

*Full Paper***The Protective Effects of a Dietary Carotenoid, Astaxanthin, Against Light-Induced Retinal Damage**

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**Abstract.** Dietary carotenoids exhibit various biological activities, including antioxidative activity. In particular, astaxanthin, a type of carotenoid, is well known as a powerful antioxidant. We investigated whether astaxanthin would protect against light-induced retinal damage. In an *in vivo* study, ddY male mice were exposed to white light at 8,000 lux for 3 h to induce retinal damage. Five days after light exposure, retinal damage was evaluated by measuring electroretinogram (ERG) amplitude and outer nuclear layer (ONL) thickness. Furthermore, expression of apoptotic cells, 8-hydroxy-deoxyguanosine (8-OHdG), was measured. In an *in vitro* study, retinal damage was induced by white light exposure at 2,500 lux for 24 h, and propidium iodide (PI)-positive cells was measured and intracellular reactive oxygen species (ROS) activity was examined. Astaxanthin at 100 mg/kg inhibited the retinal dysfunction in terms of ERG and ONL loss and reduced the expression of apoptotic and 8-OHdG-positive cells induced by light exposure. Furthermore, astaxanthin protected against increases of PI-positive cells and intracellular reactive oxygen species (ROS) activity in 661W cells. These findings suggest that astaxanthin has protective effects against light-induced retinal damage via the mechanism of its antioxidative effect.

**Keywords:** astaxanthin, photoreceptor, oxidative stress, age-related macular degeneration, retinitis pigmentosa

**Introduction**

Age-related macular degeneration (AMD) is the leading cause of adult vision loss in the Western world. AMD occurs as a result of photoreceptor degeneration, causing a decline not only of quality of vision (QOV), but also of quality of life (QOL). AMD mainly injures the macula, which detects photoreceptors. Two types of AMD are known to exist: dry AMD, in which age causes macular degeneration and failing visual acuity, and wet AMD, in which the macula is impaired by angiogenesis. Recently, angiogenic inhibitors, such as ranibizumab and pegaptanib, have been used for useful medical treatments.

Almost all retinitis pigmentosa (RP) patients have genetic factors; this disorder occurs at a rate of one

in 4,000 – 8,000 people. Night blindness occurs first, and constriction of the visual field presents gradually. Currently, treatment approaches to improve retinal function and block the progression of RP do not exist; there are only supportive cares, such as light-resistant eyeglasses, vitamin A, cardiovascular agents, and vitamin B<sub>12</sub> (1). Therefore, there is a need for elucidation of the pathophysiology and therapeutic medicine.

Previous reports have suggested that excessive light exposure is one of the factors of AMD occlusion and that it promotes AMD progression (2). In addition, it has been reported that genetic aberrance causing retinal degeneration elevates photoreceptor fragility against light in RP (3). Finally, in both AMD and RP, excessive light exposure evokes photoreceptor apoptosis in the same manner (4, 5). As such, a light-induced photoreceptor degeneration model has been widely used to clarify the mechanism of photoreceptor injury in AMD and RP. ROS are produced easily in the retina, as the retina is routinely

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exposed to light (6, 7). It is known that ROS is produced by light exposure in the retina, and it evokes photoreceptor degeneration (8, 9); thus, antioxidants such as dimethylthiourea (10), phenyl-*N*-tert-butyl nitron (11), and 2,2,6,6-tetramethyl-4-piperidinol-1-oxyl (12) have been reported to be effective in animal experiments.

Astaxanthin, a carotenoid, is present in many well-known seafoods such as salmon, trout, red sea-bream, shrimp, lobster, and fish eggs. Astaxanthin exhibits various pharmacological activities, including antioxidative (13–17), antitumor (18), anti-inflammatory (19), antidiabetic (20), hepatoprotective (21), and immunomodulatory effects (18, 22). Moreover, one of the characteristics of astaxanthin is its high degree of safety (23).

In the retina, astaxanthin has protective effects against choroidal neovascularization (24), and our laboratory previously reported that *N*-methyl-D-aspartate (NMDA)-induced retinal ganglion cell death was inhibited by astaxanthin (25). However, the effect of astaxanthin against the light-induced photoreceptor degeneration model, which is the model of dry AMD and RP, has not been clarified yet. The aim of this study was to elucidate the protective effects of astaxanthin against light-induced photoreceptor degeneration.

## Materials and Methods

### Animals

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and they were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. Male albino ddY mice (Japan SLC, Hamamatsu), aged 8–10 weeks, were used in this study. They were kept under controlled lighting conditions (12:12 h light/dark).

### Exposure to light

After dark adaptation for 24 h, the pupils were dilated with 1% cyclopentolate hydrochloride eye drops (Santen Pharmaceuticals Co., Ltd., Osaka) 30 min before exposure to light. The non-anesthetized mice were exposed to 8,000 lux of white fluorescent light (Toshiba, Tokyo) for 3 h in cages with reflective interiors. The temperature during the exposure to light was maintained at  $25^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$ . After the exposure to light, all the mice were placed in the dark for 24 h and then returned to the normal light/dark cycle.

Astaxanthin (Asahi Kasei Pharma Corp., Tokyo) at 100 mg/kg was dissolved in olive oil just before use and was administered orally eight times (at 6 h before and at 0, 6, 12, 24, 36, 48, and 72 h after light irradiation) for histological analysis, six times (at 6 h before and at 0,

6, 12, 24, and 36 h after light irradiation) for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining analysis, four times (at 6 h before and at 0, 6, and 12 h after light irradiation) for immunostaining analysis, or two times (at 6 h before and just before light irradiation) with a volume of 0.05 mL / 10 g body weight.

### Electroretinogram

ERG readings were recorded 5 days after the light exposure. Thirty-two mice in total were used in this experiment. The mice were maintained in a completely dark room for 24 h, after which they were intraperitoneally anesthetized with a mixture of ketamine (120 mg/kg) (Daiichi-Sankyo, Tokyo) and xylazine (6 mg/kg) (Bayer Health Care, Tokyo). The pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen Pharmaceuticals). Flash ERG was recorded in the left eyes of the dark-adapted mice by placing a golden-ring electrode (Mayo, Aichi) in contact with the cornea and a reference electrode (Nihon Kohden, Tokyo) through the tongue. A neutral electrode (Nihon Kohden) was inserted subcutaneously near the tail. All procedures were performed under dim red light. The amplitude of the a-wave was measured from the baseline to the maximum a-wave peak, and the b-wave was measured from the maximum a-wave peak to the maximum b-wave peak. The a-wave shows the function of the photoreceptors, and the b-wave reflects bipolar cell and Müller cell function.

### Histological analysis

Fifty-seven mice in total were used in this experiment. The mice were euthanized by cervical spine fracture dislocation. Each eye was enucleated and kept immersed for at least 24 h at  $4^{\circ}\text{C}$  in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (thickness, 5  $\mu\text{m}$ ) cut from the optic disc of each eye were prepared in the standard manner, and stained with hematoxylin and eosin. The damage induced by light exposure was then evaluated, with the six sections from each eye used for morphometric analysis, as described below. Light-microscopy images were photographed, and the thickness of the outer nuclear layer (ONL) from the optic disc was measured at 240- $\mu\text{m}$  intervals on the photographs. Data from three sections (selected randomly from the six sections) were averaged for each eye.

### TUNEL staining

TUNEL staining was performed according to the manufacturer's protocols (In Situ Cell Death Detection kit; Roche Biochemicals, Mannheim, Germany) to detect the retinal cell death induced by exposure to light. Twenty-eight mice in total were used in this experiment.

The eyes were enucleated, fixed overnight in 4% paraformaldehyde, and immersed for 2 days in 25% sucrose with 0.01 M phosphate-buffered saline (PBS). The eyes were then embedded in a supporting medium for frozen-tissue specimens (OCT compound; Tissue-Tek, Miles Laboratories, Naperville, IL, USA). Retinal sections (10- $\mu$ m thick) were cut on a cryostat at  $-25^{\circ}\text{C}$  and stored at  $-80^{\circ}\text{C}$  until staining. After being washed with PBS twice, sections were incubated with methanol containing 3%  $\text{H}_2\text{O}_2$  for 10 min, 0.1% sodium citrate aqueous containing 0.1% Triton X-100 for 10 min, and TUNEL reaction mixture, 10% terminal deoxyribonucleotidyl transferase (TdT) enzyme solution diluted in fluorescein-dUTP mixture solution, at  $37^{\circ}\text{C}$  for 1 h. The sections were washed in PBS for 5 min three times at room temperature. Two eye sections per eye (two images per one section between 285 – 715  $\mu$ m from the optic disc) were photographed and counted for TUNEL-positive cells in the ONL. The average of the four images was used as the data per eye.

#### Immunohistochemistry

Light exposure was performed as described in the exposure to light section. Thirty mice in total were used in this experiment. The eyes were enucleated, fixed overnight in 4% paraformaldehyde, and immersed for two days in 25% sucrose with PBS. The eyes were then embedded in a supporting medium for frozen tissue specimens (optimum cutting temperature compound, Tissue-Tek) and kept at  $-80^{\circ}\text{C}$ . Retinal sections were cut at 10- $\mu$ m thickness on a cryostat at  $-20^{\circ}\text{C}$  and stored at  $-80^{\circ}\text{C}$  until staining. Immunohistochemical staining was performed in accordance with the following protocol. Briefly, tissue sections were washed in 0.01 M PBS for 30 min, followed by preincubation with 10% normal goat serum in 0.01 M PBS for 1 h. Then, they were incubated overnight at  $4^{\circ}\text{C}$  with 8-OHdG monoclonal antibody diluted 1:20 in a solution of 10% goat serum in 0.01 M PBS containing 0.3% (v/v) Triton X-100. After washing with 0.01 M PBS, the sections were incubated for 30 min at room temperature with a mixture of an Alexa Fluor 488 labeled F(ab')<sub>2</sub> fragment of goat anti-rabbit IgG (H+L) (1:1000 dilution) (A11070; Invitrogen, Carlsbad, CA, USA). We confirmed the staining by comparison with the negative control. Immunofluorescence images were taken using a microscope (BX50; Olympus, Tokyo) with a cooled charge-coupled device camera (DP30BP, Olympus) at  $1360 \times 1024$  pixels via MetaMorph software (Molecular Devices, Sunnyvale, CA, USA), and the 8-OHdG-positive cells were counted in the ONL at a distance of 285 – 715  $\mu$ m from the optic disc on the images. Three eye sections per eye (two images per one section) were

photographed and the average of the six images was used as the data per eye.

#### RNA isolation

To examine the changes in gene expressions of endogenous antioxidant after light exposure, non-treated and light-exposed retinas were obtained. Thirty mice in total were used in this experiment. Mice were euthanized by cervical-spine dislocation, and the eyeballs were quickly removed. The retinas were carefully separated from the eyeballs and rapidly frozen in liquid nitrogen. RNA was isolated from retinas with the aid of a NucleoSpin<sup>®</sup> RNA II (Macherey-Nagel GmbH & Co. KG, Dürren, Germany).

#### Real-time RT-PCR

RNA concentrations were determined spectrophotometrically at 260 nm. RT-PCR was performed using the Thermal Cycler Dice Real Time System (Takara, Shiga). The reverse transcription reaction was performed at  $50^{\circ}\text{C}$  for 15 min using a PrimeScript RT reagent kit (Perfect Real Time; Takara) and Thermal Cycler Dice Real Time System (Takara). The target cDNA was amplified by 40 cycles of PCR using SYBR Premix Ex Taq (Takara) and a TP 8000 Thermal Cycler Dice Real Time system (Takara). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the reference standard, and relative levels of *Sod* (superoxide dismutase) *I*, *MT* (metallothionein)-*II*, *MT-III* compared to that of *GAPDH* were calculated. The following primers were used: *SodI*, 5'-AGCATTCCATCATTTGGCCGTA-3' (forward) and 5'-TACTGCGCAATCCCAATCACTC-3' (reverse); *MT-II*, 5'-CCTGTGCCTCCGATGGAT-3' (forward) and 5'-ACTTGTCGGAAGCCTCTTTG-3' (reverse); *MT-III*, 5'-CTGAGACCTGCCCCTGTC-3' (forward) and 5'-TTCTCGGCCTCTGCCTTG-3' (reverse); *GAPDH*, 5'-TGTGTCCGTCGTGGATCTGA-3' (forward), 5'-TTGCTGTTGAAGTCGCAGGAG-3' (reverse).

#### Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-Acetyl-L-cysteine (NAC) was purchased from Wako (Osaka). Fetal bovine serum (FBS) was purchased from Valeant (Costa Mesa, CA, USA). Dimethyl sulfoxide (DMSO) and olive oil were purchased from Nacalai Tesque, Inc. (Kyoto). Penicillin and streptomycin were purchased from Meiji Seika Kaisha, Ltd (Tokyo). Hoechst 33342 and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H<sub>2</sub>DCFDA) were purchased from Molecular Probes (Eugene, OR, USA). Astaxanthin was the free form derived from *Paracoccus carotinifaciens*. The purity of

the astaxanthin and carotenoid were 60% and 99%, respectively.

#### *Cell culture*

The mouse retinal cone-cell line 661W, a transformed mouse cone cell line derived from mouse retinal tumors, was provided by Dr. Muayyad R. Al-Ubaidi (University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA). The cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The 661W cells were passaged by trypsinization every 3–4 days.

#### *Exposure of mouse retinal cone-cell line 661W cells to white light*

The 661W cells were seeded in 96-well plates, at  $1 \times 10^3$  cells per well, and then incubated for 24 h. The entire medium was then replaced with fresh medium containing 1% FBS. Astaxanthin was added, and 24 h following treatment, the cells were exposed to 2,500 lux of white light (C-FPS115D; Nikon, Tokyo) for 24 h at 37°C. NAC was added 1 h before white light irradiation. Nuclear staining assays were performed immediately after light exposure.

#### *Nuclear staining assays*

At the end of the culture period, Hoechst 33342 ( $\lambda_{\text{ex}} = 360$  nm,  $\lambda_{\text{em}} > 490$  nm) and PI ( $\lambda_{\text{ex}} = 535$  nm,  $\lambda_{\text{em}} > 617$  nm) were added to the culture medium for 15 min at final concentrations of 8.1 and 1.5  $\mu$ M, respectively. Hoechst 33342 freely enters living cells and stains the nuclei of viable cells, as well as those that have suffered apoptosis or necrosis. Propidium iodide is a membrane-impermeable dye that is generally excluded from viable cells. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus). We counted the total number of cells and calculated the percentage of PI-positive cells as a measure of dead cells.

#### *Reactive oxygen species detection*

Intracellular radical activation within 661W cells was measured using CM-H<sub>2</sub>DCFDA. CM-H<sub>2</sub>DCFDA that is taken up into the cell is converted to dichlorodihydrofluorescein (DCFH) by an intracellular esterase. Non-fluorescent DCFH was oxidized by the ROS to fluorescent DCFH. At the end of the light exposure period, CM-H<sub>2</sub>DCFDA was added to the culture medium and incubated at 37°C for 1 h, at a final concentration of 10  $\mu$ M. The 96-well plate was loaded onto a plate in a fluorescence spectrophotometer, and the reaction was

carried out at 37°C. Cell fluorescence was determined by Hoechst 33342 staining and was used to calculate ROS production per cell (26).

#### *Statistical analyses*

Data were presented as the mean  $\pm$  S.E.M. Statistical comparisons were made with Dunnett's test or Student's *t*-test, using statistical analysis software (StatView version 5.0; SAS Institute, Cary, NC, USA).  $P < 0.05$  was considered to indicate statistical significance.

## **Results**

#### *Retinal dysfunction and histological damages after light exposure in mice*

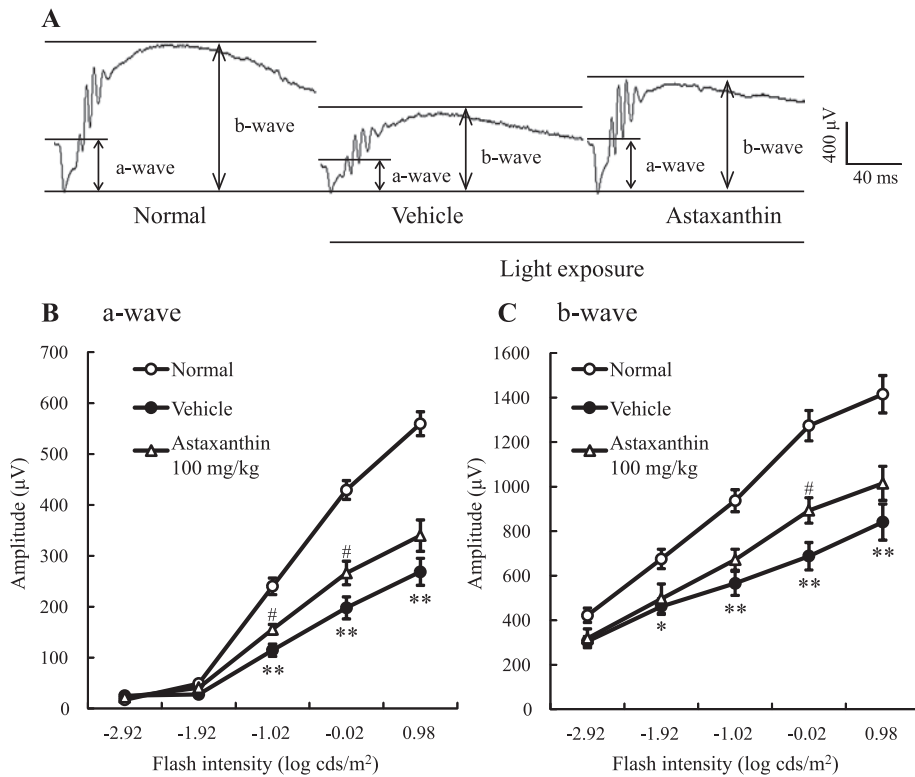
The mice were exposed to 8,000-lux light for 3 h, and retinal functions were recorded by ERG 5 days after light exposure and evaluated by both a- and b-wave amplitudes ( $n = 10$  or  $11$ ). A-wave represents photoreceptor function, and b-wave represents secondary neurons, such as Müller cell and bipolar cell functions. In the light-exposed group, the a- and b-wave amplitudes had significantly decreased when compared 5 days after exposure to light, at  $-0.02$  log cds/m<sup>2</sup>, with the non-treated retinas. Astaxanthin (100 mg/kg, p.o.) inhibited the reduction of these amplitudes by maximums of 47% and 61%, respectively (Fig. 1).

The effects of astaxanthin on light-induced retinal damage were further examined by histological analysis ( $n = 18 - 20$ ). Light damage mainly occurred in the ONL, which includes the rods and cones. ONL thickness was significantly decreased at 5 days after light exposure compared with the non-exposed retinas. Astaxanthin (100 mg/kg, p.o.) prevented the reduction of ONL thickness by a maximum of 57% (Fig. 2).

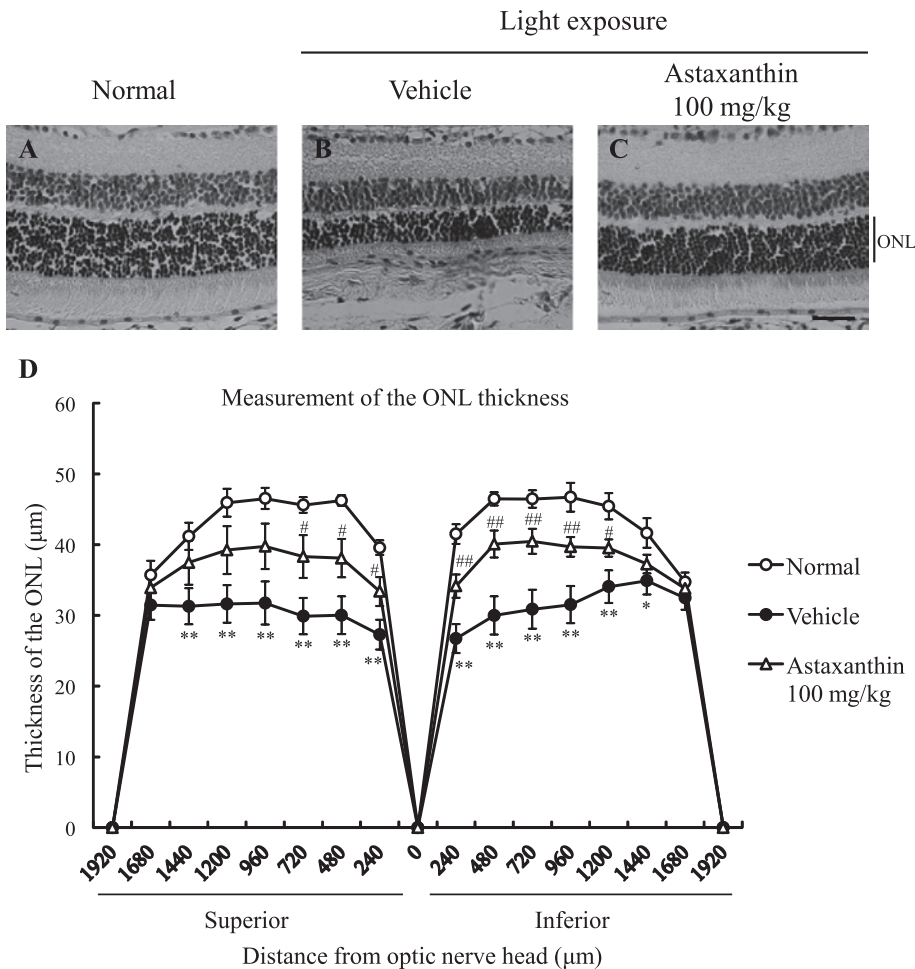
#### *Apoptosis after light exposure*

TUNEL staining is used to detect apoptosis. To investigate light-induced apoptotic cell death and the effect of astaxanthin on cell death, we performed TUNEL staining ( $n = 7 - 11$ ) and assessed the numbers of TUNEL-positive cells (Fig. 3). Several studies (27) performed TUNEL staining at 48 h after light exposure and we followed these time courses. TUNEL-positive cells appeared in the ONL. At the same time, TUNEL-positive cells were not observed in any retinal area in the normal retina. Quantitative analysis (Fig. 3D) showed that light irradiation to mouse retina significantly increased the number of TUNEL-positive cells in ONL compared with the normal retina. Astaxanthin (100 mg/kg, p.o.) significantly reduced the number of TUNEL-positive cells compared with the vehicle-treated group, and the inhibition was approximately 28%.

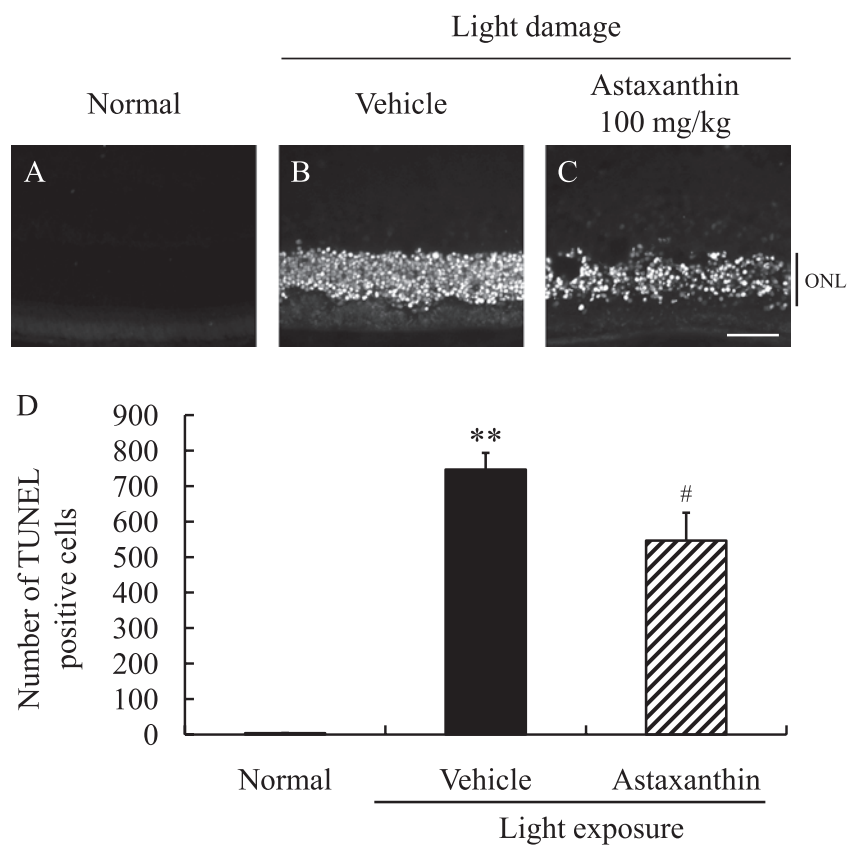




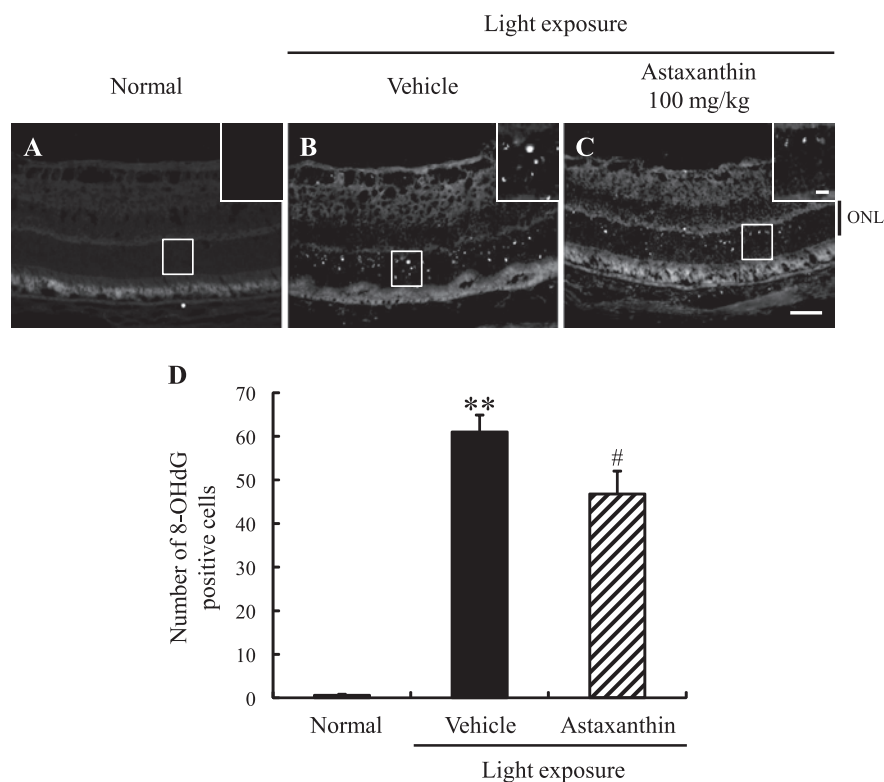
**Fig. 1.** Measurement of dark-adapted ERG amplitudes at 5 days after exposure to light in the mouse retina. A) Typical traces of dark-adapted ERG responses measured at 5 days after exposure to light. Stimulus flashes were used from 0.98 log cds/m². B and C) Amplitudes of a- and b-waves of light exposure (8,000 lux), plus the vehicle-treated group vs. light exposure, plus the astaxanthin-treated group (100 mg/kg, p.o.). Data are shown as the mean  $\pm$  S.E.M. ( $n = 10$  or  $11$ ). \* $P < 0.05$ , \*\* $P < 0.01$  vs. normal group; # $P < 0.05$ , ## $P < 0.01$  vs. vehicle group ( $t$ -test).



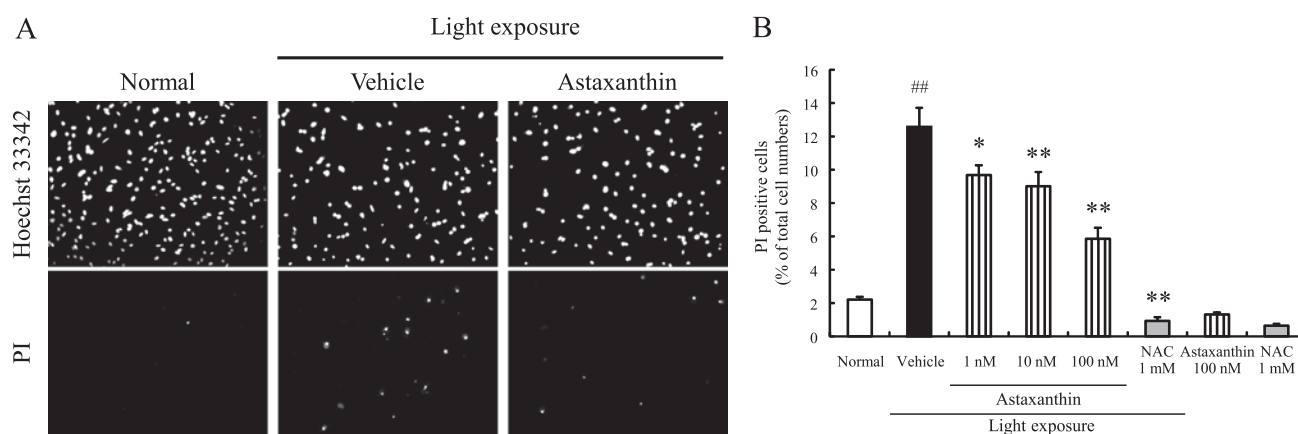
**Fig. 2.** Effects of oral administration of astaxanthin on retinal damage induced by exposure to light in mice. A) Nontreated, B) light exposure (8,000 lux, 3 h) plus vehicle-treated, and C) light exposure plus astaxanthin-treated (100 mg/kg, p.o.) retinal cross-sections at 5 days after light exposure in mice. D) Measurement of thickness in the outer nuclear layer at 5 days after light exposure. Data are shown as the mean  $\pm$  S.E.M. ( $n = 18 - 20$ ). \* $P < 0.05$ , \*\* $P < 0.01$  vs. normal group; # $P < 0.05$ , ## $P < 0.01$  vs. vehicle group ( $t$ -test). The horizontal scale bar represents 50  $\mu$ m.



**Fig. 3.** Effects of oral administration of astaxanthin on expression of TUNEL-positive cells induced by exposure to light in mice. A) Nontreated, B) light exposure (8,000 lux, 3 h) plus vehicle-treated, and C) light exposure plus astaxanthin-treated (100 mg/kg, p.o.) retinal cross-sections at 48 h after light exposure in mice. D) Measurement of TUNEL-positive cells in the outer nuclear layer. Data are shown as the mean  $\pm$  S.E.M. ( $n = 7 - 11$ ). \*\* $P < 0.01$  vs. normal group, # $P < 0.05$  vs. vehicle-treated group ( $t$ -test). Scale bar: 50  $\mu$ m.



**Fig. 4.** Effects of oral administration of astaxanthin on expression of 8-OHdG-positive cells induced by exposure to light in mice. A) Nontreated, B) light exposure (8,000 lux, 3 h) plus vehicle-treated, and C) light exposure plus astaxanthin-treated (100 mg/kg, p.o.) retinal cross-sections at 24 h after light exposure in mice. D) Measurement of 8-OHdG-positive cells in the outer nuclear layer. Data are shown as the mean  $\pm$  S.E.M. ( $n = 10$ ). \*\* $P < 0.01$  vs. normal group, # $P < 0.05$  vs. vehicle-treated group ( $t$ -test). Scale bars: 50  $\mu$ m (low magnification), 10  $\mu$ m (small square; high magnification).



**Fig. 5.** Effects of astaxanthin on 661W cell damage induced by light exposure. A – C) Representative fluorescence microscopy of Hoechst 33342 staining, moments after 24-h light exposure. A) Non-treated cells showed normal nuclear morphology. B) Light-induced degeneration occurred. C) Pretreatment with 100 nM astaxanthin. D) PI-positive cells were assessed by immersing them in Hoechst 33342 and PI complex. Astaxanthin and Trolox significantly inhibited the light-induced retinal cell damage.  $^*P < 0.05$ ,  $^{**}P < 0.01$  vs. vehicle;  $^{##}P < 0.01$  vs. control; data are expressed as the mean  $\pm$  S.E.M. ( $n = 12$ ).

#### Oxidative stress after light exposure in mice

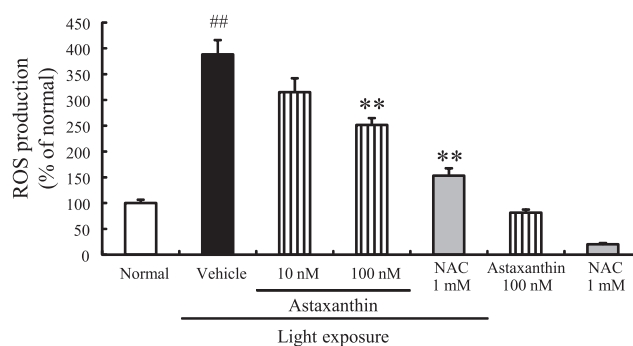
To clarify whether the protective effect of astaxanthin is related to its antioxidative effect, immunostaining was performed ( $n = 10$ ). Because 8-hydroxy-2-deoxyguanosine (8-OHdG) was a marker of DNA oxidative damage, we evaluated the numbers of these cells in the ONL. At 24 h after light exposure, 8-OHdG-positive cells were observed in the ONL; no 8-OHdG-positive cells were observed in the normal retinas (Fig. 4). Treatment with astaxanthin significantly decreased the number of 8-OHdG-positive cells; the inhibition was approximately 23%.

#### Expression of antioxidant genes

To clarify changes in mRNA levels of endogenous antioxidant, we investigated mouse retinas after 6 h of light exposure by real-time RT-PCR (Fig. 5,  $n = 10$ ). We were referred to the previous report (28) to determine the timing of sampling retinas. The mRNA expression of *Sod1*, *MT-II*, and *MT-III* were elevated significantly after light exposure (20.7-, 1.7-, 1.2-fold increase, respectively). Astaxanthin (100 mg/kg, p.o.) had no effect against *Sod1*, *MT-II*, or *MT-III* compared to vehicle-treated retinas.

#### Effects of astaxanthin against light-induced 661W cell death

We examined the effect of astaxanthin on light-induced photoreceptor degeneration. Representative photographs of Hoechst 33342 and PI staining are shown in Fig. 4A. Hoechst 33342 stains all cell nuclei (live and dead cells), while PI stains only dead cells. Pretreatment with astax-



**Fig. 6.** Effects of astaxanthin on light-induced ROS production in 661W cells. Cellular radical intensity was quantified by fluorescence microscopy of the CM-H<sub>2</sub>DCFDA probe. Light exposure causes ROS production, which was partly prevented by the astaxanthin and NAC treatment.  $^{**}P < 0.01$  vs. vehicle,  $^{##}P < 0.01$  vs. control; data are expressed as the mean  $\pm$  S.E.M. ( $n = 6$ ).

anthin at concentrations of 1 – 100 nM protected the cells from light-induced cell death in a concentration-dependent manner. NAC at 1 mM inhibited cell death (Fig. 5B).

#### Effects of astaxanthin on the intracellular oxidation of DCFH induced by various types of ROS

CM-H<sub>2</sub>DCFDA, a ROS-sensitive probe, was used as a radical scavenging capacity assay. Light irradiation increased during ROS production, and astaxanthin (100 nM) significantly reduced the production and astaxanthin at 10 nM had a tendency to reduce it, but not significantly (Fig. 6).

## Discussion

In the previous report from our laboratory, astaxanthin prevented retinal damage induced by NMDA intravitreal injection in vivo and RGC-5 degeneration induced by serum deprivation and addition of  $H_2O_2$  in vitro (25). This report suggests that astaxanthin has a protective effect against glaucoma via prevention of oxidative stress. We hypothesized that astaxanthin shows protection against retinal diseases related with oxidative stress, but the effects of light-induced retinal degeneration, which is the model of dry AMD and RP, is still unknown and there are no reports about this so far. In the present study, we demonstrated that astaxanthin protected against light-induced retinal damage, and inhibited oxidative stress of the photoreceptors.

Previous reports have studied the concentrations of free astaxanthin in plasma after single-dose oral gavage with free astaxanthin (29, 30). According to astaxanthin (500 mg/kg, p.o.) treatment in emulsion in mice,  $C_{max}$  was approximately 400 nM (29). In this study, astaxanthin was orally administered four times a day, at a dose of 100 mg/kg, indicating that a total of about 400 mg/kg astaxanthin was administered per day. This finding indicates that the maximal plasma concentration of astaxanthin in this study could be at least 100 nM. This concentration corresponded to the in vitro assay, wherein astaxanthin reduced intracellular ROS activity and 661W-induced cell damage at concentrations of 1 – 100 nM (Figs. 5 and 6).

Some reports have indicated that dietary astaxanthin treatment is safe in animals (31, 32) and humans (33, 34). In this study, astaxanthin caused no apparent abnormality, and there were no body weight changes in the light-exposure group compared with the vehicle-treated group. This observation suggests that the present study provides evidence that eight times treatment with astaxanthin at 100 mg/kg within four days causes no ill effects, in agreement with other reports published thus far.

Previous reports have indicated that light exposure induces Sod1 (8) and that the retinas of Sod-1-knockout mice are likely to be impaired by light exposure (9), suggesting that light exposure induces ROS in the retina and evokes retinal degeneration. ROS is related to the regulation of many principal cell functions such as activation of transcription factor (35), gene expression (36), and cell proliferation (37). On the other hand, excessive production of ROS induces cell death in various cells (37). In this study, 8-OHdG-positive cells appeared in the ONL layer, but astaxanthin reduced their number (Fig. 4). 8-OHdG is a biomarker of DNA oxidative stress (38), indicating that astaxanthin protected the retinal cells via reducing oxidative stress. Visible-light exposure

to the retina evokes the photooxidation of *N*-retinylidene-*N*-retinylethanolamine (A2E) and all-*trans*-retinal dimer, which are the efficient producers of singlet oxygen ( $O_2^{\cdot-}$ ) (39).  $O_2^{\cdot-}$ , an ROS, is  $O_2$  in an excited electronic state which provokes the peroxidation of lipids, cellular membrane damage, and DNA damage when it changes to its ground state (40).  $O_2^{\cdot-}$  is also related to retinal cell death (41). Carotenoids have been known to detoxify  $O_2^{\cdot-}$  catalytically; a single beta carotene molecule can remove 250 – 1000 molecules of  $O_2^{\cdot-}$  (42). Astaxanthin, one of the carotenoids, is well known to possess the capacity to detoxify  $O_2^{\cdot-}$  catalytically (43). Therefore, the findings of this study indicate that  $O_2^{\cdot-}$  produced by light irradiation might have been prevented by the existence of astaxanthin, oxidative stress was weakened, and retinal cell death was reduced.

Astaxanthin (100 mg/kg, p.o.) significantly prevented retinal damages induced by light irradiation (Figs. 1 – 4). The levels of these inhibitory effects are different in these evaluations. In retinal function and histological analysis, approximately 50% damage was prevented (Figs. 1 and 2). However, astaxanthin prevented retinal damage by only 28% (Fig. 3). These findings suggest that photoreceptor damage is related to not only cellular apoptosis, but also necroptosis (44) or autophagy (45), and they are not strictly separated, for example, apoptosis and autophagy (46). There is a possibility that the protective effect of astaxanthin administration may also involve in these pathways of cell death. Additionally, the ratio of the protective effect against apoptosis is not consistent with the inhibitory effect of 8-OHdG (Figs. 3 and 4). Several factors are involved in light-induced retinal apoptosis via oxidative stress, for example, DNA damage, lipid peroxidation (12), and inflammation (19). Astaxanthin might have protective effect against not only DNA oxidative stress or apoptotic degeneration, but also inflammation or lipid peroxidation or other effects. However, further investigations are needed to solve these issues.

We clarified that astaxanthin has protective effect against oxidative stress. However, whether endogenous antioxidant is influenced by astaxanthin is not apparent. Therefore, we investigated this by measuring the mRNA expression of three antioxidants. It has been reported that Sod1 is involved in the change of superoxide anion into oxygen and hydrogen peroxide and induced in the retina after light exposure (47). MT is expressed in the tissues and central nervous system, and it has a high content of sulfhydryls, which targets it to scavenge superoxide anion and hydroxyl radicals with an affinity more than 300-fold higher than that of reduced glutathione (48). In a previous report, *Sod1*, *MT-II*, and *MT-III* mRNAs were found to be increased in the retina 6 h after light exposure



(28), and our data was consistent with this. However, astaxanthin did not influence *Sod1*, *MT-II*, and *MT-III* mRNA. Therefore our observations suggest that astaxanthin does not interact with endogenous antioxidants.

One of the constructive characteristics of astaxanthin is that it has polar ionone rings at the ends and a non-polar zone of conjugated carbon-carbon bonds in the middle. It has been suggested that the inner membrane and surface are protected from ROS because the polar end groups overlap the polar boundary zones of the bi-layer membrane, and the nonpolar middle chain fits the membrane's nonpolar interior (49, 50). In this study, astaxanthin actually reduced intracellular ROS production (Fig. 6). These findings suggest that astaxanthin might inhibit intracellular DNA degeneration caused by ROS more strongly than similar carotenoids and that it exhibits protective effects against light-induced photoreceptor degeneration.

In conclusion, these findings suggest that dietary astaxanthin treatment is effective against light-induced retinal cell death, indicating that astaxanthin intake leads to the prevention and inhibition of the progression of RP and dry AMD.

## References

- Hamel C. Retinitis pigmentosa. *Orphanet J Rare Dis*. 2006;1:40.
- Hirakawa M, Tanaka M, Tanaka Y, Okubo A, Koriyama C, Tsuji M, et al. Age-related maculopathy and sunlight exposure evaluated by objective measurement. *Br J Ophthalmol*. 2008; 92:630–634.
- Paskowitz DM, LaVail MM, Duncan JL. Light and inherited retinal degeneration. *Br J Ophthalmol*. 2006;90:1060–1066.
- Noell WK, Walker VS, Kang BS, Berman S. Retinal damage by light in rats. *Invest Ophthalmol*. 1966;5:450–473.
- Nickells RW, Zack DJ. Apoptosis in ocular disease: a molecular overview. *Ophthalmic Genet*. 1996;17:145–165.
- Yang JH, Basinger SF, Gross RL, Wu SM. Blue light-induced generation of reactive oxygen species in photoreceptor ellipsoids requires mitochondrial electron transport. *Invest Ophthalmol Vis Sci*. 2003;44:1312–1319.
- Godley BF, Shamsi FA, Liang FQ, Jarrett SG, Davies S, Boulton M. Blue light induces mitochondrial DNA damage and free radical production in epithelial cells. *J Biol Chem*. 2005;280: 21061–21066.
- Yamashita H, Horie K, Yamamoto T, Nagano T, Hirano T. Light-induced retinal damage in mice. Hydrogen peroxide production and superoxide dismutase activity in retina. *Retina*. 1992;12: 59–66.
- Hashizume K, Hirasawa M, Imamura Y, Noda S, Shimizu T, Shinoda K, et al. Retinal dysfunction and progressive retinal cell death in SOD1-deficient mice. *Am J Pathol*. 2008;172: 1325–1331.
- Organisciak DT, Darrow RA, Barsalou L, Darrow RM, Lininger LA. Light-induced damage in the retina: differential effects of dimethylthiourea on photoreceptor survival, apoptosis and DNA oxidation. *Photochem Photobiol*. 1999;70:261–268.
- Ranchon I, Chen S, Alvarez K, Anderson RE. Systemic administration of phenyl-N-tert-butyl nitrone protects the retina from light damage. *Invest Ophthalmol Vis Sci*. 2001;42:1375–1379.
- Tanito M, Li F, Elliott MH, Dittmar M, Anderson RE. Protective effect of TEMPOL derivatives against light-induced retinal damage in rats. *Invest Ophthalmol Vis Sci*. 2007;48:1900–1905.
- Kurashige M, Okimasu E, Inoue M, Utsumi K. Inhibition of oxidative injury of biological membranes by astaxanthin. *Physiol Chem Phys Med NMR*. 1990;22:27–38.
- O'Connor I, O'Brien N. Modulation of UVA light-induced oxidative stress by beta-carotene, lutein and astaxanthin in cultured fibroblasts. *J Dermatol Sci*. 1998;16:226–230.
- Iwamoto T, Hosoda K, Hirano R, Kurata H, Matsumoto A, Miki W, et al. Inhibition of low-density lipoprotein oxidation by astaxanthin. *J Atheroscler Thromb*. 2000;7:216–222.
- Kobayashi M. In vivo antioxidant role of astaxanthin under oxidative stress in the green alga *Haematococcus pluvialis*. *Appl Microbiol Biotechnol*. 2000;54:550–555.
- Aoi W, Naito Y, Sakuma K, Kuchide M, Tokuda H, Maoka T, et al. Astaxanthin limits exercise-induced skeletal and cardiac muscle damage in mice. *Antioxid Redox Signal*. 2003;5: 139–144.
- Chew BP, Park JS, Wong MW, Wong TS. A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice in vivo. *Anticancer Res*. 1999;19:1849–1853.
- Ohgami K, Shiratori K, Kotake S, Nishida T, Mizuki N, Yazawa K, et al. Effects of astaxanthin on lipopolysaccharide-induced inflammation in vitro and in vivo. *Invest Ophthalmol Vis Sci*. 2003;44:2694–2701.
- Uchiyama K, Naito Y, Hasegawa G, Nakamura N, Takahashi J, Yoshikawa T. Astaxanthin protects beta-cells against glucose toxicity in diabetic db/db mice. *Redox Rep*. 2002;7:290–293.
- Kang JO, Kim SJ, Kim H. Effect of astaxanthin on the hepatotoxicity, lipid peroxidation and antioxidative enzymes in the liver of CCl4-treated rats. *Methods Find Exp Clin Pharmacol*. 2001; 23:79–84.
- Okai Y, Higashi-Okai K. Possible immunomodulating activities of carotenoids in in vitro cell culture experiments. *Int J Immunopharmacol*. 1996;18:753–758.
- Miyawaki H, Takahashi J, Tsukahara H, Takehara I. Effects of astaxanthin on human blood rheology. *J Clin Biochem Nutr*. 2008;43:69–74.
- Izumi-Nagai K, Nagai N, Ohgami K, Satofuka S, Ozawa Y, Tsubota K, et al. Inhibition of choroidal neovascularization with an anti-inflammatory carotenoid astaxanthin. *Invest Ophthalmol Vis Sci*. 2008;49:1679–1685.
- Nakajima Y, Inokuchi Y, Shimazawa M, Otsubo K, Ishibashi T, Hara H. Astaxanthin, a dietary carotenoid, protects retinal cells against oxidative stress in-vitro and in mice in-vivo. *J Pharm Pharmacol*. 2008;60:1365–1374.
- Matsunaga N, Imai S, Inokuchi Y, Shimazawa M, Yokota S, Araki Y, et al. Bilberry and its main constituents have neuro-protective effects against retinal neuronal damage in vitro and in vivo. *Mol Nutr Food Res*. 2009;53:869–877.
- Imai S, Inokuchi Y, Nakamura S, Tsuruma K, Shimazawa M, Hara H. Systemic administration of a free radical scavenger, edaravone, protects against light-induced photoreceptor degeneration in the mouse retina. *Eur J Pharmacol*. 2010;642:77–85.
- Tsuruma K, Shimazaki H, Ohno Y, Inoue Y, Honda A, Imai S,

- et al. Metallothionein-III deficiency exacerbates light-induced retinal degeneration. *Invest Ophthalmol Vis Sci.* 2012;53:7896–7903.
- 29 Showalter LA, Weinman SA, Osterlie M, Lockwood SF. Plasma appearance and tissue accumulation of non-esterified, free astaxanthin in C57BL/6 mice after oral dosing of a disodium disuccinate diester of astaxanthin (Heptax). *Comp Biochem Physiol C Toxicol Pharmacol.* 2004;137:227–236.
  - 30 Mercke Odeberg J, Lignell A, Pettersson A, Hoglund P. Oral bioavailability of the antioxidant astaxanthin in humans is enhanced by incorporation of lipid based formulations. *Eur J Pharm Sci.* 2003;19:299–304.
  - 31 Stewart JS, Lignell A, Pettersson A, Elfving E, Soni MG. Safety assessment of astaxanthin-rich microalgae biomass: Acute and subchronic toxicity studies in rats. *Food Chem Toxicol.* 2008;46:3030–3036.
  - 32 Ono A, Sekita K, Saitoh M, Umemura T, Ogawa Y, Furuya T, et al. [A 13-week subchronic oral toxicity study of haematococcus color in F344 rats]. *Kokuritsu Iyakuhin Shokuhin Eisei Kenkyusho Hokoku.* 1999;91–98. (text in Japanese)
  - 33 Spiller GA, Dewell A. Safety of an astaxanthin-rich *Haematococcus pluvialis* algal extract: a randomized clinical trial. *J Med Food.* 2003;6:51–56.
  - 34 Satoh A, Tsuji S, Okada Y, Murakami N, Urami M, Nakagawa K, et al. Preliminary clinical evaluation of toxicity and efficacy of a new astaxanthin-rich *Haematococcus pluvialis* extract. *J Clin Biochem Nutr.* 2009;44:280–284.
  - 35 Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *Embo J.* 1991;10:2247–2258.
  - 36 Lo YY, Cruz TF. Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. *J Biol Chem.* 1995;270:11727–11730.
  - 37 Murrell GA, Francis MJ, Bromley L. Modulation of fibroblast proliferation by oxygen free radicals. *Biochem J.* 1990;265:659–665.
  - 38 Shigenaga MK, Ames BN. Assays for 8-hydroxy-2'-deoxyguanosine: a biomarker of in vivo oxidative DNA damage. *Free Radic Biol Med.* 1991;10:211–216.
  - 39 Wu Y, Yanase E, Feng X, Siegel MM, Sparrow JR. Structural characterization of bisretinoid A2E photocleavage products and implications for age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2010;107:7275–7280.
  - 40 Fukuzawa K, Inokami Y, Tokumura A, Terao J, Suzuki A. Rate constants for quenching singlet oxygen and activities for inhibiting lipid peroxidation of carotenoids and alpha-tocopherol in liposomes. *Lipids.* 1998;33:751–756.
  - 41 Sparrow JR, Zhou J, Ben-Shabat S, Vollmer H, Itagaki Y, Nakanishi K. Involvement of oxidative mechanisms in blue-light-induced damage to A2E-laden RPE. *Invest Ophthalmol Vis Sci.* 2002;43:1222–1227.
  - 42 Johnson EA, Schroeder WA. Microbial carotenoids. *Adv Biochem Eng Biotechnol.* 1996;53:119–178.
  - 43 Hama S, Takahashi K, Inai Y, Shiota K, Sakamoto R, Yamada A, et al. Protective effects of topical application of a poorly soluble antioxidant astaxanthin liposomal formulation on ultraviolet-induced skin damage. *J Pharm Sci.* 2012;101:2909–2916.
  - 44 Chang Q, Peter ME, Grassi MA. Fas ligand-Fas signaling participates in light-induced apoptotic death in photoreceptor cells. *Invest Ophthalmol Vis Sci.* 2012;53:3703–3716.
  - 45 Wenzel A, Grimm C, Samardzija M, Reme CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. *Prog Retin Eye Res.* 2005;24:275–306.
  - 46 Bursch W. The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death Differ.* 2001;8:569–581.
  - 47 Yamamoto M, Lidia K, Gong H, Onitsuka S, Kotani T, Ohira A. Changes in manganese superoxide dismutase expression after exposure of the retina to intense light. *Histochem J.* 1999;31:81–87.
  - 48 Thornalley PJ, Vasak M. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim Biophys Acta.* 1985;827:36–44.
  - 49 Pashkow FJ, Watumull DG, Campbell CL. Astaxanthin: a novel potential treatment for oxidative stress and inflammation in cardiovascular disease. *Am J Cardiol.* 2008;101:58D–68D.
  - 50 Kidd P. Astaxanthin, cell membrane nutrient with diverse clinical benefits and anti-aging potential. *Altern Med Rev.* 2011;16:355–364.