Effects of Astaxanthin Supplementation on Lipid Peroxidation

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Abstract: Astaxanthin, the main carotenoid pigment in aquatic animals, has greater antioxidant activity *in vitro* (protecting against lipid peroxidation) and a more polar configuration than other carotenoids. We investigated the effect of three-month astaxanthin supplementation on lipid peroxidation in healthy non-smoking Finnish men, aged 19–33 years by using a randomized double-blind study design. Also absorption of astaxanthin from capsules into bloodstream and its safety were evaluated. The intervention group received two 4-mg astaxanthin (Astaxin®) capsules daily, and the control group two identical-looking placebo capsules. Astaxanthin supplementation elevated plasma astaxanthin levels to 0.032 μmol/L ($p < 0.001$ for the change compared with the placebo group). We observed that levels of plasma 12- and 15-hydroxy fatty acids were reduced statistically significantly in the astaxanthin group ($p = 0.048$ and $p = 0.047$ respectively) during supplementation, but not in the placebo group and the change of 15-hydroxy fatty acid was almost significantly greater ($p = 0.056$) in the astaxanthin group, as compared with the placebo group. The present study suggests that intestinal absorption of astaxanthin delivered as capsules is adequate, and well tolerated. Supplementation with astaxanthin may decrease *in vivo* oxidation of fatty acids in healthy men.

Key words: Astaxanthin, absorption, safety, lipid peroxidation, C18 hydroxy fatty acids, free F_2 isoprostanes

Introduction

Lipid peroxidation has an important role in the etiology of many pathological conditions including atherosclerosis [1–3]. Reactive oxygen species (ROS) such as hydroxyl and peroxyl radicals that are produced during normal metabolic processes promote lipid peroxidation in the body. Dietary antioxidant carotenoids such as astaxanthin are believed to prevent lipids from becoming oxidized [4].

Astaxanthin (3,3'-dihydroxy-β, β-carotene-4,4'-dione) is a naturally occurring carotenoid with strong antioxidant properties both *in vitro* and *in vivo*. Astaxanthin belongs to the xanthophyll group characterized by its hydroxyl and keto endings [5, 6] (Figure 1). Astaxanthin has been reported to be a ten-fold more potent antioxidant than other carotenoids such as lutein, zeaxanthin, cantaxanthin, and β-carotene [7]. As with the other carotenoids, astaxanthin can also absorb the excited energy of singlet oxygen onto its carotenoid chain, which

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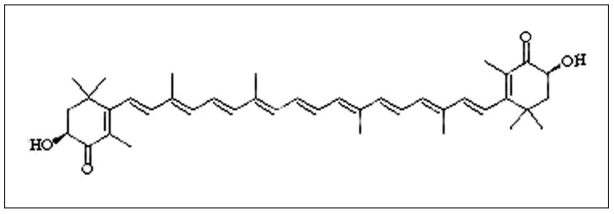

Figure 1: Chemical structure of astaxanthin.

evokes degradation of the carotenoid molecule but prevents other molecules or tissues from suffering radical-induced damage [4]. Astaxanthin is a more effective scavenger of hydroxyl radicals than β-carotene [8]. Due to the unique structure of the terminal ring moiety (hydroxyl and keto endings), the terminal ring of astaxanthin is able to scavenge radicals both at the surface and in the interior of the phospholipid membrane. The unsaturated polyene chain traps radicals only in the membrane [9]. In experimental animals astaxanthin has also been shown to induce the xenobiotic metabolizing enzyme system, the cytochrome P450 enzymes [10]. A disodium disuccinate astaxanthin derivative has been claimed to provide cardioprotection and myocardial salvage; it was reported to reduce the infarct size in rats [11] and dogs [12].

The main dietary sources of astaxanthin are aquatic animals including salmon, trout, red seabream, shrimp, lobster, and fish eggs [4]. About 450 g of farmed rainbow trout or 800 g of wild salmon contain 4 mg of naturally occurring astaxanthin [13]. However, there are no previous randomized placebo-controlled clinical trials that have evaluated the effect of astaxanthin supplementation on lipid peroxidation and inflammation markers in humans.

The measurement of lipid peroxidation in human samples is complicated. Not only are there no generally applied methods; there is not even any consensus about what measurements represent adequate markers of lipid damage [14, 15]. In this work, we used the plasma C18 hydroxy fatty acids and plasma free F_2 isoprostanes as biomarkers of lipid peroxidation *in vivo.* The hydroxy fatty acid assay measured the concentration of hydroxy derivatives of oleic acid, linoleic acid, and linolenic acid.

The purpose of the present study was to investigate the effect of supplementation on healthy male volunteers with a relatively high daily dose (8 mg) of astaxanthin on the absorption and plasma levels of astaxanthin and other antioxidants, serum lipids, the susceptibility of the combined fraction of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) to oxidation, the tendency for lipid peroxidation *in vivo,* and the safety of the supplement, as compared with placebo.

Subjects and Methods

Study design and subjects

The subjects of the study were healthy non-smoking male volunteers aged 19–33 years with no severe diseases or malabsorption. The study was a randomized, double-blind trial based on comparison of two parallel identically sized groups; the intervention group received 4 mg of astaxanthin (Astaxin®, AstaReal AB, Sweden) capsules twice per day (n = 20) and the placebo group two identical-looking placebo capsules (microcrystalline cellulose) (n = 20). The Astaxin capsule contained nutrients, fatty acids, astaxanthin (monoesters, diesters, and free form), minerals, and amino acids. Nutrient content per capsule was approximately as follows: crude fat 86 mg, crude protein 20 mg, crude fiber 17 mg, soluble carbohydrates 60 mg, and starch 28 mg. The amounts of these components in the capsule were inadequate to have any effects on lipid peroxidation. The treatment period was three months. The subjects were required not to take any astaxanthin supplementation at baseline. One subject in the placebo group was disqualified from the statistical analysis because his plasma astaxanthin concentration was high at the baseline measurement. It is most probable that this subject had been consuming astaxanthin supplements against instructions prior to the treatment period. The subjects were advised to keep their exercise and dietary habits unchanged during the study. Four-day food recordings were carried out at the beginning and at the end of the study to monitor any changes in the dietary intake of astaxanthin and other antioxidants.

Measurement of astaxanthin and other carotenoids

Analysis of *trans*-astaxanthin was performed using liquid-liquid extraction for sample preparation and high-performance liquid chromatography (HPLC) with visible spectrometric detection. Plasma samples and control plasma were thawed at room temperature under conditions of reduced light. To extract the carotenoids, 4 mL of acetone was added to 0.5 mL of plasma in 10-mL glass tubes. After mixing vigorously for 10 seconds, the tubes were left on a reciprocating shaker for 1 hour whilst protected from light. Thereafter 4.0 mL hexane was added and the samples mixed vigorously for 10 seconds before two-phase separation for 1 hour. The upper phase was then removed and dried down under a stream of nitrogen. The dried extract was dissolved in 75–150 µL chloroform:methanol (1:1, v/v). A Rheodyne injection system (model 7010) was used to inject 25 µL of sample into a re-

verse-phase column (ReproSil-Pur 120 C18-AQ). The carotenoids were eluted using a linear gradient with methanol:water:ethyl acetate, 82 : 8 : 10 at the start and 29:1:7 after 20 minutes, using a Merck-Hitachi L6200A Intelligent pump. Astaxanthin was detected at 474 nm by a Merck-Hitachi L4200 detector. Integration was performed using CSW version 1.5 software (DataApex Ltd., the Czech Republic).

Quality control plasma samples and calibration standards were prepared from stock solutions of *trans*-astaxanthin (> 98% pure, Sigma-Aldrich) in acetone, 30 µg/mL. The lower limit of quantification (LLOQ), based on peak area, was 20 ng/mL with linearity demonstrated to 1000 ng/mL. Intra-assay precision values, based upon coefficients of variation of quality control samples, were less than or equal to 14.0%.

Measurement of other vitamins

Plasma samples for simultaneous determination of alpha-tocopherol and retinol were extracted with ethanol and hexane. The concentrations were measured by reversed-phase HPLC [16]. Briefly, 200 µL of heparinized plasma was extracted with 5 mL of hexane and 1 mL of ethanol containing alpha-tocopherol acetate as an internal standard. After centrifugation, the hexane layer was separated and evaporated to dryness with a gentle stream of nitrogen. The residue was reconstituted in 200 µL of the mobile phase. The mobile phase consisted of a mixture of acetonitrile-methanol-chloroform (47 : 47 : 6, v/v/v). A reversed-phase C18 column was used, and peaks were detected at wavelengths of 292 nm for alpha-tocopherol and 325 nm for retinol (model 168; Beckman Instruments, Fullerton, CA, USA). Pure analytes (Sigma, St Louis, MO) were used as primary standards and their concentrations were determined spectrophotometrically according to Thurnham *et al* [17]. Ascorbic acid was analyzed by HPLC with the ion-exchange method described by Parviainen *et al* [18].

Erythrocyte and plasma folate levels were measured by radioimmunoassay (Quanta phase II, Bio-Rad, Hercules, California, USA). The whole blood sample for erythrocyte folate determination was hemolyzed and stabilized with ascorbic acid immediately after blood drawing and kept frozen until measured in batches within three months.

Measurement of serum lipids, lipoproteins and fatty acids

Serum total cholesterol (Konelab, Espoo, Finland) and triglycerides (Roche Diagnostics, Mannheim, Germany) were analyzed using enzymatic colorimetric methods. Serum high-density lipoprotein (HDL) cholesterol was measured after magnesium chloride dextran sulfate precipitation. Serum LDL cholesterol concentration was determined using polyvinyl sulfate precipitation. Sixteen serum fatty acids were analyzed after chloroform-methanol extraction and methylation with sulfuric acid–methanol. The methylated fatty acids were analyzed by a gas chromatograph (HP 5890; Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector and an NB-351 capillary column (HNU-Nordion, Helsinki, Finland) [15].

Measurement of the safety parameters

Activities of alanine aminotransferase (Reference range: men 10–70 U/L) and gamma-glutamyltransferase (Reference range: men 10–80 U/L) (Laboratory of the Research Institute of Public Health, University of Kuopio, Finland) were measured by an automatic analyzer (Konelab, Espoo, Finland). Blood cell profile [hemoglobin, hematocrit, erythrocytes, leukocytes, mean corpuscular volume (MCV), and thrombocyte count] was analyzed by a Cell-Dyn 610 blood cell counter (Mountain View, CA, USA).

Measurement of the resistance of LDL and VLDL to oxidation

VLDL and LDL were separated together from EDTA plasma using a rapid one-step gradient ultracentrifugation. Since EDTA blocks the lipoprotein reaction with Cu^{2+}, EDTA was removed from the LDL fraction by using small gel filtration PD-10 columns (Pharmacia, Uppsala, Sweden). Briefly, VLDL+LDL was diluted with oxygen-saturated phosphate-buffered saline to a protein concentration of 0.05 mg/mL. The formation of conjugated dienes was initiated by adding 33.5 µL of 0.1 mM copper chloride (Merck) to 2 mL of diluted VLDL+LDL fraction and the reaction was followed by spectrophotometry at 234 nm [19, 20]. The lag time and the maximum oxidation rate (Vmax) were determined at +37 °C by a temperature controlled Beckman Du 640i spectrophotometer with an enzyme kinetics data system (Beckman Co., Fullerton, CA, USA). The lag time was defined as the time from the start of the reaction to the beginning of the steepest slope and was computed by means of the least squares regression (LSR) equation. Vmax was computed also by using the LSR method from the slope of the absorbance curve during the propagation phase [21]. The lag time and Vmax were standardized against a plasma pool with previously determined values for the lag time and the Vmax.

Measurement of plasma hydroxy fatty acids

Plasma C18 hydroxy fatty acids (8-, 9-, 10-, 11-, 12-, 13-, 15-, and 16-mono hydroxy fatty acids) were measured using a gas chromatograph/mass spectrometer (Agilent Technologies, Espoo, Finland). Plasma fatty acids and fatty acid hydroperoxides were stabilized by hydrogenation using platinum as a catalyst, saponified, and esterified by diazomethane, and finally, hydroxy fatty acids were separated from fatty acids by extraction through solid-phase mini-columns. Prior to the analysis, hydroxy groups were methylated with tetramethylammonium hydroxide. Levels of different (methoxy) monohydroxy fatty acid (OHFA) methyl esters were determined by electron impact mass spectrometer. C17 and C19 OHFAs were used as internal standards. Plasma 12-hydroxy fatty acid is known to be sensitive to contamination [15, 22]. Two subjects in the placebo group at the baseline level were omitted due to this contamination problem.

Measurements of plasma free F_2-isoprostanes

Plasma samples for F_2-isoprostane measurements were frozen at – 80 °C immediately after blood drawing. Two milliliters of EDTA plasma were needed to analyze F_2-isoprostanes by gas chromatography/mass spectrometry (GC/MS) methods. A deuterated prostaglandin F_2 internal standard was added to plasma, and F_2-isoprostanes were extracted with C18 and silica Sep-Pak mini-columns (Waters, Milford, Massachusetts, USA) following pentafluorobenzyl esterification and thin layer chromatography (TLC) purification. After trimethylsilyl ether derivatization, the F_2 isoprostane concentrations were analyzed by a GC/MS assay [23].

Measurement of endogenous antioxidants

Serum paraoxonase (PON) activity was measured based on its capacity to hydrolyze paraoxon. The formation of p-nitrophenol was monitored at 405 nm in Tris-HCl buffer, pH 8.0, in the presence of Ca^{2+} on a microtiter plate (Thermo Electron Oy, Vantaa, Finland) [24]. Uric acid was measured using an enzymatic colorimetric method (Randox Laboratories Ltd., UK).

Measurement of inflammation markers

The levels of serum interleukin-6 and interleukin-2-receptors were analyzed by a solid-phase Enzyme Amplified Immunoassay (EASIA) on the microtiter plate (BioSource Europe SA, Nivelles, Belgium). Plasma C-reactive protein (CRP) was determined with a high-sensitivity particle-enhanced immunoturbidimetric assay (CRP Latex HS, Roche/Hitachi 911, Roche Diagnostics GmbH, Mannhein Germany).

Measurement of blood pressure

Resting blood pressure was measured in the morning by a trained nurse with a random-zero mercury sphygmomanometer (Hawksley, Lancing, United Kingdom). After the subjects had rested for 5 minutes, 3 measurements were taken at 2-minute intervals with the subjects seated. The mean of all 3 measurements was used to determine the systolic and diastolic blood pressures.

Assessment of nutrient intake

The consumption of foods was assessed at the time of blood sampling during the baseline phase and at the end of this study. Subjects were instructed on the use of household measures for quantitative recording of their food intake during the 4-day data collection. A nutritionist gave instructions and checked the completed food intake records. Dietary intake of nutrients and foods was calculated using NUTRICA software (version 2.5; The Social Insurance Institution of Finland, Turku, Finland). This software is compiled using mainly Finnish values of nutrient composition of foods, and takes into account losses of vitamins in food preparation. In total, the database contains comprehensive data for 1300 food items and dishes, and 30 nutrients [25].

Table I: Baseline characteristics

	Placebo n = 19	Astaxanthin n = 20	p for the differences between the groups
Age (years)	25.7 ± 3.3	23.1 ± 2.3	0.008
BMI, body mass index (kg/m²)	23.8 ± 2.3	23.8 ± 2.2	0.972
Alanine aminotransferase (U/L)	12 ± 9	15 ± 7	0.383
Gamma-glutamyltransferase (U/L)	24 ± 8	28 ± 13	0.184
Hemoglobin (g/L)	148 ± 8	151 ± 5	0.151
Systolic blood pressure (mm Hg)	123 ± 10	127 ± 13	0.305
Diastolic blood pressure (mm Hg)	83 ± 9	83 ± 9	0.978

Statistics

Results are expressed as means ± S.D. Differences between baseline and three-month values with the pooled groups were tested by paired samples *t*-test. Means were compared across the study groups by independent samples *t*-test. Differences with $p < 0.05$ were considered statistically significant. SPSS software (version 11.5; SPSS, Inc., Chicago, IL, USA) was used for statistical analyses.

Results

Baseline characteristics are described in Table I. Age was significantly lower in the astaxanthin group at study baseline when compared with the placebo group. There was no significant difference in the diet between the study groups at baseline or during the study (Table II). Astaxanthin was well absorbed from the capsules into systemic blood circulation. In the astaxanthin group, mean levels of plasma astaxanthin were elevated to 0.032 μmol/L ($p < 0.001$) during the three-month supplementation and it was also well tolerated. There was no gastrointestinal tract distress or any other side effects. Astaxanthin levels of plasma were not detectable at baseline in either group, this being the same after supplementation in the placebo group. There were no significant differences in the changes of plasma ascorbic acid, alpha-tocopherol, retinol, or carotenoids such as lycopene, β-carotene, zeaxanthin + lutein, cantaxanthin, and β-cryptoxanthin

Table II. Mean ± SD values of carotenoids, vitamins, lipids, and lipid peroxidation before and after 3-month supplementation with astaxanthin

	Placebo n = 19 Baseline	3-month	Astaxanthin n = 20 Baseline	3-month	p for difference in changes between groups
Plasma ascorbic acid (μmol/L)	71.6 ± 22.8	63.0 ± 15.9	67.0 ± 17.8	61.5 ± 19.4	0.410
Plasma astaxanthin (μmol/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.032 ± 0.012	< 0.001
Plasma α-tocopherol (μmol/L)	23.3 ± 4.0	24.5 ± 5.4	22.9 ± 5.3	21.3 ± 3.8	0.054
Plasma retinol (μmol/L)	1.97 ± 0.56	1.87 ± 0.57	2.04 ± 0.55	1.71 ± 0.36	0.181
Plasma lutein-zeaxanthin (μmol/L)	0.16 ± 0.083	0.17 ± 0.067	0.16 ± 0.060	0.15 ± 0.075	0.113
Plasma cantaxanthin (μmol/L)	0.030 ± 0.014	0.042 ± 0.026	0.042 ± 0.030	0.062 ± 0.021	0.285
Plasma lycopene (μmol/L)	0.73 ± 0.29	0.75 ± 0.31	0.84 ± 0.24	0.83 ± 0.29	0.721
Plasma β-cryptoxanthin (μmol/L)	0.27 ± 0.14	0.21 ± 0.098	0.26 ± 0.14	0.22 ± 0.15	0.325
Plasma β-carotene (μmol/L)	0.52 ± 0.25	0.49 ± 0.18	0.51 ± 0.19	0.56 ± 0.27	0.187
Serum total cholesterol (mmol/L)	4.68 ± 0.66	4.76 ± 0.87	4.52 ± 0.67	4.44 ± 0.83	0.291
Serum HDL cholesterol (mmol/L)	1.27 ± 0.19	1.22 ± 0.18	1.26 ± 0.33	1.28 ± 0.31	0.146
Serum LDL cholesterol (mmol/L)	2.80 ± 0.62	2.96 ± 0.84	2.58 ± 0.57	2.54 ± 0.73	0.169
Serum triglycerides (mmol/L)	1.08 ± 0.34	1.06 ± 0.43	1.21 ± 0.83	1.08 ± 0.31	0.543
Copper-induced lag time (min)	70 ± 8	67 ± 7	67 ± 8	66 ± 5	0.583
Copper-induced Vmax (mabs/min)	11.3 ± 1.5	11.8 ± 1.4	11.3 ± 1.3	11.3 ± 1.6	0.330
Plasma total hydroxy fatty acids (μmol/L)	1.26 ± 0.75	1.06 ± 0.29	1.15 ± 0.42	1.02 ± 0.44	0.728
Plasma 8-hydroxy fatty acids (μmol/L)	0.05 ± 0.05	0.06 ± 0.05	0.05 ± 0.06	0.04 ± 0.05	0.373
Plasma 9-hydroxy fatty acids (μmol/L)	0.29 ± 0.08	0.31 ± 0.10	0.34 ± 0.13	0.33 ± 0.16	0.362
Plasma 10-hydroxy fatty acids (μmol/L)	0.24 ± 0.06	0.24 ± 0.05	0.25 ± 0.07	0.23 ± 0.10	0.506
Plasma 11-hydroxy fatty acids (μmol/L)	0.11 ± 0.04	0.11 ± 0.04	0.13 ± 0.06	0.10 ± 0.06	0.244
Plasma 12-hydroxy fatty acids (μmol/L)*	0.11 ± 0.06 (n=17)	0.09 ± 0.04 (n=17)	0.11 ± 0.10	0.07 ± 0.05	0.246
Plasma 13-hydroxy fatty acids (μmol/L)	0.23 ± 0.10	0.24 ± 0.09	0.26 ± 0.13	0.25 ± 0.14	0.492
Plasma 15-hydroxy fatty acids (μmol/L)	0.04 ± 0.08	0.05 ± 0.08	0.05 ± 0.08	0.02 ± 0.05	0.056
Plasma 16-hydroxy fatty acids (μmol/L)	0.02 ± 0.04	0.01 ± 0.03	0.01 ± 0.02	0.01 ± 0.02	0.549
Plasma free F_2 isoprostanes (pg/mL)	46 ± 12	49 ± 12	50 ± 11	50 ± 12	0.257
Energy intake (kJ)	10396 ± 1912	9962 ± 2934	10420 ± 2427	10122 ± 2766	0.875
Ascorbic acid intake (mg/4d)	131 ± 45	124 ± 71	131 ± 78	126 ± 65	0.903
Beta-carotene intake (μg/4d)	2718 ± 1929	2789 ± 1896	2719 ± 1571	2024 ± 1404	0.232
Folate intake (μg/4d)	327 ± 100	340 ± 128	308 ± 106	307 ± 74	0.666

* Two subjects were omitted because of contamination
4d = 4 days

concentrations between the astaxanthin and placebo group (Table II).

The levels of plasma 12- and 15-hydroxy fatty acids declined (by 36% and 60%, respectively) significantly in the astaxanthin group ($p = 0.048$ and $p = 0.047$, respectively) during supplementation, but not in the placebo group. There was no difference in the levels of 12-hydroxy fatty acids between placebo and astaxanthin treated groups at three months. The concentration of plasma 15-hydroxy fatty acid decreased by 22% ($p = 0.056$) in the astaxanthin group compared with the placebo group. There were no other statistically significant differences in serum lipids, copper-induced lipid peroxidation, plasma free F_2-isoprostanes (Table II), or serum fatty acid profiles between the study groups (data not shown).

The levels of erythrocyte and plasma folate increased statistically significantly in the astaxanthin group (changes of 8% and 14%, respectively) ($p = 0.016$ and $p = 0.003$, respectively). However, there were no statistically significant differences in their values between the groups. The increase in the levels of an endogenous antioxidant, serum uric acid, was statistically significant in the astaxanthin group (change 8%, $p = 0.045$), but no significant difference between the study groups was observed. No change was found in plasma paraoxonase activity. No significant differences in the changes among the study groups were found with respect to the levels of serum inflammation markers: interleukin-2-receptor, interleukin-6, and highly sensitive CRP (Table III).

The levels of plasma alanine aminotransferase and gamma-glutamyl transferase increased statistically significantly in the placebo group compared with baseline levels (changes of 10% and 25%, respectively) ($p = 0.024$ and $p = 0.029$, respectively). There were no changes in their values in the astaxanthin group. However, there was one subject in the astaxanthin group whose alanine aminotransferase (96 U/L) and gamma-glutamyl transferase (254 U/L) values were elevated over the reference range after the three-month supplementation, for unknown reasons. The changes in liver enzymes did not differ statistically significantly between astaxanthin and placebo groups. Neither the changes in basic blood profile (hemoglobin, hematocrit, erythrocytes, leukocytes, MCV, and thrombocyte count) nor changes in blood pressure were statistically significant between the study groups (data not shown).

Discussion

The present study clearly indicates that astaxanthin supplements are absorbed well from capsules into the systemic blood circulation. There was no effect of astaxanthin on the susceptibility of the combined fraction of plasma VLDL and LDL to copper-induced oxidation lag time, Vmax as compared with placebo. However, we found a trend towards reduction in the levels of plasma 12- and 15-hydroxy fatty acids during astaxanthin supplementation as compared with the situation in the placebo group. Plasma 15-hydroxy fatty acid is formed by oxidation of polyunsaturated linolenic and linoleic acids, which are the fatty acids most sensitive to oxidation. In a previous study, we evaluated the reliability of different C18 hydroxy fatty acids as markers of plasma lipid peroxidation *in vitro* [22]. We found that during either auto-oxidation or mildly induced oxidation, there is no association between the increase in plasma concentration and the increase in any single C18 hydroxy fatty acid and the initial monounsaturated or polyunsaturated fatty acids in plasma. Theoretically plasma 12- and 15-hydroxy fatty acids are auto-oxidation products of linoleic or linolenic

Table III: Mean ± SD values of folate, endogenous antioxidants, and inflammation markers before and after 3-month supplementation with astaxanthin

	Placebo n = 19 Baseline	3-month	Astaxanthin n = 20 Baseline	3-month	p for difference in changes between groups
Erythrocyte folate (nmol/L)	457.3 ± 132.2	483.8 ± 138.1	438.6 ± 125.2	474.8 ± 146.5	0.568
Plasma folate (nmol/L)	10.6 ± 4.2	11.5 ± 4.4	9.9 ± 2.9	11.5 ± 3.7	0.539
Serum high sensitive CRP (mg/L)	1.40 ± 3.54	1.18 ± 1.43	1.37 ± 2.28	2.17 ± 5.60	0.513
Serum interleukin-6 (pg/mL)	4.99 ± 7.75	3.27 ± 0.94	8.58 ± 11.95	6.83 ± 6.80	0.993
Serum IL-2 receptor (U/mL)	304.25 ± 90.04	315.56 ± 89.61	476.92 ± 359.34	544.53 ± 488.62	0.544
Serum paraoxonase (U/L)	173.6 ± 120.5	171.6 ± 116.5	179.6 ± 108.1	181.6 ± 106.3	0.271
Serum uric acid (μmol/L)	267 ± 50	277 ± 65	259 ± 50	280 ± 47	0.445

acids. C18 hydroxy fatty acids are good indicators at the first step peroxidation of e.g., LDL lipids [26]. F_2 isoprostanes are end products of lipid peroxidation *in vivo* [27]. They are formed from arachidonic acid by free radical-based oxidation. Their biosynthesis is enzyme-independent. Arachidonic acid is esterified to phospholipids and then released in free form by phospholipases. It has been observed that when the supplementation period is long, there should be equilibrium between esterified and free pools of F_2 isoprostanes in the blood circulation, making a relevant measurement of lipid peroxidation from the free pool possible [23]. It may be obvious that the three-month supplementation period was too short to affect significant changes in F_2 isoprostane concentration due to the fact that F_2 isoprostanes are secondary products of lipid peroxidation [28].

We propose that astaxanthin is a safe dietary supplement; it did not evoke any gastrointestinal tract distress or any other side effects in our study. The changes in plasma levels of alanine aminotransferase and gamma-glutamyltransferase or basic blood cell count did not differ between the astaxanthin and placebo groups. Astaxanthin did not affect inflammation markers or blood pressure, although the number of subjects in our trial was too small to study in a reliable manner any changes in blood pressure. These findings are consistent with the published literature. A recent randomized clinical study suggested that astaxanthin supplementation (6 mg per day) had no significant effect on blood pressure or blood chemistry parameters [4]. In another study, healthy males (n = 32) received a single dose of 40 mg of astaxanthin in a lipid-based formulation [6]. The elimination half-life was 16 hours and the maximum concentration was achieved within 8 hours. The high dose was well tolerated and the most common adverse event was headache, which was thought unlikely to be attributable to the treatment. Coral-Hinostroza and co-workers [29] studied the plasma appearance of astaxanthin (doses of 10 mg and 100 mg after 4 weeks) in three middle-aged male volunteers (41 – 50 years). In that study, the maximum plasma level was reached 11.5 hours after administration of unesterified astaxanthin. Safety tests did not revealed any clinically important changes in blood pressure or blood chemistry and no adverse effects were reported. Also, Osterlie *et al* and Spiller *et al* were unable to detect any adverse effects [30, 31]. In a recent experimental animal study, long-term administration of large doses of astaxanthin (50 mg/kg) for 5 weeks was claimed to have beneficial effects in protection against hypertension and stroke in spontaneously hypertensive rats [32].

In plasma, polar carotenoids such as astaxanthin, lutein, or zeaxanthin are transported with VLDL, LDL, and HDL [29]. It is suggested that their function is to protect lipid molecules from oxidation. Oxidation of LDL is associated with the development of atherosclerosis [33, 34]. Iwamoto and co-workers [35] determined the *in vitro* and *ex vivo* effects of astaxanthin on LDL oxidation. Oxidation was performed by using 400 μmol/L of 2,2'-azobis (4-methoxy-2,4-dimethylvaleronitrile) *in vitro* with astaxanthin tested at increasing concentrations (12.5, 25.0, and 50.0 μg/mL). The oxidation lag time was prolonged in a concentration-dependent manner (31.5, 45.4, 65.0 min) compared with the placebo (19.9 min). There were 24 volunteers in that *ex vivo* study. The LDL lag time increased dose-dependently at astaxanthin doses of 1.8, 3.6, 14.4, and 21.6 mg for 14 days compared with the control group. Therefore, these results may suggest that consumption of marine animals as sources of astaxanthin can inhibit LDL oxidation and theoretically might contribute to the prevention of atherosclerosis.

The effect of astaxanthin and alpha-tocopherol or carotenoids on lipid peroxidation has been examined in many studies [36-39]. The protective effects of astaxanthin and cantaxanthin against Cu^{2+}-initiated peroxidation in large phosphatidylcholine unilamellar vesicles were studied. It was found that both carotenoids increased the lag period, though astaxanthin exhibited more potent activity due to its unique structure [36]. The singlet oxygen quenching of alpha-tocopherol and carotenoids such as β-carotene, astaxanthin, cantaxanthin, and lycopene in liposomes for inhibiting lipid peroxidation were determined. The quenching rate constants of alpha-tocopherol and all the carotenoids tested in two photosensitizing systems were almost identical [37]. These results revealed that alpha-tocopherol or carotenoids, when combined with astaxanthin, might not be able to prevent lipid peroxidation more than that achieved by astaxanthin alone. In another study, the effect of xanthophylls and alpha-tocopherol in decreasing UVB-induced lipid peroxidation and stress signalling in human lens epithelial cells was evaluated. Data obtained in that study suggested that xanthophylls such as astaxanthin are more potent than alpha-tocopherol at protecting human lens epithelial cells against UVB insult [38].

Lipid peroxidation has also been studied in laboratory animals. Jakobsson and co-workers [39] evaluated the influence of alpha-tocopherol and astaxanthin on LDL oxidation and atherosclerotic lesion formation in Watanabe heritable hyperlipidemic (WHHL) rabbits. Concentrations of plasma lipids, lipoproteins, and lag time in copper-induced LDL oxidation were followed for 24 weeks. In that study, alpha-tocopherol, but not astaxanthin, prolonged the lag time in LDL oxidation. The effect of an astaxanthin-rich algal meal together with vitamin C on lipid peroxidation was examined in mice. Lipid peroxidation was significantly decreased in mice treated

with the astaxanthin-rich algal meal and vitamin C compared with that of animals not treated at all or those treated with the control meal [40].

In the present study, the intervention group received 4 mg of astaxanthin twice daily for three months. The dose lowered plasma 12- and 15-hydroxy fatty acid levels, whereas there was no effect on the lag time of combined LDL + VLDL fraction *ex vivo*. There is a previous study in which astaxanthin doses were greater than 8 mg/day and LDL lag time increased significantly as compared with placebo [35]. The same effect was observed also with laboratory animals (WWHL rabbits) [39]. It may be possible that the astaxanthin dose must be higher than 8 mg to achieve any impact on the lag time.

Conclusion

In conclusion, the present study indicates clearly that astaxanthin was absorbed well from capsules and was safe. In addition, we found decreases in the levels of plasma 12- and 15-hydroxy fatty acids following long-term astaxanthin supplementation. Astaxanthin did not affect more markers of lipid peroxidation, possibly because the subjects were young and healthy and were not exposed to any oxidative stress. We propose that astaxanthin supplementation may decrease *in vivo* lipid peroxidation in healthy men.

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