tokyo, Japan). Apoprotein (apo AI, AII, B, CII, CIII, E) levels were measured by turbidometric immunoassay (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). The concentrations of serum α -tocopherol and β -carotene concentration were measured by HPLC (36, 37). Serum ascorbic

acid was measured by colorimetry (38). Serum uric acid and bilirubin concentration were measured enzymatically (Toyobo Co., Ltd., Azwell Co., Ltd., Osaka Japan) and the albumin level was measured by the BCG method (Sankojunyak Co., Ltd., Tokyo Japan).

Results

In vitro study

To evaluate the antioxidant effects of astaxanthin on LDL oxidation, LDL was incubated with increasing concentrations of astaxanthin and submitted to oxidation with V-70. As shown in Fig. 1, astaxanthin produced a dose dependent prolongation of oxidation of LDL (31.5, 45.4, 65.0 min). The oxidation lag time was also prolonged by lutein and α -tocopherol (116.2, 71.0 min.) (Fig. 2), although this was less effective than astaxanthin (241.2 min)

Ex vivo study

Serum cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol and apolipoprotein levels did not change significantly during the study as shown in Tables 1, 2.

The LDL lag time increased after consuming astaxanthin at doses of 1.8, 3.6, 14.4 and 21.6 mg for 14 days (Fig. 3), but no difference in oxidation of LDL between day 0 (lag time 59.9 ± 7.2 min) and day 14 (57.2 ± 6.0 min) was observed in the control group.

The mean serum α -tocopherol and β -carotene concentrations, shown in Table 3, did not change significantly. The serum ascorbic acid level significantly decreased from 1.22 to 1.13 mg/day after astaxanthin consumption 21.6 mg/day for 14 days. Additionally no changes were seen for serum uric acid, albumin and bilirubin levels, which were also act as antioxidants in the human body (Table 3).

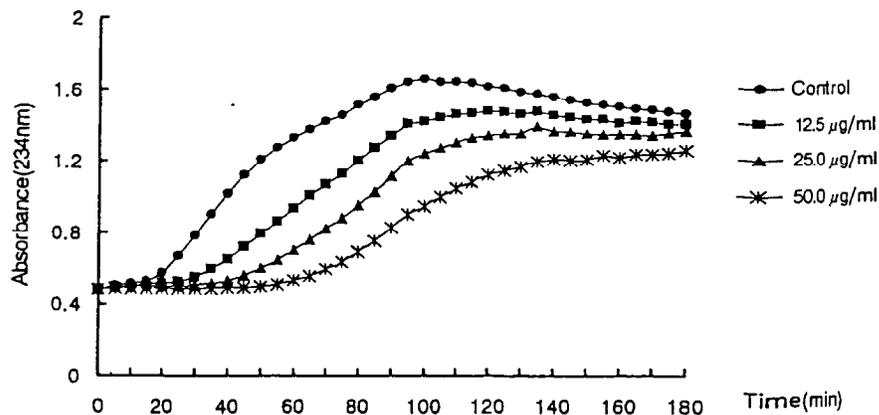


Fig. 1. Effects of astaxanthin on the lag phase of conjugated dienes formation in LDL incubated *in vitro*. LDL (70 μ g of LDL protein/ml) was incubated with increasing concentrations of the astaxanthin (0, 12.5, 25.0, 50.0 μ g/ml), and oxidation initiated by the addition of V-70 (final 400 μ M). The formation of conjugated dienes was determined by changes in the light absorbance at 234 nm every 5 min.

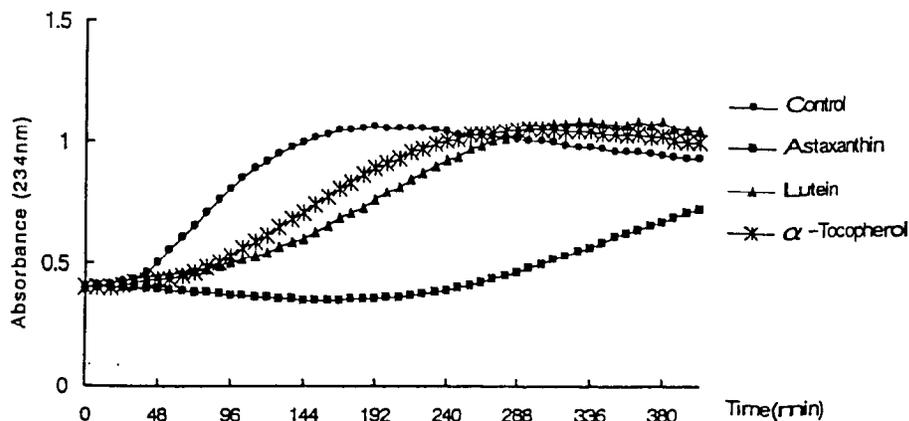


Fig. 2. Formation of conjugated dienes in LDL fraction in the presence of 10 μ g/ml various carotenoids. Oxidation of LDL (35 μ g/ml protein) catalyzed by the addition of V-70 (final 200 μ M) was monitored continuously by changes in the diene formation at 234 nm.

Table 1. The effects of astaxanthin on serum lipids and lipoproteins.

		Control	1.8 mg/day	3.6 mg/day	14.4 mg/dl	21.6 mg/day
Total cholesterol (mmol/l)	0	4.81±0.26	5.79±0.49	4.29±0.44	4.45±0.26	5.20±0.59
	14 days	5.04±0.21	5.22±0.26	4.32±0.34	4.50±0.28	5.30±0.62
Triglyceride (mmol/l)	0	0.77±0.18	1.50±0.60	0.79±0.19	0.62±0.10	0.91±0.23
	14 days	0.91±0.23	0.79±0.23	0.82±0.19	0.86±0.26	1.07±0.49
LDL cholesterol (mmol/l)	0	2.76±0.27	3.35±0.51	2.44±0.39	2.54±0.17	3.12±0.54
	14 days	2.90±0.19	3.16±0.28	2.44±0.33	2.51±0.23	3.05±0.53
HDL cholesterol (mmol/l)	0	1.70±0.19	1.74±0.23	1.50±0.13	1.63±0.19	1.66±0.20
	14 days	1.73±0.14	1.69±0.18	1.51±0.15	1.60±0.15	1.77±0.24

Values indicate mean±SE.

Table 2. The effects of astaxanthin on serum apoproteins.

		Control	1.8 mg/day	3.6 mg/day	14.4 mg/day	21.6 mg/day
Apo-AI (mg/dl)	0	151±10	155±7	138±8	144±11	153±12
	14 days	158±7	144±7	140±8	143±11	153±9
Apo-AII (mg/dl)	0	34.7±1.9	39.8±3.7	31.4±2.3	35.3±4.7	35.4±2.5
	14 days	37.5±2.6	33.8±1.6	31.4±2.3	35.7±5.6	35.6±4.0
Apo-B (mg/dl)	0	79.7±8.7	98.4±14.1	75.6±13.1	70.7±5.7	83.2±14.9
	14 days	83.3±8.7	87.2±8.2	76.2±11.0	66.7±8.8	80.6±16.5
Apo-CII (mg/dl)	0	3.9±0.6	3.7±0.8	2.5±0.8	2.2±0.9	3.7±2.1
	14 days	4.2±0.7	3.1±0.4	2.8±0.7	2.3±0.5	3.7±2.0
Apo-CIII (mg/dl)	0	11.8±1.0	14.2±3.7	8.0±2.1	7.6±2.0	12.3±5.4
	14 days	12.5±1.8	10.0±1.3	8.0±2.0	8.3±2.2	12.3±7.1
Apo-E (mg/dl)	0	5.2±0.5	6.8±0.8	4.8±0.5	4.1±0.4	5.6±1.6
	14 days	6.3±0.7	5.5±0.5*	4.0±0.5	4.1±0.3	5.9±1.8

Values indicate mean±SE.

* $p < 0.05$ vs. before experiment (0).

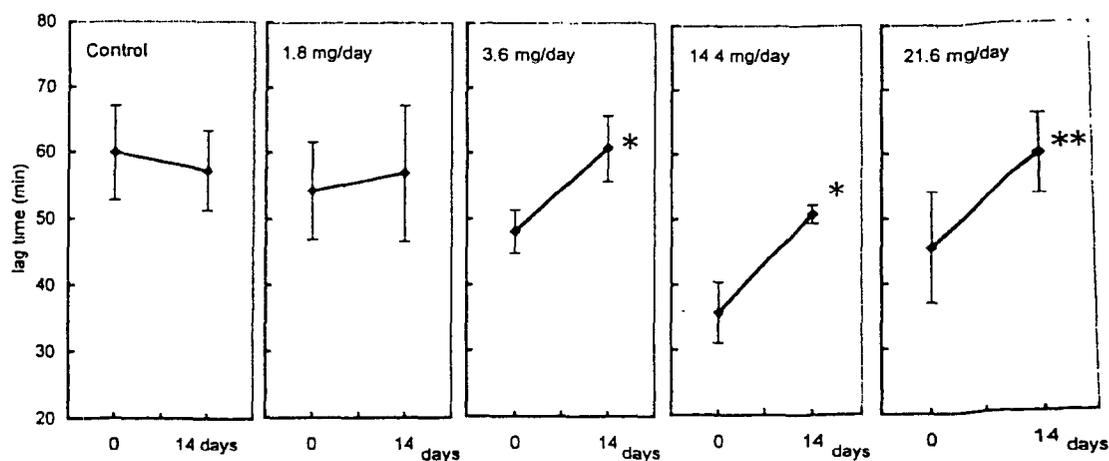


Fig. 3. Values indicate mean±SD.

Changes in LDL lag time after 14 days consumption of astaxanthin (1.8, 3.6, 14.4, 21.6 mg/day).

* $p < 0.05$, ** $p < 0.01$ vs. before experiment (0).

Total energy intake did not change significantly during the experimental period. (Table 4), and the intake of vitamins A, E and C in both the control and experimental groups remained stable during the experimental period. The intake of polyunsaturated, monounsaturated, saturated fatty acids and cholesterol also did not change.

Discussion

In this study we have demonstrated both *in vitro* and *ex vivo* that astaxanthin reduces LDL oxidizability. When compared with α -tocopherol and lutein, astaxanthin was the stronger antioxidant. These observations are consistent with the findings of Miki *et al.* (23) who investigated

Table 3. The effects of astaxanthin on serum antioxidants.

		Control	1.8 mg/day	3.6 mg/day	14.4 mg/day	21.6 mg/day
α -tocopherol (μ g/ml)	0	11.0 \pm 1.6	14.2 \pm 2.8	10.9 \pm 1.9	9.2 \pm 1.4	10.6 \pm 0.16
	14 days	11.4 \pm 2.5	12.0 \pm 0.7	10.5 \pm 1.0	8.7 \pm 3.0	9.1 \pm 0.25
β -carotene (μ g/ml)	0	0.16 \pm 0.03	0.10 \pm 0.01	0.10 \pm 0.01	0.10 \pm 0.02	0.11 \pm 0.01
	14 days	0.15 \pm 0.03	0.10 \pm 0.01	0.10 \pm 0.01	0.11 \pm 0.03	0.11 \pm 0.01
Ascorbic acid (mg/dl)	0	1.32 \pm 0.19	1.16 \pm 0.06	1.14 \pm 0.18	1.02 \pm 0.04	1.22 \pm 0.06
	14 days	1.28 \pm 0.16	1.12 \pm 0.13	1.11 \pm 0.15	1.05 \pm 0.07	1.13 \pm 0.06*
Uric acid (mg/dl)	0	5.0 \pm 1.1	5.8 \pm 1.8	5.4 \pm 1.1	5.0 \pm 0.5	5.7 \pm 1.8
	14 days	5.2 \pm 1.4	5.3 \pm 1.5	5.3 \pm 1.6	5.4 \pm 0.7	5.3 \pm 1.6
Albumin (g/dl)	0	4.3 \pm 0.3	4.8 \pm 0.3	4.9 \pm 0.2	4.9 \pm 0.5	4.9 \pm 0.2
	14 days	4.9 \pm 0.6	4.7 \pm 0.3	4.8 \pm 0.3	4.7 \pm 0.4	4.8 \pm 0.3
T-Bilirubin (mg/dl)	0	0.8 \pm 0.3	0.8 \pm 0.3	0.7 \pm 0.2	0.8 \pm 0.2	0.9 \pm 0.3
	14 days	0.7 \pm 0.2	0.9 \pm 0.2	0.6 \pm 0.2	0.6 \pm 0.1	1.0 \pm 0.3

Values indicate mean \pm SD.

* $p < 0.05$ vs. before experiment (0)

Table 4. Daily nutrient intakes.

		Control		Astaxanthin intake	
		Before	Experimental period	Before	Experimental period
Energy	(kcal)	1,762 \pm 101	1,785 \pm 70	1,533 \pm 71	1,677 \pm 110
Protein	(g)	66.2 \pm 5.2	83.3 \pm 5.2	57.3 \pm 2.9	65.7 \pm 4.6
Carbohydrate	(g)	201.0 \pm 12.3	210.0 \pm 18.2	185.2 \pm 7.4	197.3 \pm 13.7
Fat	(g)	58.9 \pm 4.3	66.5 \pm 5.3	52.2 \pm 3.9	58.4 \pm 4.7
SFA	(g)	17.4 \pm 1.6	18.9 \pm 1.1	16.3 \pm 1.5	18.7 \pm 1.7
MUFA	(g)	22.3 \pm 2.1	25.3 \pm 2.5	19.7 \pm 1.6	21.5 \pm 1.7
PUFA	(g)	13.5 \pm 1.1	14.2 \pm 1.5	10.5 \pm 0.7	11.4 \pm 1.2
Cholesterol	(mg)	356 \pm 25	422 \pm 52	269 \pm 35	308 \pm 38
Vitamin A	(IU)	2,527 \pm 255	2,130 \pm 322	1,784 \pm 574	1,293 \pm 181
Vitamin E	(mg)	7.9 \pm 0.5	9.3 \pm 1.0	6.2 \pm 0.5	6.7 \pm 0.7
Vitamin B1	(mg)	0.83 \pm 0.08	1.22 \pm 0.15*	0.82 \pm 0.06	0.87 \pm 0.08
Vitamin B2	(mg)	1.26 \pm 0.12	1.52 \pm 0.12	1.01 \pm 0.09	1.11 \pm 0.11
Vitamin C	(mg)	80 \pm 10	80 \pm 14	64 \pm 6	58 \pm 8

Values indicate mean \pm SE.

* $p < 0.01$ vs. before.

six dominant carotenoids using a system containing protoporphyrin IX with ferric iron as a free radical source and linoleic acid as the acceptor. They showed that astaxanthin was 10 times more potent as an inhibitor of lipid peroxidation than α -tocopherol. The difference in potency is probably attributable to different mechanisms associated with the structures of β -carotene and α -tocopherol. It is thought that β -carotene reacts directly with a lipid peroxy radical through a free radical chain reaction, and becomes a carbon-center radical stabilized by the resonance (39). Whereas astaxanthin and β -carotene act at the beginning of the chain reaction, the step at which the radical is generated, β -tocopherol inhibits lipid peroxidation by scavenging the intermediate peroxy radicals, thereby arresting the chain reaction (40).

Terao (30) studied the effect on the oxidation of various carotenoids using AMVN (2, 2'-azobis(2, 4-dimethylvaleronitrile)) as a radical initiator. A free-radical chain reaction was generated with methyl linoleate and AMVN, and the generation of the hydroperoxide was examined. Canthaxanthin and astaxanthin, possessing the con-

jugated carbonyl group, retarded the hydroperoxide formation more efficiently than β -carotene and zeaxanthin. The presence of a conjugated carbonyl group presumably enhances the stability of the trapped radical by decreasing its destiny for continued chain-propagation reaction.

Having confirmed the LDL antioxidant activity of astaxanthin *in vitro*, we then examined the effect of astaxanthin supplementation on the susceptibility of LDL to oxidation, *ex vivo*. The volunteers consuming astaxanthin for 14 days showed a lag time that was extended significantly with increasing dose of astaxanthin.

Although it is thought that the antioxidative effect is restricted to the free form of astaxanthin, the esterified form is more stable and absorbed more efficiently in the intestine. The majority of astaxanthins in krill exist in the ester form, and are cleaved by intestinal hydrolytic enzymes and converted to the free, antioxidant form of astaxanthin.

Astaxanthin exists in crustaceans, salmon roe and salmon. The astaxanthin content of salmon is 1.7~2.6 mg/100 g. We calculated that an intake of 3.6 mg asta-

xanthin is equivalent to approximately 165 g of salmon meat.

It has been also reported that interactions can occur between carotenoids and tocopherols. Lim *et al.* (25) examined the potential of astaxanthin as an antioxidant defense in chicks fed astaxanthin at 0.5 g/kg diets for 4 weeks. An accumulation of astaxanthin was associated with a decrease in plasma α -tocopherol and zeaxanthin/lutein level. It is reasonable to assume that astaxanthin compensates for the loss of α -tocopherol and zeaxanthin/lutein in the antioxidant defenses of the plasma. The level of PC-OOH accumulated by the exposure to the free radical generator for 4 h was not significant in the astaxanthin supplemented chicks indicating the existence of peroxyl radical-trapping activity.

In our study, however, there was no relationship between dose and either plasma β -carotene or α -tocopherol levels, with all doses resulting in roughly similar plasma levels of both nutrients. However, a more detailed investigation will be needed to solve the mechanisms of these interactions.

Within the living body some antioxidants exist that prevent formation of free radicals. Uric acid, albumin and bilirubin were reported to be efficient antioxidants *in vitro* (41, 42). Levels of these substances were not altered during this study.

Our endogenous antioxidant defenses appear inadequate to completely prevent oxidative damage. Hence, sources of dietary antioxidants may be especially important to us.

Our results support a conclusion that consumption of marine fauna containing astaxanthin inhibits LDL oxidation and may contribute to the prevention of atherosclerosis.

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