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Chlorella is an Effective Dietary Source of Lutein for Human Erythrocytes

Taiki Miyazawa[1], Kiyotaka Nakagawa[1], Fumiko Kimura[1], Yuya Nakashima[2], Isao Maruyama[2], Ohki Higuchi[3] and Teruo Miyazawa[1]*

[1] *Food and Biodynamic Chemistry Laboratory, Graduate School of Agricultural Science, Tohoku University (Sendai 981-8555, Japan)*
[2] *Department of Research & Development, Chlorella Industry Co., Ltd. (Fukuoka 833-0056, Japan)*
[3] *Biodynamic Plant Institute Co., Ltd. (Hokudai Business Spring 101, Sapporo 001-0021, Japan)*

Abstract: *Chlorella* contains a high amount of carotenoids, especially lutein, and has received attention as a possible dietary source for improving carotenoid levels in human blood. In the present study, we performed a 2-month single arm human study, and investigated the efficacy of *Chlorella* supplementation (9 g *Chlorella*/day; equivalent to 32 mg lutein/day) on lutein and other carotenoid concentrations in plasma as well as erythrocytes of 12 healthy subjects. Following *Chlorella* supplementation, lutein was the predominant carotenoid in erythrocytes, showing a 4-fold increase (from 14 to 54 pmol/mL packed cells). After the one month without *Chlorella* ingestion, erythrocyte lutein then decreased to a basal level (17 pmol/mL packed cells). Erythrocyte carotenoid (lutein, zeaxanthin, α-carotene, and β-carotene) levels were proportional to plasma carotenoid levels. The results suggest the transfer of *Chlorella* carotenoids, especially lutein, from plasma lipoprotein particles to the erythrocyte membrane. *Chlorella* intake would be effective for improving and maintaining lutein concentrations in human erythrocytes.

Key words: *Chlorella*, carotenoids, lutein, erythrocytes

1 INTRODUCTION

Chlorella (*Chlorella vulgaris*), a unicellular green alga, is used as a traditional food and is recognized as a potential source of various nutrients, such as proteins, lipids, minerals, and vitamins[1, 2]. To date, developments in mass production technology have facilitated large scale cultivation of *Chlorella* for therapeutic purposes[3]. In recent animal and human studies, *Chlorella* has been reported to show anti-oxidative, anti-hypertensive, anti-inflammatory, immune-modulatory, and anti-diabetic activities[4–7], and these beneficial effects are considered to be attributed to *Chlorella* contents, including ascorbic acid, tocopherol, and L-arginine[8].

In addition to these ingredients, *Chlorella* is rich in bioactive carotenoids, especially lutein[9–11]. A few days after a single oral dose of 6 g *Chlorella* tablets (containing 15 mg lutein) to healthy volunteers, blood serum lutein concentration increased and reached a maximum of average 850 pmol/mL serum (about one and a half-fold from the fasting conditions)[12], and the concentration was maintained over a 3-day period. *Chlorella*, therefore, appears to be a good dietary source for improving lutein bioavailability.

On the other hand, carotenoids are known to be present in both human plasma and erythrocytes[13]. As a polar carotenoid, lutein is predominant in erythrocyte membranes[14]. Previous animal and human studies suggested that lutein supplementation resulted in improved erythrocyte antioxidant status and decreased oxidative stress, which may contribute to the prevention of life-threatening diseases, especially senile dementia[15]. We previously observed that senile dementia's erythrocytes contain much higher concentration of lipid hydroperoxides than normal subjects[21]. And we observed pure lutein supplementation decreased the erythrocyte hydroperoxides in normal subjects[22]. While the importance of erythrocyte lutein has been suggested, there is little information from human trials regarding food sources that could efficiently supplement lutein to erythrocyte membranes.

In this study, we hypothesized that continuous dietary intake of *Chlorella* might increase the lutein level of human erythrocytes. To evaluate the hypothesis, we performed a 2-month single arm human study with *Chlorella* supplementation (9 g *Chlorella*/day).

*Correspondence to: Teruo Miyazawa, Food and Biodynamic Chemistry Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, JAPAN
E-mail: miyazawa@biochem.tohoku.ac.jp
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2 EXPERIMENTAL PROCEDURES

2.1 Materials

Lutein, zeaxanthin, β-cryptoxanthin and lycopene were purchased from Extrasynthese (Lyon, France). Echinenone, α-carotene, β-carotene, pyrogallol, methanol, ammonium acetate, and methyl *tert*-butyl ether (MTBE) were obtained from Wako (Osaka, Japan). *Chlorella* tablets, consisting of *Chlorella* powder (Biorinck®), were kindly provided by Chlorella Industry (Fukuoka, Japan). Each tablet (200 mg) contained 0.71 mg lutein, 0.06 mg zeaxanthin, 0.01 mg α-carotene, and 0.03 mg β-carotene for carotenoids, and 0.04 mg α-tocopherol for vitamin E. All other reagents were of analytical grade.

2.2 Methods

2.2.1 Supplementation study

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 Chlorella Industry C., Ltd. (ethics no. PMKK-2011-001). Exclusion criteria included pregnancy, lactation, and severe medical illness. Twelve healthy subjects (aged 29-61 years; six men and six women) participated in this study. Written informed consent was obtained from all subjects according to the criterion of the ethics committee.

Subjects took 15 tablets (containing 10.67 mg lutein, 0.99 mg zeaxanthin, 0.12 mg α-carotene, 0.51 mg β-carotene and 0.53 mg α-tocopherol) 3 times per day (at breakfast, lunchtime, and dinner) for 2 months. Thus, the daily dose of *Chlorella* was 9 g/day (equivalent to 32 mg lutein/day). At one month and 2 months after the *Chlorella* supplementation, blood was collected into a tube with ethylenediamine tetraacetic acid as an anticoagulant. One month after completing the ingestion trial (one-month observation period), blood was also collected. The blood samples were subjected to centrifugation at 1000 g for 10 min at 4℃. After the plasma and buffy coat were removed, erythrocytes were washed 3 times with phosphate buffered saline (pH 7.4) to prepare packed cells. The packed cells were immediately subjected to determination of carotenoids. Plasma samples were stored at −80℃ until analysis.

2.2.2 Extraction of carotenoids from erythrocytes

Two mL of packed cells were diluted with 2 mL of water, and were mixed with 4 mL of 80 mmol/L ethanolic pyrogallol, 0.8 mL of 1.8 mol/L aqueous KOH, and 125 μL of 1 μmol/L ethanolic echinenone (an internal standard). After addition of 1 mL of 0.1 mol/L aqueous sodium dodecyl sulfate, the sample was mixed with 12 mL of hexane/dichloromethane (5:1, v/v, containing 2.4 mmol/L butylated hydroxytoluene) for extraction of erythrocyte carotenoids. The extract was purified by Sep-Pak silica cartridge (Waters, Milford, MA), and then subjected to HPLC coupled with ultraviolet (UV) detection[14]. These extraction procedures were conducted under subdued (yellow) light to minimize photo degradation of the carotenoids.

2.2.3 Measurement of carotenoids and blood chemistries

A C30 carotenoid column (4.6×250 mm, 5 μm; YMC, Kyoto, Japan) was used, and the column was eluted using a binary gradient consisting of the following HPLC solvents: A, methanol/MTBE/water (83:15:2 v/v/v, containing 3.9 mmol/L ammonium acetate), and B, methanol/MTBE/water (8:90:2 v/v/v, containing 2.6 mmol/L ammonium acetate). The gradient profile was as follows: 0-12 min, 10-55% B linear; 12-20 min, 55-100% B linear; 20-25 min, 100% B linear; and 25-27 min, 100-10%. The flow rate was adjusted to 1 mL/min, and the column temperature was maintained at 30℃. The column eluent was sent to a UV detector (UV-2075 PLUS, JASCO, Tokyo, Japan) for monitoring lutein and other carotenoids at 463 nm. Concentrations of erythrocyte carotenoids were calculated using an equation corresponding to the standard curve of each carotenoid and were adjusted by the percentage recovery of the added echinenone (the internal standard).

Plasma lutein and other carotenoids were determined by HPLC-UV[14]. Plasma α-tocopherol was measured by HPLC with fluorescence detection[16]. Blood characteristics were analyzed using standardized methods.

2.2.4 Statistical analyses

Data were expressed as mean values and standard deviations. All data were analyzed by a normality test and a Friedman test followed by Scheffe's method with non-normally distributed data. Meanwhile, comparisons of data collected before and after supplementation were analyzed by paired t-tests with normally distributed data, and Wilcoxon signed-rank test for non-normally distributed data. Correlative relationships were assessed with Spearman's rank correlation coefficient with abnormally distributed data. These statistical analyses were conducted using Excel Toukei 2010 (Social Survey Research Information, Tokyo, Japan).

3 RESULTS

Blood biochemical measurements before and after the 2-month *Chlorella* ingestion trial are shown in Table 1. Among these parameters, plasma α-tocopherol was significantly increased by *Chlorella* ingestion, not surprising given the substantial amount of α-tocopherol present in *Chlorella*. Significant differences were observed in albumin, alkaline phosphatase (ALP), HDL cholesterol, platelets, leucocytes, erythrocytes, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and hematocrit after *Chlorella* ingestion. However, these changes were small and were found to be within normal ranges.

Before *Chlorella* supplementation, six carotenoids

Table 1 Physical and haematological parameters before and after the administration of *Chlorella* tablets (9 g/day/subject, 32 mg lutein/day/subject) (Mean values and standard deviations).

	Before ingestion		2 month		
Parameters	Mean	SD	Mean	SD	*p*
Age (years)	49	11			
Total number of subjects	12				
Men	6				
Women	6				
Height (cm)	166.0	11.0			
Weight (kg)	59.1	15.0	58.8	14.9	0.318*
BMI (kg/m^2)	21.5	3.2	21.5	3.1	0.920*
Systolic blood pressure (mmHg)	115.1	14.0	115.9	11.3	0.723*
Diastolic blood pressure (mmHg)	73.4	10.3	74.9	7.0	0.512*
Total protein (g/dL)	7.1	0.3	7.1	0.3	0.236*
Albumin (g/dL)	4.4	0.2	4.5	0.2	0.001*
Creatinine (mg/dL)	0.7	0.2	0.8	0.2	0.367*
Creatine kinase (U/L)	127.7	72.1	118.7	52.5	0.442*
GOT (U/L)	18.7	2.9	18.3	3.4	0.596*
GPT (U/L)	16.1	4.8	16.1	4.8	1.000*
ALP (U/L)	203.3	49.2	219.5	57.0	0.004*
γ-GTP (U/L)	16.7	5.5	17.3	8.2	0.582*
Urea (mg/dL)	13.5	3.7	14.5	3.5	0.339*
α-Tocopherol (nmol/mL)	18.1	5.0	31.3	7.0	0.002†
β-Tocopherol (nmol/mL)	0.2	0.1	0.3	0.1	0.012†
γ-Tocopherol (nmol/mL)	3.4	1.3	4.7	1.5	0.007†
Total cholesterol (mg/dL)	211.3	31.3	215.2	28.5	0.452*
HDL cholesterol (mg/dL)	62.5	12.4	65.9	13.5	0.022*
LDL cholesterol (mg/dL)	132.6	33.4	132.7	31.1	0.988*
TG (mg/dL)	94.5	43.3	106.5	60.6	0.242*
Fasting glucose (mg/dL)	78.8	7.2	77.6	9.1	0.640*
Platelets (x10^4/μL)	21.9	3.6	23.6	3.4	0.036*
Leucocytes (/μL)	4933.3	701.1	5383.3	873.7	0.011*
Erythrocytes (x10^4/μL)	463.3	39.6	468.0	33.0	0.0001*
Hb (g/dL)	13.9	1.6	14.1	1.4	0.067*
MCV (fL)	90.6	2.9	92.7	2.9	0.0002*
MCH (pg)	29.8	1.3	30.0	1.2	0.155*
MCHC (%)	33.0	0.9	32.4	0.8	0.002*
Hematocrit (%)	42.0	4.2	43.4	3.8	0.001*

* Paired *t*-test among groups.
† Wilcoxon signed-rank test among groups.

(lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, and lycopene) were detected in erythrocytes and plasma (Fig. 1). Among these carotenoids, lutein was predominant in erythrocytes, and showed a 4-fold increase (from 14 to 54 pmol/mL packed cells) after 1 month of *Chlorella* ingestion (Table 2). The concentration was then maintained during the supplementation period. Erythrocyte lutein decreased to a basal level (17 pmol/mL packed cells) 1 month after cessation of *Chlorella* ingestion (one-month observation period). These results indicate that daily *Chlorella* intake would be effective for improving and maintaining erythrocyte lutein concentrations in humans.

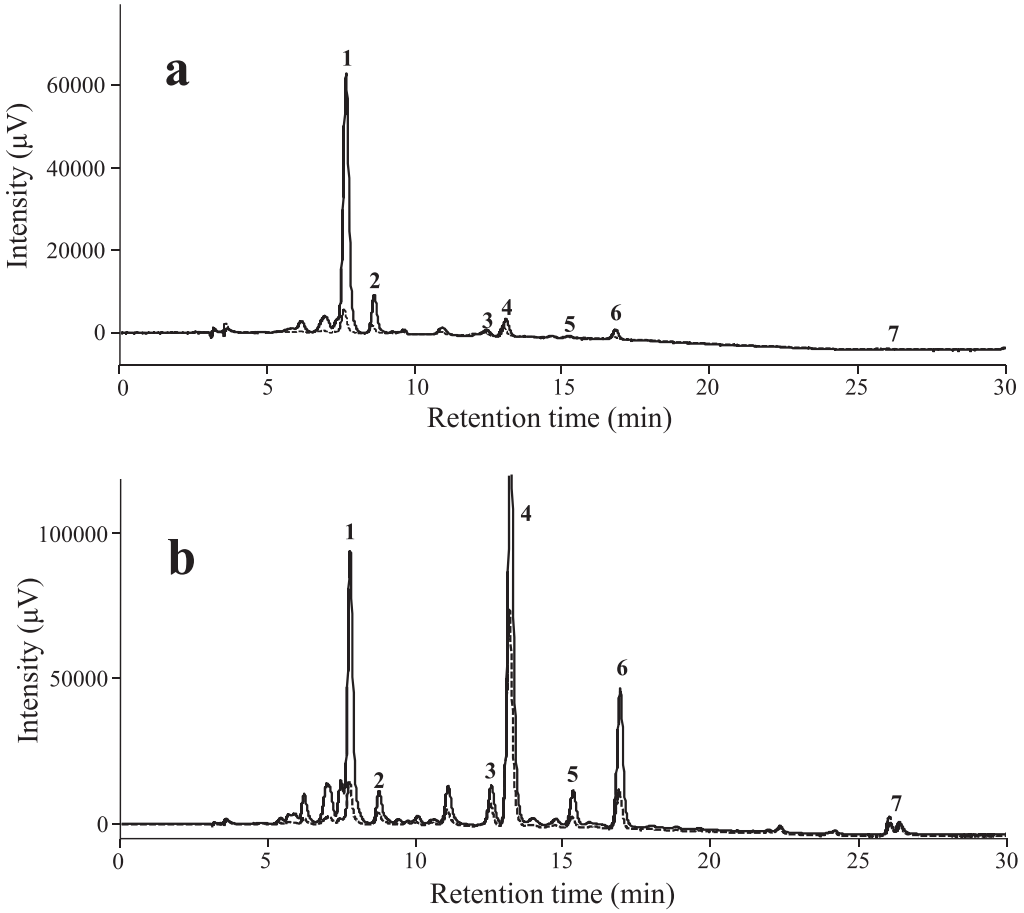

Fig. 1 UV-HPLC chromatograms of erythrocyte (a) and plasma carotenoids (b) taken before (dotted line) and after 2-month supplementation (solid line) of *Chlorella* tablets (9 g *Chlorella*/day; equivalent to 32 mg lutein/day). Peaks are identified as follows: 1, lutein; 2, zeaxanthin; 3, β-cryptoxanthin; 4, echinenone (internal standard); 5, α-carotene; 6, β-carotene; 7, lycopene.

Like lutein, zeaxanthin increased in erythrocytes (Table 2), likely due to the presence of zeaxanthin in the *Chlorella* tablets. Although not significant, erythrocyte α-carotene and β-carotene showed increases. The carotenoid (lutein, zeaxanthin, α-carotene, and β-carotene) levels were proportional between erythrocytes and plasma (Table 3 and Fig. 2). It is therefore likely that ingested carotenoids were absorbed from the intestine and incorporated into blood plasma and erythrocytes. Polar carotenoids such as lutein and zeaxanthin would likely be transferred from plasma lipoprotein particles to erythrocyte lipid membranes.

4 DISCUSSION

Lutein, a polar carotenoid, has received attention as a potential nutraceutical agent[17]. *Chlorella* algae contains high amounts of lutein[11], and a previous study suggested that *Chlorella* intake influences serum lutein levels[12]. With a single oral dose of *Chlorella* (a 6 g *Chlorella* tablet, containing 15 mg lutein), serum lutein concentration reportedly increased about one and a half-fold, but erythrocyte lutein concentrations were not investigated. On the other hand, daily intake of mixed frozen spinach and corn (containing a total of 11 mg lutein) for 2 months increased serum lutein levels by 2-fold[18], but characterization of erythrocytes was not conducted. Supplementation of 1 egg yolk (containing 0.14 mg lutein) for 1 month was also effective at increasing serum lutein concentrations by 1.25-fold[19]. Thus, it is likely that lutein-rich food (e.g., *Chlorella*, spinach, and corn) would be useful for improving lutein bioavailability. However, the effect of long-term intake of *Chlorella* on serum and erythrocyte lutein has not been thoroughly investigated. Thus, it has not been determined whether *Chlorella* can efficiently supply lutein to human erythrocytes. In this study, we conducted a 2-month single arm human study, and examined the efficacy of 1 or 2 months of *Chlorella* ingestion on erythrocyte and plasma lutein levels. The subjects received a rather high dose of *Chlorella* lutein (32 mg/day) because we felt it would facili-

Table 2 Changes in carotenoids contents in erythrocytes and plasma taken before and after administration of *Chlorella* (32 mg lutein/day/subject) (Mean values and standard deviations).

	before administration		1 month		2 month		1 month observation period		
Parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD	p‡
Erythrocyte carotenoids (pmol/mL packed cells)									
Lutein	14.3	11.3	54.1 **††	28.6	56.2 **††	27.8	16.6	6.4	0.0001
Zeaxanthin	5.4	5.1	9.5 ††	5.2	8.3 †	4.9	3.4	1.5	0.0002
β-Cryptoxanthin	5.5	6.4	3.5	3.3	5.8	6.4	4.3	4.4	0.494
α-Carotene	0.6	0.8	0.6	0.9	0.9	0.7	0.7	0.6	0.079
β-Carotene	2.3	1.7	3.7	3.0	3.7	2.6	3.2	2.8	0.079
Lycopene	1.8	1.8	3.2	4.9	1.3	2.3	0.7	0.4	0.139
Plasma carotenoids (pmol/mL plasma)									
Lutein	442	230	1256 **††	491	979 *††	526	425	183	0.0001
Zeaxanthin	90	43	138 †	59	103	52	72	43	0.026
β-Cryptoxanthin	159	109	153	132	256	316	471	561	0.139
α-Carotene	184	122	243	171	188 †	83	139	81	0.019
β-Carotene	454	309	715 †	458	595 †	326	392	242	0.001
Lycopene	262	176	132	59	70 **§	72	78	47	0.001

Mean values were significantly different in the Scheffe's method between before administration and after administration. *p<0.05, **p<0.01
Mean values were significantly different in the Scheffe's method between observation period and after administration. †p<0.05, ††p<0.01
Mean values were significantly different in the Scheffe's method between 1 month and 2 month administration. § p<0.05
‡ Friedman test among groups.

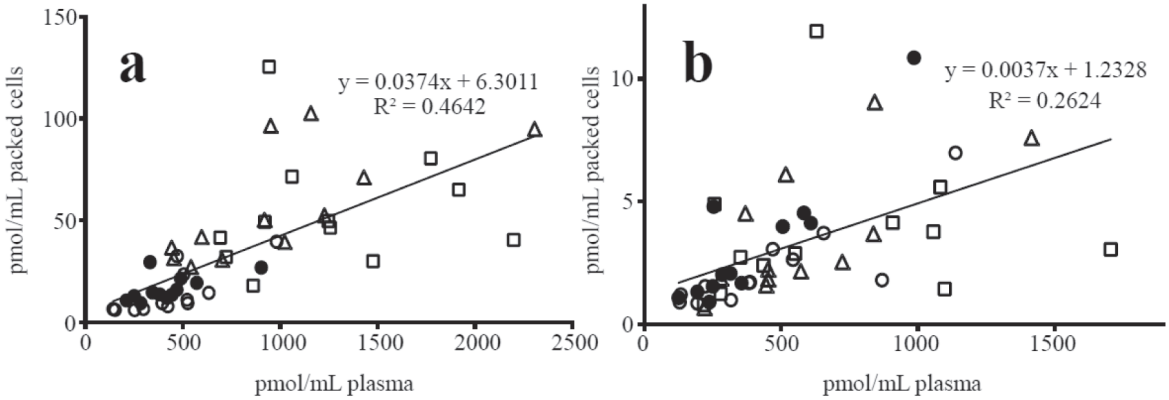

Fig. 2 Correlation between erythrocyte and plasma lutein (a) or β-carotene (b). Open circles, before administration; triangle, 1 month after administration; square, 2 months after administration; black circle, 1 month after administration was completed (1-month observation period).

tate our analysis of the transport of lutein from plasma lipoprotein to erythrocyte lipid membranes.

Chlorella intake resulted in an increase of plasma α-tocopherol concentration. No meaningful changes were observed in other parameters of blood biochemistry (Table 1). These results may suggest the safety of *Chlorella* supplementation. After *Chlorella* ingestion, plasma lutein concentration increased and reached a maximum of 1260 pmol/mL plasma (about 3-fold above basal levels) (Fig. 1 and Table 2). Considering the results of previous studies[12, 18, 19] and our findings (Fig. 1 and Table 2), daily intake of *Chlorella* leads to increased plasma lutein in normal subjects. These results confirm that *Chlorella* is a good dietary source for improving lutein levels in blood. Interestingly, compared to plasma lutein concentrations, there was a greater increase in erythrocyte lutein. Erythrocyte lutein showed an increase of 4-fold after the *Chlorella* ingestion (Table 2). However, we found that erythrocyte lutein returned to basal levels after 1 month without consuming *Chlorella*. Similar changes were observed in plasma lutein. Therefore, continuous *Chlorella* intake would be important for maintaining erythrocyte lutein at significant levels.

In addition to lutein, we assessed other carotenoids in erythrocytes and plasma. The relative amounts of the carotenoids were in the following order: lutein, β-cryptoxanthin, zeaxanthin, β-carotene, lycopene and α-carotene (Table 2). These data support our previous finding that polar carotenoids are the most prevalent carotenoids in human erythrocytes[14]. *Chlorella* tablets contained lutein, zeaxanthin, α-carotene, and β-carotene, and these carotenoids were distributed between plasma and erythrocytes in fairly predictable ratios (Table 3 and Fig. 2). It is plausible that polar and nonpolar carotenoids are located in the outer and inner regions of plasma lipoproteins, respectively, facilitating the transfer of outer lutein from lipoprotein to erythrocyte outer lipid bilayers[20].

Table 3 Correlations between erythrocytes and plasma carotenoids.

Carotenoid	r (n=12)	*p*
Lutein	0.83	0.0001
Zeaxanthin	0.57	0.0001
β-Cryptoxanthin	0.30	0.039
α-Carotene	0.43	0.002
β-Carotene	0.69	0.0001
Lycopene	0.51	0.0001

Analyzed by Spearman's rank correlation coefficient test.

5 CONCLUSIONS

In conclusion, we performed a 2-month single arm human study and provided evidence that *Chlorella* is an effective dietary source of carotenoids. In particular, it elevated lutein levels in plasma and erythrocytes. We previously found that there was a higher accumulation of phospholipid hydroperoxide, an oxidative stress marker, in erythrocytes of dementia patients[15, 21]. 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. In the present study, orally administered *Chlorella* lutein was incorporated into erythrocytes at high concentrations. Such lutein may have the potential to act as an important antioxidant for erythrocytes, and it might contribute to the prevention of dementia. This possibility warrants the testing of *Chlorella* in *in vivo* dementia models with an eventual goal of use in human therapy.

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