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Antioxidant effect of astaxanthin on phospholipid peroxidation in human erythrocytes

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Abstract

Phospholipid hydroperoxides (PLOOH) accumulate abnormally in the erythrocytes of dementia patients, and dietary xanthophylls (polar carotenoids such as astaxanthin) are hypothesised to prevent the accumulation. In the present study, we conducted a randomised, double-blind, placebo-controlled human trial to assess the efficacy of 12-week astaxanthin supplementation (6 or 12 mg/d) on both astaxanthin and PLOOH levels in the erythrocytes of thirty middle-aged and senior subjects. After 12 weeks of treatment, erythrocyte astaxanthin concentrations were higher in both the 6 and 12 mg astaxanthin groups than in the placebo group. In contrast, erythrocyte PLOOH concentrations were lower in the astaxanthin groups than in the placebo group. In the plasma, somewhat lower PLOOH levels were found after astaxanthin treatment. These results suggest that astaxanthin supplementation results in improved erythrocyte antioxidant status and decreased PLOOH levels, which may contribute to the prevention of dementia.

Key words: Astaxanthin: Phospholipid hydroperoxides: Erythrocytes: Dementia

We have previously confirmed that higher levels of phospholipid hydroperoxides (PLOOH), the primary oxidation products of phospholipids (PL)[1,2], accumulate abnormally in the erythrocytes of dementia patients[3]. Such erythrocytes with high levels of lipid hydroperoxides have been postulated to have a decreased ability to transport oxygen to the brain, which may impair blood rheology, thus facilitating dementia[4–8]. Recently, we have developed an HPLC method to determine erythrocyte carotenoid content[9]. Using this method, we gathered evidence that accumulation of polar oxygenated carotenoids (xanthophylls) occurs predominantly in human erythrocytes[9], and that a decrease in xanthophylls and an increase in PLOOH levels in erythrocytes correlate with the severity of dementia[10]. These findings led to the hypothesis that xanthophyll supplementation may minimise the accumulation of erythrocyte PLOOH, and that xanthophylls could be used therapeutically as drugs or functional foods to prevent the disease. Although there is still scarce information on whether orally administered xanthophylls are distributed to human erythrocytes and actually inhibit erythrocyte PLOOH formation, our recent human study has revealed antioxidant properties of the xanthophyll lutein towards erythrocyte PLOOH formation[11]. Animal studies have also supported this hypothesis[12,13].

Among xanthophylls, astaxanthin has recently received attention for its potent antioxidant activity[14,15]. Astaxanthin is naturally synthesised by plants and algae, and is now commercially available as a food supplement from *Haematococcus* alga[16]. The recommended daily intake is estimated to be 1–12 mg/d; however, there is not much information regarding the bioavailability of astaxanthin in humans. To the best of our knowledge, the occurrence and antioxidant roles of astaxanthin in human erythrocytes have not been reported.

In this investigation of whether administered astaxanthin is distributed to erythrocytes and inhibits erythrocyte PLOOH formation, we conducted a randomised, double-blind, placebo-controlled human trial. The efficacy of 12-week astaxanthin supplementation (6 or 12 mg) on both astaxanthin and PLOOH levels in the erythrocytes of thirty middle-aged and senior subjects was investigated. For erythrocyte astaxanthin analysis, a newly developed HPLC coupled with tandem MS (MS/MS) method was applied. Our findings (the inhibitory effect of astaxanthin on erythrocyte PLOOH)

Abbreviations: CL, chemiluminescence; MRM, multiple reaction monitoring; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; PL, phospholipid; PLOOH, phospholipid hydroperoxide.

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would provide new insights into the possible application of astaxanthin as an anti-dementia agent.

Subjects and methods

Subjects and materials

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 Anti-Aging Science (Tokyo, Japan; ethics no. I030807). All subjects were recruited from the Anti-Aging Science volunteer database, and written informed consent was obtained from all subjects. Exclusion criteria included pregnancy, lactation and severe medical illness.

Two doses of the test materials were prepared by filling soft capsules with astaxanthin-rich *Haematococcus pluvialis* oil (Puresta®; Yamaha Motor Company, Limited, Shizuoka, Japan)[17]. Compositions of the test materials were 75 mg Puresta oil 80 and 145 mg olive oil/capsule for the low dose (equivalent to 6 mg astaxanthin dialcohol); and 150 mg Puresta oil 80 and 70 mg olive oil/capsule for the high dose (equivalent to 12 mg astaxanthin dialcohol). To prepare placebo capsules, 150 mg maize oil was used instead of 150 mg Puresta oil 80. Placebo capsules were coloured to appear identical to test capsules.

Supplementation trial

A 12-week randomised, double-blind, placebo-controlled trial was conducted. A total of thirty healthy subjects (fifteen men and fifteen women), between 50 and 69 years of age (mean 56·3 (SD 5·3) years), with a BMI of 27·5 (SD 2·1) kg/m^2 were recruited, and randomly received 0 (placebo), 6 or 12 mg astaxanthin. During the 12-week trial, subjects ingested one of the three astaxanthin-dosed (0, 6 or 12 mg) capsules with an appropriate amount of water once daily after breakfast. Before and after the supplementation period (weeks 0 and 12, respectively), anthropometric data (e.g. height, body weight and blood pressure) and blood samples were collected from the subjects after they had fasted overnight, adverse effects were assessed by interviews and self-reports, and compliance was checked by self-reports and returned capsule counts. Throughout the study period, subjects were instructed to maintain their usual lifestyle (avoid excessive eating and drinking, intense exercise and lack of sleep). Dietary intake, alcohol consumption and physical activity (pedometer count) during the 3 d before each blood collection (weeks 0 and 12) were also assessed by self-reports.

Measurement of erythrocyte astaxanthin and other carotenoids

Blood samples were subjected to centrifugation at 1000 ***g*** for 10 min at 4°C. After the plasma and buffy coat were removed, erythrocytes were washed three times with PBS (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 were mixed with 5 ml of 80 mM-ethanolic pyrogallol, 1·0 ml of 1·8 M-aqueous KOH and 40 μl of 1 μM-ethanolic echinenone (internal standard)[9]. After the addition of 1·25 ml of 0·1 M-aqueous SDS, the sample was mixed with 15 ml of hexane–dichloromethane (5:1) to extract erythrocyte carotenoids. The extract was purified by a Sep-Pak silica cartridge (Waters, Milford, MA, USA), and then was subjected to HPLC-MS/MS for the determination of astaxanthin. The HPLC-MS/MS apparatus 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 optimised with an astaxanthin standard under positive atmospheric pressure chemical ionisation. The standard and erythrocyte extracts were separated with a C30 carotenoid column (4·6 × 250 mm, 5 μm; YMC, Kyoto, Japan). The column was eluted using a binary gradient consisting of the following HPLC solvents: A, methanol–methyl *tert*-butyl ether–water (83:15:2; containing 3·9 M-ammonium acetate); B, methanol–methyl *tert*-butyl ether–water (8:90:2; containing 2·6 M-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 detected in the post-column by MS/MS with multiple reaction monitoring (MRM) for the transition of the parent ion to the product ion. The concentration of erythrocyte astaxanthin was calculated using the standard curve of astaxanthin and was adjusted by the percentage recovery of the added echinenone (internal standard). For the determination of other carotenoids, erythrocyte extracts were analysed by HPLC coupled with UV diode array detection and atmospheric pressure chemical ionisation MS[9].

Measurement of erythrocyte phospholipid hydroperoxides

For the determination of erythrocyte PLOOH[1–3], total lipids were extracted from packed cells with a mixture of 2-propanol and chloroform containing butylated hydroxytoluene. PLOOH (i.e. phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH)) in the total lipids were measured by HPLC with chemiluminescence (CL) detection. The column was a 4·6 × 250 mm, 5 μm Finepak SIL NH2-5 (Japan Spectroscopic Company, Tokyo, Japan), the eluent 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 Company, 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 or PEOOH standards.

Other biochemical measurements

For plasma samples, astaxanthin, other carotenoids and PLOOH were determined by HPLC-MS/MS, HPLC-UV[9] and HPLC-CL[1–3], respectively. Tocopherols in erythrocytes

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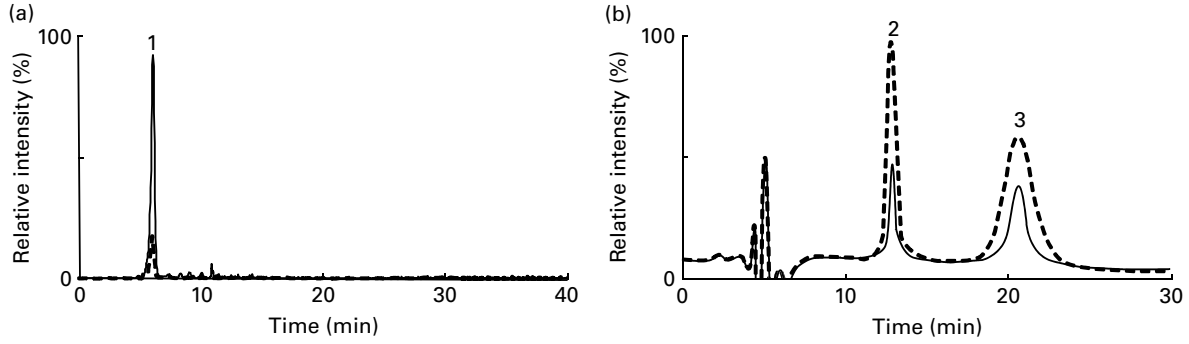

Fig. 1. Typical multiple reaction monitoring (MRM) and chemiluminescence (CL) chromatograms of (a) astaxanthin and (b) phospholipid hydroperoxides (PLOOH) in erythrocytes taken (– – –) before and (——) after 12-week supplementation of astaxanthin (12 mg/d). Erythrocyte astaxanthin and PLOOH were determined by HPLC-MS/MS with MRM and HPLC-CL, respectively. Peak identifications are as follows: 1, astaxanthin; 2, phosphatidylcholine hydroperoxide; 3, phosphatidylethanolamine hydroperoxide.

and plasma were measured by HPLC with fluorescence detection[18]. Also, blood chemistries such as haematological and blood biochemical parameters were analysed using standardised methods.

Statistical analysis

Data are expressed as means and standard deviations. One-way ANOVA was used to compare means among the three groups. If a statistically significant difference ($P<0{\cdot}05$) was detected, Dunnett's test was performed for comparison between the control group and one of the two astaxanthin groups. For comparison of baseline (week 0) and post-dosing (week 12) values in each treatment group, a paired *t* test was used. These statistical analyses were done using SPSS for Windows (SPSS Inc., Chicago, IL, USA).

Results

As mentioned in the introduction, the distribution of astaxanthin in erythrocytes has not been reported, mainly due to the lack of an analytical method. We, therefore, developed an HPLC-MS/MS method to analyse for erythrocyte astaxanthin before we conducted the 12-week astaxanthin supplementation study. In brief, an astaxanthin standard was analysed by MS/MS with flow injection, and astaxanthin showed 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 (*m/z* 597 and 147) allowed us to quantitatively determine erythrocyte astaxanthin concentrations using HPLC-MS/MS with MRM (Fig. 1(a)).

In the human trial, before administration (baseline), there were no significant differences in anthropometric parameters among the three groups (Table 1). The trial was completed without any of the subjects withdrawing. The mean compliance to the prescribed dose for the 6 mg astaxanthin, 12 mg astaxanthin and placebo groups was 99·5 (SD 3·7), 98·1 (SD 1·9) and 98·7 (SD 2·4) %, respectively. The average energy intake during the 12-week trial, as calculated from the self-reports, did not statistically differ among the groups. Furthermore, no significant differences were observed among the groups in the intake of each type of nutrient (carbohydrate, protein, fat, cholesterol and fibre), alcohol consumption and pedometer counts. Data from all thirty subjects were, therefore, included in the statistical analysis.

The results of the physical, haematological and blood biochemical measurements before and after 12 weeks of dosing are shown in Tables 2 and 3. Some parameters (i.e. Hb, haematocrit, mean corpuscular volume, mean corpuscular Hb, uric acid, total cholesterol, LDL, fasting glucose and Hb_{A1c}) showed changes from baseline in the astaxanthin groups

Table 1. Baseline characteristics of the study subjects in the 0 (placebo), 6 and 12 mg astaxanthin groups (Mean values and standard deviations)

	0 mg		6 mg		12 mg		
Background factors	Mean	SD	Mean	SD	Mean	SD	*P**
Age (years)	56·6	4·4	56·3	6·6	56·1	5·1	0·979
Total number of subjects	10		10		10		
Men	5		5		5		
Women	5		5		5		
Height (cm)	159	11	160	8	164	7	0·407
Weight (kg)	70·3	9·3	70·5	8·1	74·4	5·3	0·429
BMI (kg/m^2)	27·7	2·1	27·4	2·2	27·6	2·1	0·946
Systolic blood pressure (mmHg)	133	92	124	16	134	18	0·371
Diastolic blood pressure (mmHg)	83·1	10·9	82·3	9·8	90·7	15·0	0·250

* One-way ANOVA test among groups.

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and/or the placebo group. However, these changes were small and were observed to be within the normal range irrespective of the test materials administered. Moreover, no differences were noted among the three groups in any parameters at baseline or post-dosing. Therefore, the physical and metabolic states of the subjects were considered to be randomised homogeneously throughout the trial.

In the typical MRM chromatogram of the erythrocyte extract taken before and 12 weeks after supplementation, astaxanthin was clearly detected (Fig. 1(a)). After supplementation, the erythrocyte astaxanthin concentration significantly increased and was higher than that of the placebo group (Table 4). On the other hand, in a typical CL chromatogram of erythrocyte total lipids, PCOOH and PEOOH were identified as the predominant PLOOH forms (Fig. 1(b)). After supplementation, erythrocyte PLOOH concentration decreased and was lower than that of the placebo group (Table 4). In plasma, the only detectable PLOOH was PCOOH, and a somewhat lower PCOOH level was found after astaxanthin supplementation (Table 5). In both erythrocytes and plasma, astaxanthin supplementation did not affect the levels of other carotenoids (except for small changes in lycopene and lutein) and tocopherols. These results suggest that upon ingestion of astaxanthin, it is absorbed, distributed and accumulated in erythrocytes, where it acts as an antioxidant molecule, thereby reducing PLOOH, an index of oxidative stress.

Discussion

In recent years, medical and nutritional experts have seriously considered the antioxidant properties of food constituents, since the reactive oxygen species-mediated peroxidation of

Table 2. Changes in physical and haematological parameters before and after the 12-week administration of 0, 6 or 12 mg astaxanthin

(Mean values and standard deviations)

	0 mg		6 mg		12 mg		
Parameters	Mean	SD	Mean	SD	Mean	SD	*P*†
Weight (kg)							
Before	70·3	9·3	70·5	8·1	74·4	5·3	0·429
After	69·0	9·2	69·6	7·5	74·3	5·9	0·248
BMI (kg/m^2)							
Before	27·7	2·1	27·4	2·2	27·6	2·1	0·946
After	27·1	2·2	27·1	2·2	27·6	2·5	0·872
Systolic blood pressure (mmHg)							
Before	133	18	124	16	134	18	0·371
After	134	16	127	17	134	16	0·555
Diastolic blood pressure (mmHg)							
Before	83·1	10·9	82·3	9·8	90·7	15·0	0·250
After	81·4	9·7	84·2	9·2	86·1	9·1	0·532
Heart rate (bpm)							
Before	70·5	12·5	71·8	11·2	70·9	4·0	0·956
After	68·7	11·6	71·1	7·1	75·6	6·2	0·213
Leucocytes (×10^9/l)							
Before	4·6	0·6	5·4	1·0	5·7	1·7	0·096
After	4·9	0·8	5·1	0·8	6·1	1·9	0·112
Erythrocytes (×10^{12}/l)							
Before	4·5	0·4	4·7	0·3	4·7	0·4	0·587
After	4·5	0·4	4·7	0·3	4·7	0·5	0·375
Hb (g/l)							
Before	144	11	145	11	142	27	0·916
After	137**	11	142	13	141	23	0·799
Haematocrit (%)							
Before	44·9	3·1	45·4	3·8	44·1	6·9	0·839
After	42·8**	2·9	44·1	4·1	43·9	5·2	0·750
Corpuscular volume (fl)							
Before	99·1	6·3	96·0	3·3	94·8	12·9	0·522
After	96·3**	4·7	94·7**	3·3	94·0	12·1	0·783
Corpuscular Hb (pg/cell)							
Before	31·7	2·1	30·8	1·1	30·5	5·3	0·735
After	30·9**	1·7	30·4**	1·2	30·3*	5·1	0·193
Corpuscular Hb concentration (%)							
Before	32·0	0·8	32·1	0·8	32·0	2·1	0·983
After	32·1	0·6	32·1	0·6	32·0	2·1	0·988
Platelets (×l0^9/l)							
Before	237	47	225	41	261	67	0·320
After	212	55	228	28	264	82	0·151

bpm, Beats/min.

Mean values were significantly different in the paired *t* test between before and after astaxanthin administration: *$P < 0·05$, **$P < 0·01$.

† One-way ANOVA test among groups.

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Table 3. Changes in blood biochemical parameters before and after the 12-week administration of 0, 6 or 12 mg astaxanthin

(Mean values and standard deviations)

	0 mg		6 mg		12 mg		
Parameters	Mean	SD	Mean	SD	Mean	SD	*P*†
Total protein (g/l)							
Before	73·0	2·3	74·6	2·1	71·9	3·5	0·950
After	72·6	2·1	73·9	1·8	71·5	3·8	0·162
Albumin (g/l)							
Before	45·0	1·6	45·7	1·1	44·2	2·2	0·161
After	44·6	1·0	45·4	1·1	43·8	2·2	0·081
Total bilirubin (mg/l)							
Before	8·0	2·9	7·0	2·1	7·0	2·7	0·615
After	7·4	1·9	7·2	2·9	7·3	2·9	0·986
GOT (U/l)							
Before	22·6	6·9	25·0	9·2	23·0	8·4	0·786
After	21·0	5·6	21·3	3·9	23·6	11·0	0·700
GPT (U/l)							
Before	25·7	17·6	30·6	15·2	24·1	12·9	0·620
After	21·2	13·0	24·2	8·0	25·0	15·6	0·779
ALP (U/l)							
Before	225	67	238	55	203	72	0·489
After	225	58	236	58	197	60	0·336
γ-GTP (U/l)							
Before	40·8	23·6	35·1	15·2	41·3	31·5	0·820
After	37·3	19·4	30·7	11·4	41·6	38·5	0·638
Urea (mg/l)							
Before	129	25	144	31	146	24	0·340
After	139	29	128	25	135	29	0·671
Creatinine (mg/l)							
Before	7·0	1·5	7·3	1·4	7·8	2·1	0·543
After	7·0	1·5	7·2	1·5	8·0	1·9	0·404
Uric acid (mg/l)							
Before	52·0	11·8	62·6	19·2	57·8	16·8	0·357
After	53·1	10·4	57·9*	16·0	56·3	13·7	0·721
Total cholesterol (mg/l)							
Before	2180	440	2260	390	2030	230	0·372
After	2040*	430	2150	320	1960	150	0·401
HDL (mg/l)							
Before	588	147	688	190	588	121	0·268
After	581	130	655	147	562	105	0·250
LDL (mg/l)							
Before	1350	350	1320	370	1170	200	0·396
After	1220*	370	1240	290	1120	190	0·629
TAG (mg/l)							
Before	1250	520	1110	780	1250	730	0·869
After	1140	410	1160	670	1360	1140	0·790
Fasting glucose (mg/l)							
Before	1010	60	1020	110	1010	60	0·948
After	1040**	50	1040	110	1050	110	0·985
Hb_{Alc} (%)							
Before	5·1	0·3	5·1	0·3	5·1	0·3	0·982
After	5·2	0·3	5·2	0·2	5·2**	0·3	1·000

GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; ALP, alkaline phosphatase; γ-GTP, γ-glutamic transpeptidase.

Mean values were significantly different in the paired *t* test between before and after astaxanthin administration: *$P<0·05$, **$P<0·01$.

† One-way ANOVA test among groups.

biological molecules (e.g. lipids) has been postulated to induce a variety of pathological events such as atherogenesis, ageing and dementia. Although many *in vitro* studies on the antioxidant properties of food constituents have been reported, little is known about the biological functions of dietary antioxidants *in vivo* (especially in humans), except for a few major antioxidants (e.g. tocopherols and ascorbic acid). Since the bioavailability of food constituents is limited by their digestibility and metabolic fate, oral administration trials are favoured in evaluating their biological functions.

The present randomised, double-blind, placebo-controlled human trial shows that when human subjects ingest astaxanthin, it is absorbed, distributed and accumulated in erythrocytes, where it exhibits antioxidative effects (inhibition of erythrocyte PLOOH). It is interesting to note that the antioxidative effect observed in the present study was

Table 4. Changes in carotenoids, phospholipid hydroperoxides (PLOOH) and tocopherol contents in erythrocytes taken before and after the 12-week administration of 0, 6 or 12 mg astaxanthin
(Mean values and standard deviations)

	0 mg		6 mg		12 mg		
Parameters	Mean	SD	Mean	SD	Mean	SD	*P*‡
Carteonoids (pmol/ml packed cells)							
Astaxanthin							
Before	4·0	1·7	2·9	1·3	2·8	1·0	0·112
After	3·6	1·2	35·7**††	11·1	44·9**††	24·0	0·000
Lutein							
Before	43·3	6·8	39·8	7·5	39·5	6·1	0·391
After	43·2	8·5	42·1*	8·0	40·6	7·4	0·765
Zeaxanthin							
Before	7·8	2·1	7·1	1·4	7·6	2·1	0·662
After	7·8	2·6	6·9	1·2	7·4	1·8	0·603
β-Cryptoxanthin							
Before	10·5	2·6	9·9	3·0	9·6	2·1	0·735
After	10·1	2·4	9·5	2·7	9·4	2·6	0·802
α-Carotene							
Before	1·2	0·4	0·9	0·4	1·1	0·3	0·234
After	1·1	0·4	0·9	0·3	1·1	0·3	0·151
β-Carotene							
Before	3·9	0·3	3·8	0·4	3·7	0·4	0·678
After	3·9	0·4	3·8	0·5	3·8	0·6	0·902
Lycopene							
Before	0·7	0·1	0·6	0·1	0·7	0·2	0·231
After	0·7	0·1	0·6	0·1	0·6*	0·2	0·273
Xanthophylls§							
Before	65·5	10·3	59·5	11·1	59·5	9·1	0·407
After	64·7	11·4	94·2**††	20·9	102·4**††	20·6	0·000
Non-polar carotenoids‖							
Before	5·7	0·6	5·3	0·7	5·5	0·7	0·350
After	5·7	0·7	5·3	0·6	5·5	0·9	0·421
Total carotenoids							
Before	71·3	10·6	64·8	11·6	65·1	9·8	0·397
After	70·4	11·7	99·4**††	21·5	107·9**††	20·5	0·000
PLOOH (pmol/ml packed cells)							
PCOOH							
Before	8·8	6·6	8·7	4·6	12·3	7·0	0·340
After	9·1	6·3	5·2**	2·7	6·6**	2·3	0·122
PEOOH							
Before	5·1	2·4	4·6	3·6	6·3	6·5	0·706
After	5·8	2·6	2·8*†	1·8	3·0†	2·5	0·011
Total PLOOH¶							
Before	13·9	7·6	13·3	6·1	18·6	11·6	0·353
After	14·9	8·3	8·0**†	3·8	9·7**†	4·0	0·031
Tocopherols (nmol/ml packed cells)							
α-Tocopherol							
Before	7·8	2·4	7·2	2·6	6·9	1·0	0·663
After	8·4	1·9	7·8	0·9	7·6	0·8	0·365
γ-Tocopherol							
Before	0·9	0·4	0·8	0·5	0·9	0·3	0·872
After	0·8	0·3	0·8	0·4	0·8	0·3	0·998
Total tocopherols							
Before	8·7	2·3	8·0	2·6	7·8	1·1	0·652
After	9·2	2·0	8·6	1·0	8·4	0·9	0·372

PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide.
Mean values were significantly different in the paired *t* test between before and after astaxanthin administration: **P*<0·05, ***P*<0·01.
Mean values were significantly different in Dunnett's test between the control group and one of the two astaxanthin groups: †*P* < 0·05, ††*P*<0·01.
‡ One-way ANOVA test among groups.
§ Xanthophylls are the sum of astaxanthin, lutein, zeaxanthin and β-cryptoxanthin.
‖ Non-polar carotenoids are sum of α-carotene, β-carotene and lycopene.
¶ PLOOH are the sum of PCOOH and PEOOH.

produced by a relatively short-term supplementation with astaxanthin (12 weeks).

In the present study, since the distribution of astaxanthin in erythrocytes had not previously been reported, we developed an HPLC-MS/MS method to analyse the erythrocyte astaxanthin content before we conducted the astaxanthin supplementation study. Using MS/MS, we found that protonated astaxanthin tended to generate product ions (e.g. *m/z* 147).

Table 5. Changes in carotenoids, phospholipid hydroperoxides (PLOOH) and tocopherols contents in plasma before and after the 12-week administration of 0, 6 or 12 mg astaxanthin
(Mean values and standard deviations)

	0 mg		6 mg		12 mg		
Parameters	Mean	SD	Mean	SD	Mean	SD	*P*‡
Carteonoids (pmol/ml)							
Astaxanthin							
Before	9	4	6	3	8	6	0·383
After	8	4	86**††	30	109**††	49	0·000
Lutein							
Before	520	82	480	88	473	75	0·398
After	529	104	489	107	484	108	0·584
Zeaxanthin							
Before	140	37	127	25	137	37	0·663
After	139	46	124	22	134	33	0·607
β-Cryptoxanthin							
Before	283	71	266	80	259	56	0·740
After	273	66	256	73	253	71	0·802
α-Carotene							
Before	131	43	100	45	130	37	0·182
After	130	44	97	39	127	37	0·147
β-Carotene							
Before	673	50	662	70	651	68	0·742
After	679	63	667	80	660	103	0·883
Lycopene							
Before	153	20	131	26	136	35	0·210
After	143*	19	122*	25	128*	38	0·275
Xanthophylls§							
Before	952	174	880	183	877	156	0·549
After	950	178	956**	188	980**	202	0·931
Non-polar carotenoids‖							
Before	957	89	892	106	917	111	0·375
After	951	102	887	97	916	144	0·475
Total carotenoids							
Before	1909	238	1772	261	1794	253	0·434
After	1901	237	1843*	264	1896*	320	0·872
PLOOH (pmol/ml)							
PCOOH							
Before	26·2	8·0	18·7†	5·6	18·2*	6·4	0·021
After	27·4	7·2	15·6*††	3·4	13·9††	4·4	0·000
Tocopherols (nmol/ml)							
α-Tocopherol							
Before	44·7	9·3	42·8	17·6	36·9	5·6	0·328
After	45·1	10·5	39·5	8·5	38·9	10·7	0·326
γ-Tocopherol							
Before	4·1	2·0	3·2	0·9	3·8	1·9	0·483
After	3·8	0·8	3·1	0·8	3·8	1·4	0·302
Total tocopherols							
Before	48·8	8·7	46·0	17·4	40·6	6·6	0·314
After	48·8	10·4	42·6	8·8	42·7	11·0	0·304

PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide.
Mean values were significantly different in the paired *t* test between before and after astaxanthin administration: *$P<0·05$, **$P<0·01$.
Mean values were significantly different in Dunnett's test between the control group and one of the two astaxanthin groups: †$P<0·05$, ††$P<0·01$.
‡ One-way ANOVA test among groups.
§ Xanthophylls are the sum of astaxanthin, lutein, zeaxanthin and β-cryptoxanthin.
‖ Non-polar carotenoids are sum of α-carotene, β-carotene and lycopene.

The product ion indicated that MRM could be adapted to the HPLC-MS/MS analysis of astaxanthin. Under optimised conditions, the detection limit of the HPLC-MS/MS with the MRM method was very sensitive at 0·02 pmol astaxanthin/injection. The characteristics and advantages of our HPLC-MS/MS method are as follows. The method was selective and sensitive enough to measure astaxanthin in erythrocytes (Fig. 1(a)) as well as in the plasma. Also, the method was sufficiently simple and convenient to be applicable to a large number of samples. The method, therefore, would be a powerful tool for studying the metabolic fate of astaxanthin as well as its bioavailability.

Until now, there are few reports concerning human erythrocyte carotenoids. Some studies have successfully detected erythrocyte carotenoids (mainly β-carotene)[19], while other studies have been unable to detect these

species[20]. Incorporation of a carotenoid (β-carotene) into erythrocytes after oral supplementation has been described in some reports[21]. However, there has been no study evaluating whether administered carotenoids other than β-carotene are distributed to erythrocytes, except for our recent human study of the xanthophyll lutein[11]. In the present study, using the newly developed HPLC-MS/MS analysis method, incorporation of astaxanthin into erythrocytes after oral supplementation was established (Fig. 1(a)). Because both the erythrocyte and plasma astaxanthin concentrations increased (Tables 4 and 5), it seems likely that astaxanthin in plasma lipoprotein particles is partly transferred into erythrocytes. By this hypothesis, astaxanthin would be located on the outer region of plasma lipoproteins, which would facilitate its transfer to erythrocytes. On the other hand, the concentrations of endogenous antioxidants (i.e. carotenoids and tocopherols) showed virtually no change before and after astaxanthin supplementation (Tables 4 and 5). This is advantageous for elucidating the antioxidant contribution of astaxanthin. By the way, it was known that blood carotenoid concentration in females is somewhat higher than that in males. In the present study, we compared sex difference in blood carotenoids, but no statistical differences were observed between males and females in each carotenoid at baseline or post-dosing.

In the present study, to evaluate peroxidisability, we measured the PLOOH content. Because PLOOH are the primary oxidation products of PL, an increase in PLOOH directly reflects *in vivo* oxidative stress[1–3,22,23]. As has been observed, astaxanthin supplementation clearly reduced the erythrocyte PLOOH concentration (Fig. 1(b)), indicating that astaxanthin incorporation into erythrocytes attenuated PL peroxidation of erythrocyte membranes. On the other hand, the antioxidant effect of astaxanthin seemed to be more apparent on the erythrocyte membrane, as compared with the plasma (Tables 4 and 5). Erythrocytes are rich in PUFA in their PL bilayer, and contain high concentrations of molecular oxygen and ferrous ions as constituents of oxyhaemoglobin. The oxidation of Hb is accompanied by the formation of superoxides, a source of reactive oxygen species. Therefore, erythrocyte membrane PL would be more susceptible to peroxidation than other organelle membranes, even though they are protected by several antioxidative systems such as superoxide dismutase, catalase and glutathione peroxidase. For plasma PCOOH, unexpectedly, at baseline (week 0), groups taken 6 or 12 mg astaxanthin showed significantly less PCOOH than the group taken 0 mg astaxanthin. Because no differences were observed among the three groups in other parameters (e.g. haematological and blood biochemical values), it might be other factor(s) affecting plasma PCOOH before the start of the study. This possibility needs further investigation.

In the present study, when comparing erythrocyte PCOOH between the placebo and astaxanthin groups, PCOOH levels after astaxanthin supplementation (5·2 and 6·6 pmol/ml packed cells for 6 and 12 mg astaxanthin groups, respectively) were somewhat lower but not statistically significant as compared with those of the placebo group (9·1 pmol/ml packed cells; $P=0·122$; Table 4). In contrast, PEOOH levels after astaxanthin supplementation (2·8 and 3·0 pmol/ml packed cells for 6 and 12 mg astaxanthin groups, respectively) were significantly lower ($P=0·011$) than those of the placebo group (5·8 pmol/ml packed cells). PLOOH (sum of PCOOH and PEOOH) is, therefore, considered to show significant changes ($P=0·031$) between the placebo (14·9 pmol/ml packed cells) and astaxanthin groups (8·0 and 9·7 pmol/ml packed cells for 6 and 12 mg astaxanthin groups, respectively). Considering these, the antioxidant effect of astaxanthin appears likely to be through the reduction of erythrocyte PEOOH rather than PCOOH. This possibility needs to be clarified in future studies. On the other hand, for the efficacy of astaxanthin, inhibitory effects of the 6 mg astaxanthin group on PCOOH and PEOOH were as good as or even better than those of the 12 mg astaxanthin group (Tables 4 and 5). Concentrations of erythrocytes and plasma astaxanthin were not different between the 6 and 12 mg astaxanthin groups, suggesting that 6 mg astaxanthin is effective enough to show antioxidative benefit *in vivo*. Thus, to estimate the optimal dose of astaxanthin, we are now conducting a human study by administering 3–6 mg astaxanthin to volunteers.

Among the carotenoids (xanthophylls), astaxanthin has recently received increased scientific interest due to its potent antioxidant activity and hence possible anti-metabolic syndrome, anti-brain ageing and anti-atopic dermatitis effects[24–26]. We have previously found that there was a higher accumulation of PLOOH in the erythrocytes of dementia patients[3]. Erythrocytes high in lipid hydroperoxides have been suggested to have a decreased ability to transport oxygen to the brain and may impair blood rheology, thus facilitating dementia[4–8]. In the present study, orally administered astaxanthin was incorporated into erythrocytes, and erythrocyte PLOOH levels decreased. On the basis of these points, it seems that similar to lutein[11], astaxanthin has the potential to act as an important antioxidant in erythrocytes, and thereby may contribute to the prevention of dementia. This possibility warrants the testing of astaxanthin in other models of dementia with a realistic prospect of its use as a human therapy.

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