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Effects of Astaxanthin Supplementation on Lipid Peroxidation

Jouni Karppi¹, Tiina H. Rissanen^{1,2}, Kristiina Nyysönen¹, Jari Kaikkonen³, Anders G. Olsson⁴, Sari Voutilainen¹ and Jukka T. Salonen^{1,3}

¹ Research Institute of Public Health, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland. Fax: +358 17 162936. E-mail: jouni.karppi@uku.fi, kristiina.nyysonen@uku.fi, sari.voutilainen@uku.fi

² The Department of Public Health and Clinical Nutrition, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland. Fax: +358 17 162937. E-mail: tiina.rissanen@uku.fi

³ Oy Jurilab Ltd, Microkatu 1, FI-70210 Kuopio, Finland (www.jurilab.com). Fax: +358 17 467 8001. E-mail: jari.kaikkonen@jurilab.com, jukka.salonen@jurilab.com

⁴ Internal Medicine, Department of Medicine and Care, Berzelius Science Park, University hospital and University of Linköping, SE-58185 Linköping, Sweden. Fax: + 46 13 149991. E-mail: andol@imv.liu.se

Received for publication: November 15, 2005; Accepted for publication: April 6, 2006

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 peroxy 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

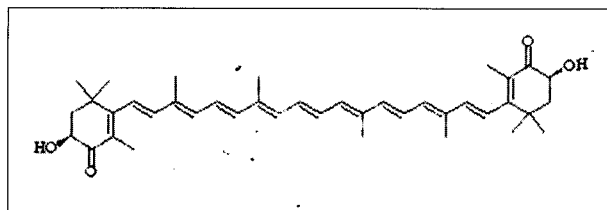


Figure 1: Chemical structure of astaxanthin.

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 μ L chloroform:methanol (1:1, v/v). A Rheodyne injection system (model 7010) was used to inject 25 μ L 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 Cu²⁺, 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 (V_{max}) 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. V_{max} was computed also by using the LSR method from the slope of the absorbance curve during the propagation phase [21]. The lag time and V_{max} were standardized against a plasma pool with previously determined values for the lag time and the V_{max}.

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 F₂-isoprostanes

Plasma samples for F₂-isoprostane measurements were frozen at -80 °C immediately after blood drawing. Two milliliters of EDTA plasma were needed to analyze F₂-isoprostanes by gas chromatography/mass spectrometry (GC/MS) methods. A deuterated prostaglandin F₂ internal standard was added to plasma, and F₂-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 F₂ 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 Ca²⁺ 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, Mannheim 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 1: 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/m ²)	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

