Erythrocytes Carotenoids after Astaxanthin Supplementation in Middle-Aged and Senior Japanese Subjects

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Abstract: A randomized, double-blind human trial was conducted to assess the effect of 4- or 12-week astaxanthin supplementation (1 or 3 mg/day) on the carotenoid compositions of erythrocytes in Japanese middle-aged and senior subjects. Erythrocyte astaxanthin concentrations after 4- or 12-weeks of supplementation (3 mg/day) was significantly higher than after placebo or 1 mg astaxanthin supplementations. No differences were observed in either the carotenoid compositions or the phospholipid hydroperoxide concentrations in erythrocytes after astaxanthin intake in both the 1 and 3 mg/day groups.

Key words: astaxanthin; carotenoids; erythrocyte compositions; human trial; phospholipid hydroperoxides

1 INTRODUCTION
Carotenoids are important cellular components in animals and humans. Experimental studies indicate that they enhance the immune system¹, inhibit mutagenesis², reduce chemically induced neoplasia³, and protect tissues from ultraviolet(UV) light-related damage⁴. Together with β-carotene, lutein, and lycopene, the potential antioxidant function of astaxanthin has been investigated⁵. Astaxanthin occurs naturally in plants and algae, and is commercially available as a food supplement from Haematococcus algae⁶. Little is known regarding the bioavailability of astaxanthin in humans, especially for Japanese middle-aged and senior subjects. Recently, we conducted a randomized, double-blind human trial to assess the efficacy of 12-week astaxanthin supplementation (6 or 12 mg/day)⁷. We confirmed that erythrocyte astaxanthin concentrations after oral intake of 6 or 12 mg/day for 12-weeks were significantly higher than erythrocyte astaxanthin concentrations observed before astaxanthin supplementation. Supplementation of astaxanthin for 12 weeks (12 mg/day) also resulted in a significant decrease in erythrocyte phospholipid hydroperoxide (PLOOH) concentrations in senior subjects⁷.

In this study, erythrocyte carotenoids and PLOOH concentrations were determined by high-performance liquid chromatography (HPLC) in Japanese middle-aged and senior individuals, after 4- and 12-weeks intake of 1 or 3 mg/day astaxanthin. Therefore, astaxanthin supplementation was lower in this study than in our previous study⁷. The principal objective was to investigate whether 1 or 3 mg of astaxanthin taken daily in Japanese subjects affects erythrocyte astaxanthin and PLOOH concentrations.

2 EXPERIMENTAL PROCEDURES
2.1 Subjects and supplementation trial
The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethics committee of TES Holdings Co. Ltd. (Tokyo; Correlation to: Teruo Miyazawa, Food and Biodynamic Chemistry Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, JAPAN
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Abbreviations: APCI, atmospheric pressure chemical ionization; CL, chemiluminescence; DAD, diode array detection; HPLC, high-performance liquid chromatography; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; MTBE, methyl tert-butyl ether; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; PLOOH, phospholipid hydroperoxides

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with a body mass index (BMI) of 24.9 (1.1) kg/m² for the 0 mg placebo group, 22.8 (1.1) kg/m² for the 1 mg group, and 24.4 (1.1) kg/m² for the 3 mg group were recruited from the TES Holdings volunteer database. Exclusion criteria included pregnancy, lactation, and severe medical illness. All subjects gave written informed consent, and were randomly divided into 3 groups.

During a 12-week trial, the subjects ingested 1 of 2 astaxanthin doses (1 or 3 mg) or a placebo in capsule form, with an appropriate amount of water, once daily after breakfast. The astaxanthin capsules were filled with astaxanthin-rich Haematococcus pluvialis oil (Puresta®, Yamaha Motor Co. Ltd., Shizuoka, Japan). The composition of the capsules was as follows: 160 mg olive oil, 60 mg corn oil, and 30 mg vitamin E/capsule for the placebo group. 20 mg Puresta oil 50, 200 mg olive oil, and 30 mg vitamin E/capsule for the low dose equivalent to 1 mg astaxanthin di-alcohol, and 60 mg Puresta oil 50, 160 mg olive oil, and 30 mg vitamin E/capsule for the high dose equivalent to 3 mg astaxanthin di-alcohol.

Throughout the study period, the subjects were instructed to maintain their usual lifestyle (avoid excessive eating and drinking, intense exercise, and lack of sleep). Adverse effects were assessed by interviews and self-reports, and compliance was checked by self-reports and returned capsule counts. Dietary intake, alcohol consumption, and physical activity (pedometer count) during the 3 days before each blood collection (0-, 4-, and 12-week) were also assessed by self-reports.

2.2 Determination of erythrocyte astaxanthin and other carotenoids

Before, during, and after the supplementation period (0-, 4- and 12-week), blood was drawn from the subjects after an overnight fast, using heparin as an anticoagulant. The blood was subjected to centrifugation at 1000 × g for 10 min at 4°C. After the plasma and buffy coat were removed, erythrocytes were washed 3 times with phosphate-buffered saline (pH 7.4) to prepare packed cells. For the determination of erythrocyte carotenoids (including astaxanthin), packed cells (2.5 mL) were diluted with 2.5 mL of water and then mixed with 5 mL of 80 mM ethanolic pyrogallol, 1.0 mL of 1.8 M aqueous potassium hydroxide (KOH), and 40 μL of 1 μM ethanolic echinenone (internal standard). After addition of 1.25 mL of 0.1 M aqueous sodium dodecyl sulfate, the sample was mixed with 15 mL of hexane and dichloromethane (5:1) to extract erythrocyte carotenoids. The extract was purified using a Sep-Pak silica cartridge (Waters, Milford, MA, USA), and then subjected to HPLC-tandem mass spectrometry (MS/MS) for the determination of astaxanthin concentration. Because astaxanthin has high polarity and tends to overlap with other erythrocYTE compounds, HPLC-MS/MS was employed to for the structure-specific detection of astaxanthin.

For the determination of astaxanthin, a stock solution was prepared by dissolving an astaxanthin standard in tetrahydrofuran at a concentration of 1 to 5 mM. The concentration of astaxanthin was checked spectrophotometrically using its extinction coefficient \(\varepsilon\). The astaxanthin standard and erythrocyte extracts were analyzed using HPLC-MS/MS equipment, which consisted of a liquid chromatograph (Shimadzu, Kyoto, Japan) and a 4000 QTRAP MS/MS instrument (Applied Biosystems, Foster City, CA, USA). The MS/MS parameters (e.g. collision energy) were optimized with an astaxanthin standard under positive atmospheric pressure chemical ionization (APCI). A C30 carotenoid column (4.6 × 250 mm, 5 μm; YMC, Kyoto, Japan) was used, and eluted with a binary gradient consisting of the following HPLC solvents: A, methanol:methyl tert-butyl ether (MTBE): water (83:15:2; containing 3.9 mM ammonium acetate) and B, methanol:MTBE:water (8:90:2; containing 2.6 mM ammonium acetate). The gradient profile was as follows: 0 − 12 min, 10 − 45% B linear; 12 − 24 min, 45 − 100% B linear; 24 − 38 min, 100% B. The flow rate was adjusted to 1 mL/min, and the column temperature was maintained at 25°C. Astaxanthin was determined in the post-column by MS/MS with multiple reaction monitoring (MRM) for the transition of the parent ion to the product ion \(7\).

For the determination of other carotenoids, standard carotenoid solutions were prepared in a similar way, and erythrocyte extracts were analyzed by HPLC coupled with UV diode array detection (DAD) and APCI MS, as previously described \(8\).

2.3 Determination of erythrocyte phospholipid hydroperoxides

For the determination of erythrocyte PLOOH concentrations, total lipids were extracted from packed cells with a mixture of 2-propanol and chloroform containing EDTA-2Na. Using erythrocyte total lipids, PLOOH levels (phosphatidylcholine hydroperoxide [PCOOH] and phosphatidyl-ethanolamine hydroperoxide [PEOOH]) were measured by HPLC coupled with chemiluminescence (CL) detection \(9\). The column was a 4.6 × 250 mm, 5-μm Finepak SIL NH2-5 column (Japan Spectroscopic Co., Tokyo, Japan), the eluent used was 2-propanol:methanol:water (135:45:20), and the flow rate was 1 mL/min. Post-column CL detection was carried out using a CLD-100 detector (Tohoku Electronic Industries Co., Sendai, Japan). A mixture of luminol and cytochrome C in 50 mM borate buffer (pH 10.0) was used as a hydroperoxide-specific post-column CL reagent. Calibration was carried out using PCOOH and PEOOH.
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2.4 Statistical analysis
Data were analyzed using IBM SPSS Statistics Ver. 19 (IBM Inc., NY, USA). Two-way mixed model analysis of variance (ANOVA) was used to test astaxanthin dose (groups) and treatment period (weeks) effects on erythrocyte carotenoid and PLOOH concentrations. In cases where interactions were identified, individual differences between means in each group were determined with the Bonferroni post-hoc test.

3 RESULTS AND DISCUSSION
When the astaxanthin standard was analyzed by MS/MS with flow injection, it displayed an intense molecular ion at m/z 597 (M+H)⁺. Product ion scanning was conducted for the ion, and astaxanthin-specific fragment ions (e.g., m/z 147) were identified. These ions, such as m/z 597 and 147, allowed the determination of erythrocyte astaxanthin concentrations using HPLC-MS/MS with MRM⁷ (Fig. 1A). In a typical MRM chromatogram of erythrocyte extract taken after supplementation, astaxanthin was clearly detected (Fig. 1B). As shown in Table 1, significant interactions (p < 0.001) were observed between astaxanthin supplementation and erythrocyte astaxanthin levels in Japanese middle-aged and senior subjects. Bonferroni post-hoc tests showed

![Typical multiple reaction monitoring (MRM) chromatograms of standard astaxanthin (1 pmol, A) and human erythrocyte astaxanthin (10 μL of erythrocyte extract prepared from astaxanthin-supplemented subject [3 mg/day for 12 weeks], B).](image)

Table 1 Carotenoid concentrations of erythrocytes in subjects after supplementation of astaxanthin.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>0 mg (n=10)</th>
<th>1 mg (n=10)</th>
<th>3 mg (n=10)</th>
<th>2-way ANOVA group x week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotenoids (nM/packed cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Astaxanthin</td>
<td>0 week</td>
<td>0.9</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.2⁺</td>
<td>0.2</td>
<td>0.8⁺</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.8⁺</td>
<td>0.9</td>
<td>0.2⁺</td>
</tr>
<tr>
<td>Lutein</td>
<td>0 week</td>
<td>37.6</td>
<td>27.8</td>
<td>28.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>28.1</td>
<td>11.6</td>
<td>18.3</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>31.7</td>
<td>16.9</td>
<td>36.1</td>
</tr>
<tr>
<td>β-Cryptoxanthin</td>
<td>0 week</td>
<td>13.0</td>
<td>11.0</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.8</td>
<td>2.3</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5.0</td>
<td>1.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Zeaxanthin</td>
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<td>8.6</td>
<td>5.0</td>
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<tr>
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<td>4</td>
<td>5.0</td>
<td>1.7</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
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<td>4.4</td>
<td>1.7</td>
<td>6.6</td>
</tr>
<tr>
<td>β-Carotene</td>
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<td>2.3</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.6</td>
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<tr>
<td></td>
<td>12</td>
<td>4.2</td>
<td>1.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Mean values with different alphabets are significantly different (p < 0.05) in the 2-way mixed model ANOVA with Bonferroni post-hoc test.
* : not significant.

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that a 3-mg dosage of astaxanthin for 4 and 12 weeks significantly increased erythrocyte astaxanthin levels ($p < 0.05$).

When standard carotenoids were subjected to HPLC-DAD by using a C30 column, they were well separated and eluted as follows: lutein, β-cryptoxanthin, zeaxanthin, echinone (internal standard), α-carotene, β-carotene, and lycopene. DAD limits for carotenoids were in the range of 0.1 to 0.25 pmol at a signal-to-noise ratio of 3. With regards to erythrocyte carotenoids in Japanese middle-aged and senior subjects, lutein, β-cryptoxanthin, zeaxanthin, and β-carotene were detected, with lutein being the most predominant (Table 1). Astaxanthin supplementation did not affect the concentrations of other carotenoids.

PCOOH and PEOOH were detected as the predominant PLOOH forms in erythrocytes. Astaxanthin supplementation (1 or 3 mg/day) did not affect erythrocyte PLOOH concentrations (sum of PCOOH and PEOOH) (Table 2). This may be due to the astaxanthin intake being lower in the current study than in our previous study. The present results suggest that supplementation of astaxanthin (3 mg/day) increases erythrocyte astaxanthin concentrations, but this dosage is not enough to prevent membrane phospholipid hydroperoxidation in erythrocytes.

There are several reports regarding the bioavailability of astaxanthin in humans, but few focus on Japanese subjects. Carotenoids are the most widespread group of hydrophobic pigments found in food. They may play a crucial role as electron-transport agents to protect organs, tissues, and cells from the damaging actions of reactive oxygen species and lipid peroxides, if they are present in sufficient amounts.

In conclusion, it was confirmed that erythrocyte astaxanthin concentrations were significantly increased in Japanese middle-aged and senior individuals supplemented for 4 and 12 weeks with 3 mg/day of astaxanthin. Significantly, this study suggests that even a low dose of astaxanthin consumption (3 mg/day) contributes to an increase in erythrocyte astaxanthin concentrations in Japanese subjects.

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References


