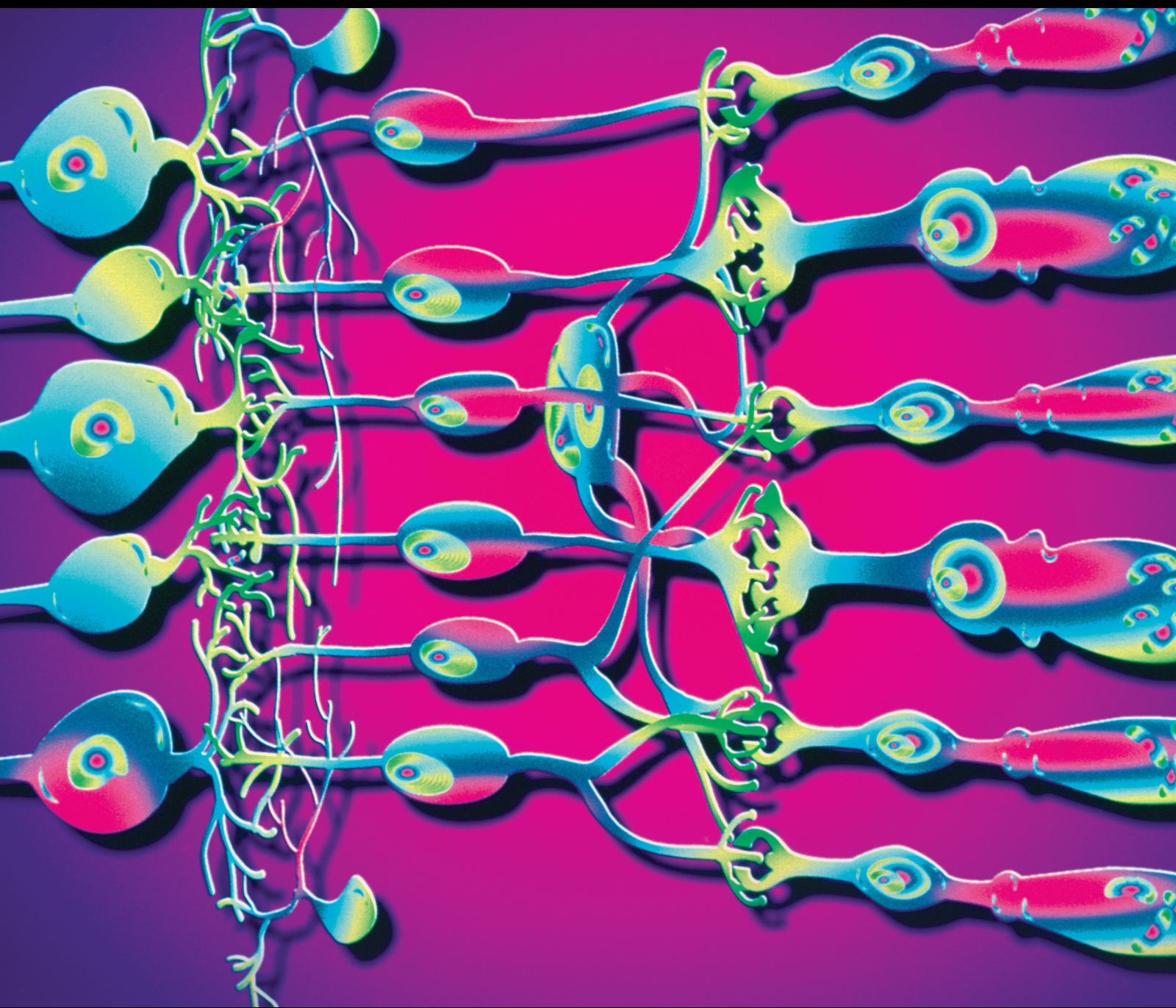
Journal of Ophthalmology

Zeaxanthin and Lutein in the Management of Eye Diseases

Guest Editors: Shun-Fa Yang, Joan E. Roberts, Qing-huai Liu, Jijing Pang, and Tadeusz Sarna



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Editorial Board

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Editorial

Zeaxanthin and Lutein in the Management of Eye Diseases

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Zeaxanthin and lutein, two carotenoid pigments of the xanthophyll subclass, are present in a high concentration in the retina, especially in the macula. They work as a filter protecting the macula from the blue light and also as structurally bound antioxidants which protect surrounding ocular cells against oxidative stress. Many observational and interventional studies have indicated that lutein and zeaxanthin might reduce the risk of various eye diseases, especially the age-related macular degeneration.

In this special issue, four review articles discuss zeaxanthin and lutein in the context of basic science studies, experimental animal studies, clinical trials, and safety and toxicological studies. J. E. Roberts and J. Dennison, in their paper "The Photobiology of Lutein and Zeaxanthin in the Eye," reviewed the oxidative stress inherently in photobiology, the reactive intermediate(s) of endogenous or exogenous photosensitizing agents in ocular tissues, and the ability of zeaxanthin and lutein to protect ocular tissues against damage. C. Xue et al. published the paper "Management of Ocular Diseases Using Lutein and Zeaxanthin: What Have We Learned from Experimental Animal Studies?" which reviews the preventive and therapeutic effects of zeaxanthin and lutein on various ocular diseases as studied in experimental animal models. This comprehensive survey provides new insights on future use of these xanthophylls for clinical management of vision-threatening diseases. N. K. Scripsema et al. authored a review article, "Lutein, Zeaxanthin, and *meso*-Zeaxanthin in the Clinical Management of Eye Disease," which looks at the current collection of epidemiological studies and clinical trials of carotenoids in various ocular diseases. These studies, especially the powerful randomized, placebo-controlled clinical trials, have confirmed the ability of zeaxanthin and lutein to modify the visual loss and risk of progression to advanced AMD, especially neovascular AMD. J. A. Edwards published an article, "Zeaxanthin: Review of Toxicological Data and Acceptable Daily Intake," which further explores zeaxanthin's safety in numerous well-organized experimental animal studies, which support its clinical use at much higher doses than what are currently recommended for the management of AMD.

Recent studies have revealed that, in addition to traditional mechanisms, lutein and zeaxanthin can influence the viability and function of cells through various signal pathways or transcription factors, such as their ability to inhibit the growth and cause apoptosis of malignant tumor cells such as ocular melanoma cells. In this issue, X. L. Xu, from the Memorial Sloan-Kettering Cancer Center, collaborated with researchers and pathologists from New York Eye and Ear infirmary to explore the "Effects of Zeaxanthin on Growth and Invasion of Human Uveal Melanoma in Nude Mouse Model." The study documents the ability of intraocularly administered zeaxanthin to significantly inhibit the growth and invasion of human uveal melanoma in nude mice. M.-C. Bi et al. in their paper, "Nonlethal Levels of Zeaxanthin Inhibit Cell Migration, Invasion, and Secretion of MMP-2 via NF-κB Pathway in Cultured Human Uveal Melanoma Cells," reported that zeaxanthin inhibited the secretion of MMP-2 along with the migration and invasion of cultured human

uveal melanoma cells. Both melanoma papers suggest that zeaxanthin may be a promising agent in the management of uveal melanoma and prevention of its spread.

The role of lutein and zeaxanthin in management of inflammation is explored by S.-C. Chao et al. and H.-Y. Lin et al. in their two papers: "Effects of Lutein and Zeaxanthin on LPS-induced Secretion of IL-8 by Uveal Melanocytes and Relevant Signal Pathways" and "Effects of Lutein on Hyperosmoticity-Induced Upregulation of IL-6 in Cultured Corneal Epithelial Cells and Its Relevant Signal Pathways." The first paper describes the ability of zeaxanthin and lutein to inhibit LPS-induced secretion of IL-8 by uveal melanocytes, which suggests a potential role for their application in the management of uveitis and other inflammatory eye diseases. The second paper shows how lutein inhibits hyperosmoticity-induced upregulation of IL-6 in cultured corneal epithelial cells and suggests that lutein may be a promising agent for the local treatment of dry eye.

Y. Tian et al. in their paper, "Lutein Leads to a Decrease of Factor D Secretion by Cultured Mature Human Adipocytes," reported that secretion of Factor D (the rate limiting enzyme of the complement alternative implicated in the pathogenesis of AMD) was significantly decreased following lutein supplementation to cultured human adipocytes. This suggests that lutein could be a useful tool for blocking the progression of AMD and other inflammatory diseases that are modulated by FD.

The one clinical study in this issue by S. M. van der Made et al., "Increased Macular Pigment Optical Density and Visual Acuity following Consumption of a Buttermilk Drink Containing Lutein-Enriched Egg Yolks: A Randomized, Bouble-Blind, Placebo-Controlled Trial," demonstrated improved visual acuity, macular pigment, and plasma lutein concentrations in elderly subjects with drusen and/or retinal pigment epithelial abnormalities following daily consumption of a dairy drink containing lutein-enriched egg yolks for one year.

Papers in this special issue have been assembled to go beyond the well-documented therapeutic effects of lutein and zeaxanthin on the AMD and explore future applications, suggested by animal and in vitro data, for diabetic retinopathy, cataract, uveal melanoma, phototoxicity, retinal detachment, uveitis, and dry eye. In addition, they demonstrate that the routes of administration of zeaxanthin and lutein could be expanded from oral administration to include local applications, such as eye drops or intravitreal injection. Finally, they point out that the dosages of zeaxanthin and lutein used in clinical trials could be increased dozenfold those currently used, if necessary for greater efficacy in severe conditions such as uveitis or uveal melanoma. It is the hope of the editors and authors that these insights may stimulate new uses for these ubiquitous but largely underappreciated components of our ocular environment.

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Clinical Study

Increased Macular Pigment Optical Density and Visual Acuity following Consumption of a Buttermilk Drink Containing Lutein-Enriched Egg Yolks: A Randomized, Double-Blind, Placebo-Controlled Trial

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Purpose. To study the effect of 1-year daily consumption of a dairy drink containing lutein-enriched egg yolks on macular pigment optical density (MPOD) and visual function parameters in elderly subjects with ocular drusen and/or retinal pigment abnormalities. *Methods*. One hundred and one subjects were recruited to participate in this randomized, double-blind, placebo-controlled parallel intervention trial. Statistical analyses were performed with 46 subjects in the lutein group and 43 in the control group. MPOD, best corrected visual acuity (BCVA, logMAR), and dark adaptation were measured at the start of the study, after 6 months and after 12 months. Plasma lutein and zeaxanthin concentrations were assessed at baseline and at the end of the study. *Results*. In the lutein group, plasma lutein concentrations increased significantly from 205 ng/mL at baseline to 399 ng/mL after twelve months of intervention. MPOD increased significantly from 0.45 to 0.52 and BCVA improved significantly from −0.04 to −0.09 LogMar. Differences in rod dark adaptation rate between both groups were not significant. *Conclusion*. Daily consumption of a dairy drink containing lutein-enriched egg yolks for one year improves visual acuity, MPOD, and plasma lutein concentration in elderly subjects with drusen and/or retinal pigment epithelial abnormalities.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness among the aged population in industrialized countries [1]. The incidence for late AMD varies from 1.4% in a Japanese study population >40 years [2] to 3.1% in a population aged 43–86 years in the US Beaver Dam Eye Study [3–5].

An increased intake of lutein via supplements has been shown to increase plasma lutein concentrations [6, 7] and to increase macular pigment level [8–10]. Although epidemiologic evidence evaluating the relation between dietary lutein and zeaxanthin intake and the risk for AMD is inconsistent [11, 12], ample evidence, including recent genetic data [13], points towards a protective effect of these carotenoids against AMD progression [14, 15]. Additionally, a study by Ma et al. [16] showed progressive improvements in macular pigment optical density (MPOD) and a trend towards improvement of best corrected visual acuity (BCVA) after lutein supplementation in early AMD patients.

Interestingly, bioavailability of lutein is shown to be two to three times higher from eggs than from spinach and lutein supplements, possibly caused by the lipid-rich matrix in which the lutein is provided [17]. From our previous study in one hundred healthy volunteers, it became clear that it is possible to significantly increase plasma lutein and zeaxanthin concentrations by twelve weeks consumption of a buttermilk drink that included lutein-enriched egg yolks [18].

Table 1: Distribution of AMD at baseline in the present study.

Grade	Criteria	Lutein group ($n = 51$)	Placebo group ($n = 49$)
0	Any small drusen	26	24
1	Soft distinct drusen	15	7
2	Indistinct drusen	5	8
3	Soft drusen/pigment changes	2	8
4	Atrophic changes	1	1
Total		49	48

MPOD did not change, which was most likely due to the relatively short intervention period. As described above, several studies have already shown an effect of lutein supplements on MPOD and visual function [19–22]; however, there are no reports yet that describe an effect of dietary lutein intake on visual function parameters. Therefore, the primary aim of this study was to assess the effects of 1-year daily consumption of a dairy drink containing lutein-rich egg yolks on MPOD and visual function parameters in elderly subjects with ocular drusen and/or retinal pigment abnormalities who had not yet been diagnosed with AMD.

2. Subjects and Methods

2.1. Subjects. One hundred and one subjects were recruited via advertisements in newspapers in the province of Limburg, the Netherlands, and 52 subjects were enrolled in the lutein group, while 49 subjects were enrolled in the control group. All participants gave their written informed consent before the screening procedure started. Eligible subjects were at least 50 years of age, showing drusen and/or retinal pigment epithelium alterations in at least one eye as evidenced by fundus photographs. Furthermore, visual acuity had to be >0.5, and eligible subjects should not have ocular media opacities, as assessed qualitatively by one of the staff ophthalmologists of our clinic, and were not allowed to use nutritional supplements containing lutein or zeaxanthin. Individuals willing to participate were excluded from taking part in the study when they used medication to treat diabetes, had cardiovascular diseases or disturbances in their lipid metabolism that demanded lipid-lowering treatment, or were allergic to eggs or egg products. One subject already withdrew after the screening and before the baseline visit. The study protocol was approved by the Medical Ethics Committee of Maastricht University Medical Centre and registered at ClinicalTrials.gov on May 14, 2009, as NCT00902408. All research and measurements followed the tenets of the Declaration of Helsinki and were performed within Maastricht University Medical Centre from October 30, 2009, through December 2, 2011.

2.2. Study Design. A one-year, randomized, double-blind, placebo-controlled intervention trial was conducted in elderly subjects with ocular drusen and/or retinal pigment abnormalities. Subjects were allocated to the control or lutein group according to a preestablished, computer generated randomization scheme. Allocation was concealed in sequentially numbered, sealed envelopes and stored by the study coordinator. Participants who were allocated to the experimental (lutein) group were asked to consume one lutein-enriched egg-yolk containing dairy drink daily (NWT-02, provided by Newtricious R&D, Oirlo, The Netherlands). The one and a half egg yolks in this drink were enriched in lutein, zeaxanthin, and DHA via the feed of laying hens and were incorporated in 80 mL buttermilk drink. The intervention products contained on average 1.38 ± 0.16 mg of lutein, 0.21 ± 0.02 mg of zeaxanthin, and 160 ± 10 mg of DHA. Subjects who were assigned to the control group received a similar buttermilk drink without the addition of 1.5 egg yolks. In order to keep participants and the study team unaware of treatment groups, the color of the control drink was matched to that of the lutein-enriched drink by adding synthetic colorants E104 and E110. Lutein, zeaxanthin, and DHA concentration were below detection limit in the control group. For one year, participants received a fresh delivery of buttermilk drinks at home every two weeks. These drinks were provided in 100 mL flasks that were packaged in carton boxes. Both flasks and boxes were coded with the randomization number of the subject.

2.3. Fundus Photography, Grading, and Classification. After maximal pupil dilatation was achieved using tropicamide 0.5% eye drops, fundus photographs were obtained using a Topcon TRC-50EX camera. Acquired images were centered on the fovea (1840×1224 pixels) and subtended 45°.

After the study finished, detailed grading for age-related maculopathy and age-related macular degeneration was performed on the fundus photographs taken from the test eye at the baseline visit according to the international classification and grading system [23, 24]. The grading was performed by an independent site, for example, at the Rotterdam Study Center. Table 1 shows the distribution of AMD at baseline.

2.4. Macular Pigment Optical Density (MPOD). MPOD was determined by heterochromatic flicker photometry (QuantifEYE; Topcon, Newbury, UK). In this device there are two light emitting diodes (blue, 470 nm, and green, 540 nm) that make up a target that flickers in counterphase. At the start of the test, the temporal flicker frequency is above the normal critical flicker fusion frequency (60 Hz) and is reduced at 6 Hz/sec. The subject fixates on the target and presses a button when flicker is detected. The luminance ratio of blue and green is then changed, incrementing blue and decrementing green. The temporal frequency is reset to 60 Hz and again ramped down at 6 Hz/sec, until the subject detects flicker and presses the response button. Starting with a green luminance

being higher than the blue luminance, this cycle continues for a series of blue-green luminance ratios until a V shaped function is obtained with a clear minimum that corresponds to the equalization of the blue and green luminance. This process of detecting flicker for a series of blue-green luminance ratios is then repeated for peripheral viewing at 6 degrees eccentricity, where again a V shaped curve is obtained which provides a minimum for the periphery. From these two curves, the MPOD is calculated according to the formula MPOD = $\log[L_c/L_p]$, where L_c and L_p are the luminance of the blue light at the minimum for central and peripheral viewing, respectively.

2.5. Visual Acuity. Best corrected visual acuity (BCVA) was measured with an internally illuminated Early Treatment Diabetic Retinopathy Study (ETDRS) logMAR chart at 4 m. Illumination of the testing room was set at 200 lux. The luminance of the charts was checked with a photometer (PR-650 SpectraScan Colorimeter). Mean luminance of the center of the charts was 180 cd/m^2. Participants were asked to read all the letters they could recognize, monocularly with the testing eye, starting from the top left letter in the first row.

2.6. Dark Adaptation. Dark adaptation (DA) was measured monocularly with an undilated pupil. Prior to the beginning of a DA test, the tested eye was bleached with an electronic Flash Gun. The other eye was covered with an eye patch. Testing distance was set to 100 cm. Stimuli generated with a VSG 2/5 card (Cambridge Research Systems Ltd., Rochester, UK) were displayed on a Sony GDM-f500 high resolution graphics display. Neutral density filters were placed in front of the screen during the time-course of the test in order to increase its luminance range. This was achieved by covering the monitor screen with a 1.3 log unit neutral density filter for the first cone stage and subsequently adding 2 other filters each of 1.3 using a sliding mechanism at different stages as sensitivity improved. Following bleaching, the subjects fixated a red fixation target (0.3 degrees) presented at 11 degrees visual angle from the testing stimulus. The testing stimulus was composed of a white illuminant C (CIE 1931 coordinates are $x = 0.31$ and $y = 0.316$) 1 degree, temporally modulated (1 Hz) stimulus. Thresholds were determined with the method of adjustment. This procedure continued at approximately 2-minute intervals until an absolute threshold was reached.

Dark adaptation data were processed using a 7-parameter model using the Nelder-Mead method implemented in MATLAB (Nantick, MA). A text file was produced to include the 7 parameters of the model fit. These data were subject to the statistical analysis. Nonrational data such as excessive sensitivity values (e.g., 3000 dB) were removed by filtering so that the sensitivity range fell within 0–200 dB. The model was reapplied to this modified data set.

2.7. Plasma Lutein and Zeaxanthin Concentrations. Fasting blood samples were taken at the start of the study (T0) and after 12 months of intervention (T12), to assess plasma lutein concentrations. Blood samples were taken from a forearm vein after an overnight fast (no food or drink after 8 PM, except for water), by the same person, and at the same location. Plasma was obtained by sampling blood into EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA), followed by low-speed centrifugation at 1300 ×g for 15 min at 4°C.

Lutein and zeaxanthin concentrations were analyzed using high performance liquid chromatography (HPLC), as previously described [25]. Briefly, on the day of analysis, the samples were thawed and mixed well. Samples were deproteinized by adding 500 μL sample to 500 μL ethanol. After this, the samples were mixed and allowed to stand for 15 minutes at room temperature to complete precipitation of proteins. Subsequently, carotenoids were extracted by adding 1.0 mL n-hexane. After centrifugation for 10 minutes at 4°C and 3.000 ×g, 0.5 mL of the upper hexane layer was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.5 mL of a mixture of methanol, acetonitrile (1 : 1), and dichloromethane and analyzed by HPLC. Separation was obtained on a C18 reversed-phase column, thermostatically controlled at 30°C. The samples were eluted by use of a mobile phase consisting of methanol, acetonitrile, 2-propanol, and water at a flow rate of 1.5 mL/min. Detection was performed with a diode array UV detector at 450 nm. Quantification was carried out by including commercially available lutein and zeaxanthin as a standard (Sigma-Aldrich, St. Louis, USA).

2.8. Statistical Analyses. Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Differences in gender distribution and smoking status over the experimental groups were tested using the Pearson Chi-square test, while baseline differences in age, plasma lutein and zeaxanthin concentrations, MPOD, and visual acuity were evaluated by an unpaired Student's t-test. A Linear Mixed Models (LMM) analysis with subject ID as grouping factor and diet and time and their interaction term as covariate was performed to evaluate differences in MPOD and VA. The same approach was used to evaluate differences within the control and lutein group regarding changes in MPOD during the 1-year intervention. Changes in plasma lutein and zeaxanthin concentrations over time were evaluated using an unpaired Student's t-test. P values were considered significant if $P < 0.05$. Results are shown as mean ± standard deviation (SD).

The expected increase in MPOD in our, earlier reported [18], one-year trial was estimated at 12%. Considering an MPOD measuring error of 17%, a significance level (α) of 5%, a power of 90%, and a 10% dropout rate, 48 subjects had to be included in the study. However, for one of the secondary outcome parameters in this study (reported elsewhere), which was flow-mediated dilation (FMD), 50 subjects should be included in both intervention groups to detect a true difference in FMD of at least 1%, assuming a standard deviation of FMD of 1.7%, a dropout rate of 10%, a power of 80%, and a significance level (α) of 5%. Therefore, a total of 101 subjects started the study which means that it was well powered to show significant effects for the measurements described here.

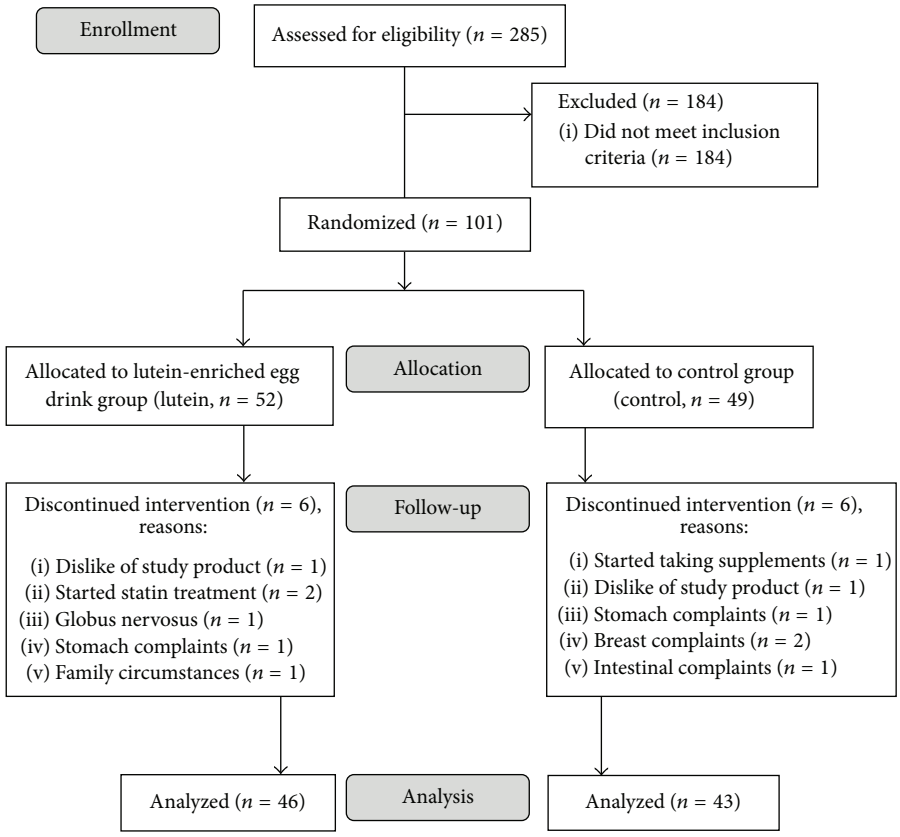

Figure 1: Subject flow chart.

3. Results

3.1. Subject Characteristics. During the 1-year follow-up of the study, twelve participants withdrew. The flow of participants throughout the study and reasons for discontinuation are shown in Figure 1. At baseline, no statistically significant differences were found between the lutein and control groups regarding age, BMI, plasma lutein concentration, MPOD, visual acuity, and dark adaptation (Table 2).

3.2. Macular Pigment, Visual Acuity, Dark Adaptation, and Plasma Lutein and Zeaxanthin. Visual acuity improved upon receiving a lutein-enriched diet ($P < 0.01$, Figure 2). A significant decrease of 0.0052 ± 0.0017 LogMAR units per month (i.e., half a line increase on ETDRS chart over one year) was observed in the lutein group as compared to the control group ($P < 0.01$).

Although the rod dark adaptation rate showed a tendency to increase in the lutein group, which represents faster recovery, and a decrease in the placebo group (i.e., a further slowing down of the rod dark adaptation rate), differences between the groups were not significant following statistical analysis ($P = 0.14$; data not shown).

We observed a significant 94% increase in plasma lutein concentrations from 205 to 399 ng/mL in the lutein group and no significant change in the control group ($P < 0.001$, Figure 3). A similar increase in plasma zeaxanthin was observed in the lutein group ($P < 0.01$). Consequently, also MPOD increased significantly in the lutein group as compared to the control group ($P < 0.05$, Figure 4) and within the lutein group as compared to baseline (Table 3). The increase in MPOD in the lutein group compared to the control group was on average 0.0041 ± 0.0019 per month.

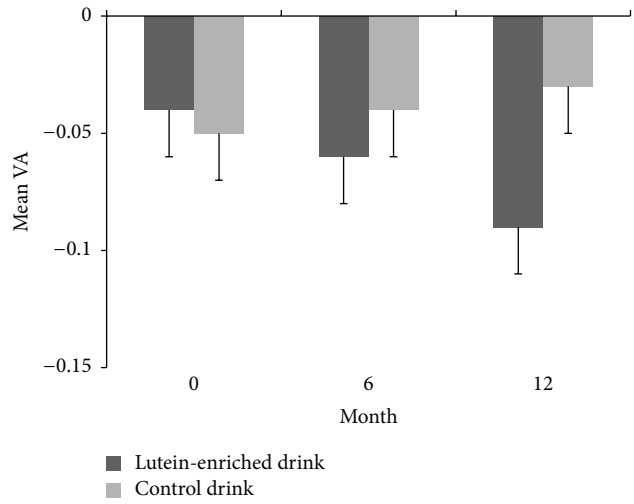

Figure 2: Mean (±SE) visual acuity (LogMAR) in time for the lutein group (dark grey) and the placebo group (light grey). Change in lutein group was significantly different from change in control group ($P < 0.01$).

Table 2: Baseline visual acuity (VA), macular pigment optical density (MPOD), and plasma lutein concentrations in lutein and control groups.

	Lutein group ($n = 51$)	Control group ($n = 49$)	P value
Gender (m/f)	17/34	15/34	0.832
Age (years)	62 ± 7	63 ± 8	0.557
BMI (kg/m^2)	26.7 ± 3.6	26.0 ± 3.8	0.375
Ever smoked (yes)	24	20	0.472
Lutein (ng/mL)	206 ± 148	199 ± 118	0.803
MPOD	0.45 ± 0.14	0.46 ± 0.16	0.859
VA (LogMar)	−0.04 ± 0.14	−0.05 ± 0.13	0.711
Dark adaptation	−0.17 ± 0.05	−0.19 ± 0.06	0.302

MPOD, macular pigment optical density; VA, visual acuity.

Table 3: Mean changes in MPOD in lutein and control groups during the time course of the study.

	Mean MPOD	Absolute change from baseline	SE	% change from baseline	P value
Lutein group					
Baseline, $n = 46$	0.45	—	0.02	—	—
6th month, $n = 41$	0.47	0.05	0.02	4.4	<0.001
12th month, $n = 45$	0.52	0.07	0.02	15.6	<0.001
Control group					
Baseline, $n = 46$	0.46	—	0.02	—	—
6th month, $n = 38$	0.48	0.02	0.03	6.7	0.18
12th month, $n = 43$	0.48	0.02	0.03	4.4	0.34

A linear mixed model approach was used to assess differences.

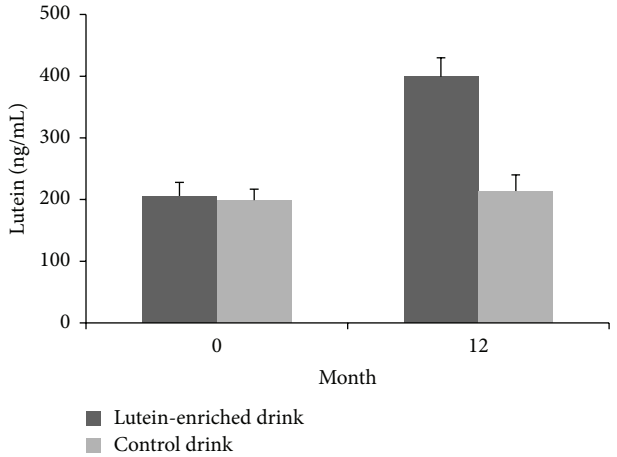

Figure 3: Mean (±SE) plasma lutein concentration in time for the lutein group (dark grey) and the placebo group (light grey). The increase in the lutein group was significantly different from the change in the control group ($P < 0.001$).

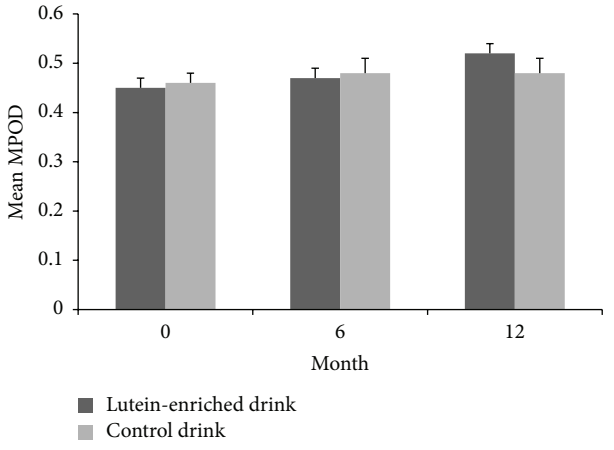

Figure 4: Mean ± SE macular pigment optical density (MPOD) in time for the lutein group (dark grey bars) and the placebo group (light grey bars). MPOD increased significantly in the lutein group as compared to the control group ($P < 0.05$).

4. Discussion

This study shows that it is possible to increase plasma lutein and improve visual acuity and macular pigment optical density by daily consumption of a dairy drink containing lutein-rich egg yolks in elderly subjects with drusen and/or retinal pigment abnormalities. To the best of our knowledge, the effect of long term consumption of a functional food on visual function parameters has not yet been reported earlier. The individuals included in our study had ocular drusen and/or retinal pigment abnormalities in at least one eye, but they had not yet been diagnosed with AMD at the time of

recruitment. Analysis of the fundus photographs by an independent referral center showed that 48% of the eyes would have been classified as AMD grades 1 to 4. In view of the small numbers we did not perform a subgroup analysis concerning MPOD or BCVA response.

Following the diet with a lutein-enriched egg drink (1.4 mg extra lutein/day), we observed an increase in MPOD concentrations after 12 months. An earlier pilot study from our group did not show an effect of a lutein-enriched egg diet on MPOD levels in healthy subjects. Besides the different study population, this is probably due to the shorter intervention period of 3 months of this previous study [18]. A study by Murray et al. [6] showed a significant increase in MPOD after twelve-month intake of 10 mg lutein ester. In this study, the increase in MPOD relative to the control group was 39.5%, which is considerably higher than the 10.7% increase observed in our study. However, in the latter study, the group supplemented with lutein had a significantly lower baseline MPOD than the placebo group [6]. Others showed an increase in MPOD in two lutein-supplemented groups that either received a daily 10 or 20 mg dose for 48 weeks [7]. Unfortunately, no comparison was made between intervention groups and placebo group, which makes it hard to draw conclusions on the true effect of lutein on MPOD in this particular study. A one-year study in subjects with early AMD showed an increase in MPOD following intake of one out of three combinations of lutein, zeaxanthin, and *meso*-zeaxanthin [26]. However, no placebo group was included in this study. Another study [27] found a 27.2% relative increase in MPOD after the daily intake of 20 mg lutein for three months followed by 10 mg daily for a time period of three months. In this study, a spectroscopic technique was used to measure MPOD, whereas in our study, and in most other studies until now, MPOD is assessed by flicker photometry [28–30]. A trial describing a twelve-month lutein supplementation in atrophic AMD patients reported a significant 36% MPOD increase in the lutein group, whereas a decrease in MPOD was observed in the control group [19]. Furthermore, a recent meta-analysis concluded that dietary supplementation of lutein leads to a significant improvement in MPOD [22]. Although the increase in MPOD as seen in our study was significant, it did not reach the relative increase as observed in earlier studies using lutein containing pill supplements. The reasons for this discrepancy are not clear but could be associated with the high baseline MPOD of the subjects in our study as compared to the other studies [6, 19, 27, 31].

Macular pigment is composed of three different carotenoids including lutein, zeaxanthin, and *meso*-zeaxanthin, whereby lutein is mainly found in the peripheral macula, whereas zeaxanthin and *meso*-zeaxanthin are present in the center. Eggs are a well-known source of both lutein and zeaxanthin and after consumption they may accumulate in the retina. Retinal *meso*-zeaxanthin will be incorporated in the macula following local bioconversion from lutein or may be acquired via other foodstuffs.

While the increase in MPOD was not as pronounced as shown in the supplement studies mentioned above, we did show a significant improvement over time in visual acuity in the lutein intervention group as compared to the control group. Our data are in agreement with earlier studies showing an increase in both MPOD and VA after twelve months of daily 10 mg nonesterified lutein or 10 mg lutein combined with a range of antioxidants and vitamins [19]. Visual acuity did not improve in the study by Murray et al. [6], which was argued to be caused by the fact that >50% of the population already had a normal or above normal VA. Indeed, in their study, a significant improvement in VA was found in a subpopulation of subjects with a low VA at baseline. Additionally, a recent meta-analysis showed a significant improvement in VA after lutein and zeaxanthin supplementation in four out of seven studies. A slightly stronger effect was found in studies with higher-dose (20 mg lutein daily) supplementation [32]. Although the amount of lutein given in our study is considerably lower than the dosages given in these studies, we found a significant improvement in VA. It should be noted that the lutein group in our study started with a relatively good VA of −0.04 logMAR. Despite the adequate VA in these subjects, we still observed a small but statistically significant improvement after the one-year intervention. This implies that the effect in our study might have even been more pronounced if we would have included subjects with a lower baseline VA. Additionally, this meta-analysis found a dose-dependent improvement in contrast sensitivity after lutein supplementation [32], which indicates that this parameter should be further explored in the target population as performing additional visual function tests to detect subtle changes in the macula is clearly important.

The quantity of lutein used in the current study (1.4 mg/day) was less than the amount used in studies that provided lutein capsules, which varied between 5 and 20 mg of lutein [33]. Still, we were able to show a significant increase in plasma lutein concentrations in the intervention group over the one-year course after consuming the lutein-enriched egg yolks that provided an average additional daily lutein intake of 1.4 mg. This underlines the finding that lutein has a high bioavailability from eggs [17], which is most probably caused by the matrix in which the lutein is incorporated [34]. Lutein bioavailability after taking a capsule supplement is dependent on the quantity of fat in the meal during which it is taken [35].

Several studies have evaluated the effect of lutein supplementation on plasma lutein concentration. A study with university students taking 10 mg lutein and 2 mg zeaxanthin over a time period of 300 days showed a 400% increase in plasma lutein concentration [36]. Other studies showed significant increases in serum lutein concentration varying from 17 to 555% change in both normal and AMD subjects after only 8 weeks intake of one out of three combinations of lutein, zeaxanthin, and *meso*-zeaxanthin [37]. These results were reproduced in a three-year study using the same intervention in forty-seven subjects with early AMD [38]. However, it must be noted that no placebo arm was included in both studies.

As reflected by the high standard deviation of plasma lutein concentrations in our study, it is clear that there is a marked difference in the individual response to lutein supplementation. Genetic polymorphisms are suggested to play a role in bioavailability of lutein and zeaxanthin and influence both serum and retina status of these carotenoids. In a recent

study by Meyers and colleagues, these genetic determinants were found to be independent of dietary lutein and zeaxanthin intake [13]. This study showed several genes to be associated with serum lutein and zeaxanthin concentration, including stAR-related lipid transfer protein 3 (*STARD3*), which is involved in xanthophyll binding in the retina; ATP-binding cassette subfamily G member 8 (*ABCG8*); and cholesteryl ester transfer protein (*CETP*), which are involved in high-density lipoprotein (HDL) transport [13]. Another study also showed a high interindividual variability (CV 75%) in postprandial chylomicron lutein response after consuming lutein-enriched meals, which was found to be genetically determined [39]. Furthermore, there might be an optimum in terms of lutein and/or zeaxanthin supplementation. It was hypothesized that there is duodenal, hepatic-lipoprotein, or retinal carotenoid competition for carotenoid uptake [40]. These findings suggest that future research into the relation between dietary lutein and improving visual performance should also take genetic background of the subjects into account.

Epidemiological and intervention studies indicate that a higher lutein intake may delay AMD development [12]. Increased consumption of dietary cholesterol from lutein-enriched eggs might lead to raised serum cholesterol concentrations [41], which in turn is associated with a higher risk of developing cardiovascular disease (CVD). However, a recent meta-analysis revealed that a higher consumption of eggs (up to one egg per day) was not associated with increased risk of coronary heart disease or stroke [42]. We assessed the possible side effects of our trial and showed that the consumption of the lutein-enriched egg-yolk containing buttermilk drink daily for a time period of 1 year did not lead to a significant increase of serum total, HDL, and LDL cholesterol, as well as the ratio of total cholesterol to HDL cholesterol [43].

In conclusion, we here show that daily consumption of a lutein-enriched egg drink for one year leads to a significant increase in visual acuity and is capable of enhancing both plasma and macular concentrations of lutein.

Disclosure

Sanne M. van der Made is currently employed at Newtricious R&D B.V.

Competing Interests

Elton R. Kelly, Aize Kijlstra, Jogchum Plat, and Tos T. J. M. Berendschot have no conflict of interests.

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Research Article

Effects of Lutein on Hyperosmoticity-Induced Upregulation of IL-6 in Cultured Corneal Epithelial Cells and Its Relevant Signal Pathways

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Dry eye is a common disorder characterized by deficiency of tear. Hyperosmoticity of tear stimulates inflammation and damage of ocular surface tissues and plays an essential role in the pathogenesis of dry eye. Cultured human corneal epithelial (CE) cells were used for the study of effects of lutein and hyperosmoticity on the secretion of IL-6 by CE cells. Cell viability of CE cells was not affected by lutein at 1–10 μM as determined by MTT assay. Hyperosmoticity significantly elevated the secretion of IL-6 by CE cells as measured by ELISA analysis. The constitutive secretion of IL-6 was not affected by lutein. Lutein significantly and dose-dependently inhibited hyperosmoticity-induced secretion of IL-6. Phosphorylated- (p)- p38 MAPK, p-JNK levels in cell lysates and NF-κB levels in cell nuclear extracts were increased by being exposed to hyperosmotic medium. JNK, p38, and NF-κB inhibitors decreased hyperosmoticity-induced secretion of IL-6. Lutein significantly inhibited hyperosmoticity-induced elevation of NF-κB, p38, and p-JNK levels. We demonstrated that lutein inhibited hyperosmoticity-induced secretion of IL-6 in CE cells through the deactivation of p38, JNK, and NF-κB pathways. Lutein may be a promising agent to be explored for the treatment of dry eye.

1. Introduction

Dry eye is a multifactorial disorder of the ocular surface characterized by symptoms of ocular discomfort and visual disturbance and is associated with reductions in the quality and/or quantity of tears. Symptoms of dry eye include eye irritation, stinging, dryness, eye fatigue, and fluctuating visual disturbances. Dry eye is a common disease and the prevalence of dry eye ranges from 5 to 33% of the adult population. The prevalence of dry eye increases with age and is more common in women. It can lead to significant functional impairment in daily life and affects quality of life and productivity. A substantial economic burden to the patients and society is derived owing to associated health care costs and loss of productivity of affected individuals [1–7].

Deficiency of tear, resulting from the decrease of aqueous tear production or excessive tear evaporation, is the essential pathological changes of dry eye. Hyperosmolarity of tear film caused by deficiency of tear can initiate inflammation and damage of ocular surface and is the main mechanism of the development of dry eye [1, 4, 8].

Inflammation plays an important role in the pathogenesis of dry eye. It has been reported that various proinflammatory cytokines and chemokines levels in the tears or ocular

surface tissues are significantly increased in dry eye patients [9–14]. IL-6 is a proinflammatory cytokine and plays an important role in the pathogenesis of autoimmune diseases [15, 16]. IL-6 levels in the tear are significantly increased in dry eye patients [9–12]. IL-6 levels rise in lacrimal and ocular surface tissues in patients with Sjögren syndrome (an autoimmune disorder and an important cause of dry eye) [13, 14]. The expression and production of IL-6 are also elevated in experimental dry eye models [8, 17–19]. It has been reported that hyperosmoticity can stimulate the expression and secretion of IL-6 in various cultured cells [8, 18, 19].

Lutein, a natural bioactive substance that belongs to the xanthophyll class of the carotenoids, is found in dark green leafy vegetables such as kale and spinach. Lutein is naturally present in the eye at a high level. It is a yellow colored pigment that can absorb high energy blue light and protects cells from phototoxicity. Lutein also works as an antioxidant and a free radical scavenger [20–23].

Lutein has been studied for its effects on the prevention and treatment of various ophthalmic diseases in vitro and in vivo, including age-related macular degeneration (AMD) and diabetic retinopathy [24–30]. Epidemiological studies and clinical trials documented that a high level of lutein in the macula is associated with lower incidence of AMD and supplementation of lutein has effects on the prevention and treatment of AMD. Therefore, lutein is widely used as nutrient supplements for the management of AMD and other eye diseases [31–34].

Recently, a number of in vivo and in vitro studies suggested that lutein had an anti-inflammatory effect in experimental animal uveitis models and in cultured cells stimulated by various proinflammatory factors [35–39]. Our previous study documented that lutein inhibited lipopolysaccharide- (LPS-) induced secretion of IL-8 in cultured uveal melanocytes, which suggested that lutein may be a promising agent to be explored for the prevention and treatment of ocular inflammation [35]. Furthermore, blood lutein levels are inversely related to IL-6 levels in normal individuals or patients with various diseases [40–42]; supplementation of lutein decreases IL-6 levels in experimental animals or patients [43–45]. To the best of our knowledge, the effects of lutein on the inflammatory processes of ocular surface have not been reported.

The purpose of the present study was to investigate hyperosmoticity-induced upregulation of IL-6 in cultured human CE cells and its relevant signal pathways.

2. Material and Methods

2.1. Cell Culture. The human corneal epithelial (CE) cells used in the present study are a SV40-adenovirus-immortalized CE cell line, which was obtained from Dr. Peter Reinach (State University of New York, New York, USA) [8]. Cells were grown in defined K-SFM medium supplemented with 10% fetal bovine serum and 50 μg/mL gentamicin (all from GIBCO, Grand Island, NY, USA) and incubated in a humidified 95% air/5% CO_2 atmosphere at 37°C [8].

2.2. MTT Assay. The effects of lutein on the cell viability of cultured CE were tested by using MTT assay as previously described [8]. For each experiment, cells were seeded into 96-well plates at a density of 5×10^3 cells/well. Lutein (Sigma, St. Louis, MO, USA) was dissolved in DMSO (Sigma) and added to the medium at different levels 24 h later. After incubation for 24 h, MTT (50 μL/well of 1 mg/mL) was added and cells were incubated for another 4 h. DMSO at 100 μL/well was added after the removal of the culture medium. The optical density as the parameter of cell viability was measured at 540 nm with a microplate reader (Multiskan EX, Thermo, Ventana, Finland). All experiments were performed in triplicate.

2.3. Effects of Lutein and Hyperosmoticity on the Secretion of IL-6 by CE Cells. For the study of secretion of IL-6 by CE cells, 5×10^4 cells were seeded on 24-well plates. After 24 h, the culture medium was replaced with serum-free isoosmotic or hyperosmotic medium and cultured for 24 h. For the preparation of hyperosmotic medium, sodium chloride was added to the medium to reach a final concentration of 90 mM with hyperosmoticity at 450 mOsM [8]. Osmette Osmometer (Precision System, Natick, MA, USA) was used to measure the osmolarity of the solution [8]. In the study of the effects of lutein, lutein at 0, 1, 3, and 10 μM was added 30 min before exposure to the hyperosmotic medium and then cultured for 24 h. Conditioned culture media from different groups were collected and centrifuged. The supernatants were stored at −80°C. All experiments were performed in triplicate.

2.4. Measurement of IL-6 Protein Levels. IL-6 protein levels in the conditioned culture medium were measured by enzyme-linked immunosorbent assay (ELISA) by using the human IL-6 Quantikine ELISA kit (R&D System, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. The optical density of the ELISA samples was measured at 450 and 540 nm using a microplate reader; IL-6 levels (pg/mL) were calculated from a standard curve and expressed as the percentage of the negative controls (cells cultured with isoosmotic medium without lutein). The sensitivity of the assay was 0.7 pg/mL. All experiments were performed in triplicate.

2.5. Effects of Lutein and Hyperosmoticity on MAPK and NF-κB Levels in CE Cells. CE cells at a density of 1×10^6 cells/well were seeded on 6-well plates and cultured for 24 h. Then, culture medium was removed and washed and cells were cultured with isoosmotic or hyperosmotic medium with and without lutein (10 μM) as described above. The cultures were washed with cold PBS 60 min later. Cells were collected and centrifuged. The pellets were cultured with cell extraction buffer (Biosource, Camarillo, CA, USA), protease inhibitor cocktail (Sigma), and PMSF (Biosource) for 30 min at 4°C with vortexing at 10 min intervals. Cultures were microcentrifuged at 4°C and the supernatants were collected and stored at −80°C until analysis for mitogen-activated protein kinase (MAPK) levels. For the measurement of nuclear factor-kappa B (NF-κB) levels in cell nucleus, cells were collected, treated with hypotonic buffer (BioSource),

and centrifuged. The pellets that contained nuclear fraction were collected, treated with cell extraction buffer (BioSource), vortexed, and centrifuged. The supernatants were stored at −80°C until analysis for NF-κB levels.

2.6. Measurement of MAPK and NF-κB Levels. ELISA was used for the measurement of MAPK and NF-κB levels. MAPK levels were measured by using various phosphorylated- (p)- MAPK kits (Biosource). P-p38 MAPK, p-extracellular signal-regulated kinases 1/2 (ERK1/2), and p-c-Jun N-terminal kinase (JNK1/2) kits were used to determine p-p38 MAPK, p-ERK1/2, and p-JNK1/2 levels in cell extracts, respectively. The test was performed according to the protocol provided by the manufacturer and expressed as the percentage of the negative controls (cells cultured with isoosmotic medium without lutein). The sensitivity of these kits was 0.8 U/mL. NF-κB levels in the nuclear portion were measured by NF-κB ELISA kits (Invitrogen) according to the manufacturer's instructions. The levels of NF-κB were expressed as percentages of the negative controls. The sensitivity of this kit was <50 pg/mL. All tests were performed in triplicate.

2.7. Effects of MAPK and NF-κB Inhibitors on Hyperosmoticity-Induced Secretion of IL-6 by CE Cells. CE cells were plated into 24-well plates at a density of 1×10^5 cells per well. After 24 h incubation, the medium was changed. Various MAPK inhibitors (Calbiochem, San Diego, CA), including UO1026 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 MAPK inhibitor) all at 10 μM, were added to the medium. For the study of the effect of NF-κB inhibitor, 5 μM BAY11-7082 (Calbiochem, San Diego, CA) was added to the medium. Thirty minutes later, sodium bicarbonate was added to the medium to archive hyperosmotic medium as described above. Cells were cultured with isoosmotic or hyperosmotic medium as described above. After 24 h incubation, the conditioned media were collected and stored. IL-6 levels were determined using the human IL-6 Quantikine ELISA kit as described above. Tests were performed in triplicate.

2.8. Statistical Analysis. Data analysis was performed using specific software (SPSS 19.0, SPSS Inc., Chicago, IL, USA). Statistical significance was analyzed using analysis of one-way ANOVA test. *P* values less than 0.05 were considered as significant.

3. Results

3.1. MTT Assay. Cell viability of cultured human CE cells was not affected by lutein at 1, 3, and 10 μM as compared to cells cultured without lutein ($P > 0.05$) (Figure 1). Therefore, we used lutein at 1–10 μM for testing its effects on hyperosmoticity-induced secretion of IL-6 and changes of various signal pathways levels in this study.

3.2. Effects of Lutein and Hyperosmoticity on Secretion of IL-6 by CE Cells. CE cells cultured in isoosmotic medium showed a constitutive secretion of IL-6 at 42.3 ± 4.7 ng/mL.

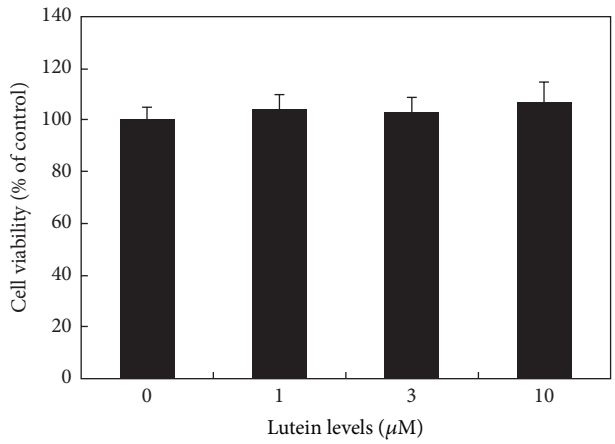

Figure 1: Effects of lutein on cell viability of cultured human CE cells. Cells were cultured with different levels of lutein and cell viability was measured by MTT assay. Lutein at 1, 3, and 10 μM did not affect the cell viability.

Hyperosmotic medium (450 mOsM) caused a significant increase of IL-6 levels in the culture medium ($P < 0.05$, Figure 2).

In cells cultured with isoosmotic medium, lutein did not significantly affect IL-6 levels in the conditioned medium ($P > 0.05$, Figure 2(a)). In cells cultured with hyperosmotic medium, IL-6 levels in the conditioned medium from lutein treated cultures were dose-dependently and significantly decreased as compared to the positive controls (cells cultured with hyperosmotic medium but without lutein) (Figure 2(b)). IL-6 levels in cells treated with lutein at 3 and 10 μM were significantly lower than that in the positive controls ($P < 0.05$, Figure 2(b)).

3.3. Effects of Lutein and Hyperosmoticity on MAPK and NF-κB Levels in CE Cell. Hyperosmoticity caused a significant increase of phosphorylated p38 MAPK and JNK1/2 in CE cells lysates ($P < 0.05$) (Figures 3(a) and 3(b)) but not p-ERK1/2 levels (Figure 3(c)). Lutein did not significantly affect p38 MAPK, JNK1/2, and ERK1/2 levels in cells cultured with isoosmotic medium (Figure 3). In cells cultured with hyperosmotic medium, lutein at 10 μM significantly reduced p-p38 MAPK and p-JNK1/2 levels, but not p-ERK1/2 levels as compared to the positive controls (cells cultured with hyperosmotic medium but without lutein) ($P < 0.05$, Figure 3).

NF-κB levels in cell nuclear extracts from cells treated with hyperosmotic medium were significantly greater than that of the negative controls (cells cultured with isoosmotic medium) ($P < 0.05$, Figure 3(d)). Lutein did not significantly affect NF-κB levels in cells cultured with isoosmotic medium ($P > 0.05$, Figure 3(d)). In cells cultured with hyperosmotic medium, lutein at 10 μM significantly reduced NF-κB levels as compared to the positive controls ($P < 0.05$, Figure 3(d)).

3.4. Effects of MAPK and NF-κB Inhibitors on Hyperosmoticity-Induced Secretion of IL-6 by CE Cells. Pretreatment of

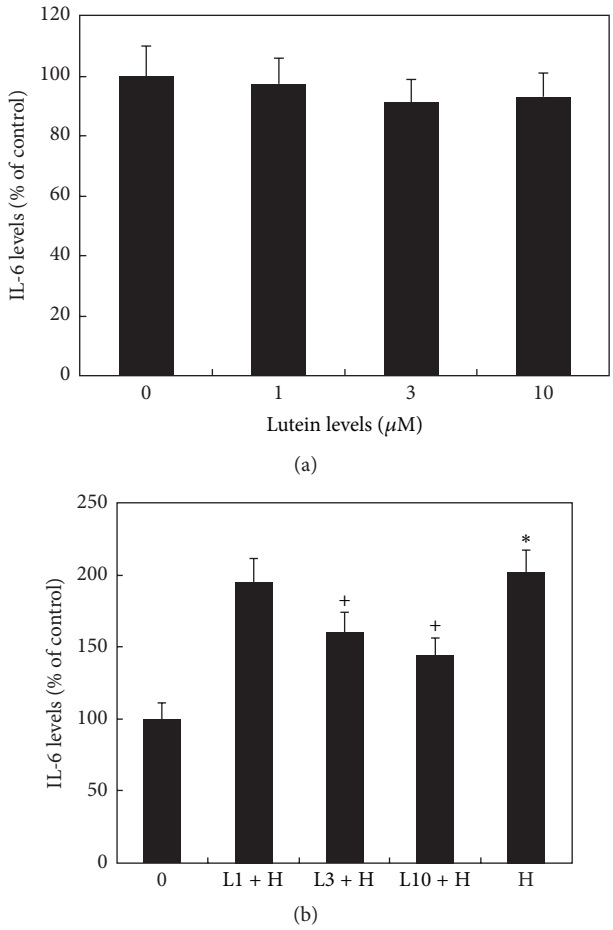

Figure 2: Effects of lutein and hyperosmotic medium on the secretion of IL-6 by cultured human CE cells. Cells were cultured with isoosmotic medium (0) or hyperosmotic medium (H) at 450 mOsM (b) with or without lutein at 1 μM (L1), 3 μM (L3), and 10 μM (L10) for 24 h. IL-6 levels of conditioned medium were measured by IL-6 ELISA kit. Hyperosmotic medium caused a significant increase of IL-6 levels ($^{*}P < 0.05$) as compared to cells cultured in isomer medium (0). Lutein at 3 and 10 μM significantly inhibited hyperosmoticity-induced increase of IL-6 secretion ($^{+}P < 0.05$).

cells with SB 203580 (p38 MAPK inhibitor) or SP 600125 (JNK inhibitor) for 30 min before the cells were exposed to hyperosmotic medium significantly decreased IL-6 levels in conditioned medium as compared to the positive controls (cells cultured with hyperosmotic medium alone) ($P < 0.05$, Figure 4). Pretreatment of cells with UO1026 (ERK inhibitor) for 30 min before the cells were exposed to hyperosmotic medium did not significantly reduce IL-6 levels in conditioned medium as compared to the positive controls ($P > 0.05$, Figure 4).

In the study of the role of NF-κB in hyperosmoticity-induced increase secretion of IL-6, cells pretreated with BAY11-7082 (NF-κB inhibitor) significantly decreased the release of IL-6 as compared to the positive controls (Figure 4).

These results suggested that p38 MAPK, JNK, and NF-κB, but not ERK, played an important role in hyperosmoticity-induced increase of IL-6 secretion by cultured CE cells.

4. Discussion

IL-6 is a pleiotropic cytokine that regulates multiple biological processes, including the development of the nervous and hematopoietic systems, acute-phase responses, and inflammation and immune responses [15, 16]. IL-6 is an important cytokine that amplifies immune and inflammatory responses and plays a critical role in the occurrence of autoimmune diseases. Dysregulation of the expression of IL-6 is associated with a variety of diseases, especially autoimmune diseases and inflammatory proliferative diseases, which include rheumatoid arthritis, glomerulonephritis, psoriasis, Crohn diseases, plasmacytoma, and myeloma [16].

It has been reported that tear IL-6 levels are significantly increased in dry eye patients [9–12] and the expression of IL-6 was upregulated in conjunctival tissues in Sjögren syndrome, a major cause of dry eye [13, 14]. Hyperosmoticity is the major pathological change in dry eye and plays an important role in the development of inflammation and damage of the ocular surface. Hyperosmoticity caused significant increase of IL-6 levels in various experimental animal models [17] and cultured cells [8, 18, 19], especially in cultured CE cells [8, 19]. In the present study, hyperosmoticity significantly increased IL-6 secretion of cultured human CE cells, which is consistent with previous reports.

Lutein, in addition to working as a blue light filter, is an antioxidant and also has an anti-inflammatory effect [35–39]. Lutein inhibits inflammation induced by various stimulators in vitro or in vivo [35–39]. In vitro study suggested that lutein inhibits LPS-induced expression of IL-6 in macrophages [39]. In experimental animals, supplementation of lutein decreased liver IL-6 levels in normal chicks or alcohol intoxicated rats [43, 44]. In normal individuals or patients with various diseases such as atherosclerosis and undertaken peritoneal dialysis, blood lutein or lutein/zeaxanthin levels were inversely associated with the elevation of IL-6 levels [40–42]. Supplementation of lutein decreased serum IL-6 levels in atherosclerosis patients [45]. In experimental ophthalmology, lutein inhibited LPS-induced uveitis in rats and mice [35–38] and decreased IL-6 levels in the aqueous humor [38]. In the present study, lutein significantly and dose-dependently inhibited hyperosmoticity-induced increase of the secretion of IL-6 by cultured human CE cells. This result is consistent with the results obtained from previous studies.

It has been reported that hyperosmoticity induced the expression of proinflammatory cytokines through various signal pathways. MAPK and NF-κB pathways have been linked with this process [8, 18]. In the present study, hyperosmotic medium caused the increase of p-p38 MAPK, p-JNK1/2 levels, but not p-ERK1/2 levels, and also elevated NF-κB levels in nuclear extracts. Also p38 MAPK, JNK1/2, and NF-κB inhibitors significantly reduced hyperosmoticity-induced secretion of IL-6 by CE cells, whereas ERK1/2 inhibitor did not. These results suggested that hyperosmoticity increased IL-6 secretion of cultured CE cells by activation of p38 MAPK, JNK1/2, and NF-κB pathways. This is consistent with previous reports stating that hyperosmoticity increased IL-6 secretion by cultured CE cells via p38 MAPK, JNK1/2, and NF-κB pathways [8].

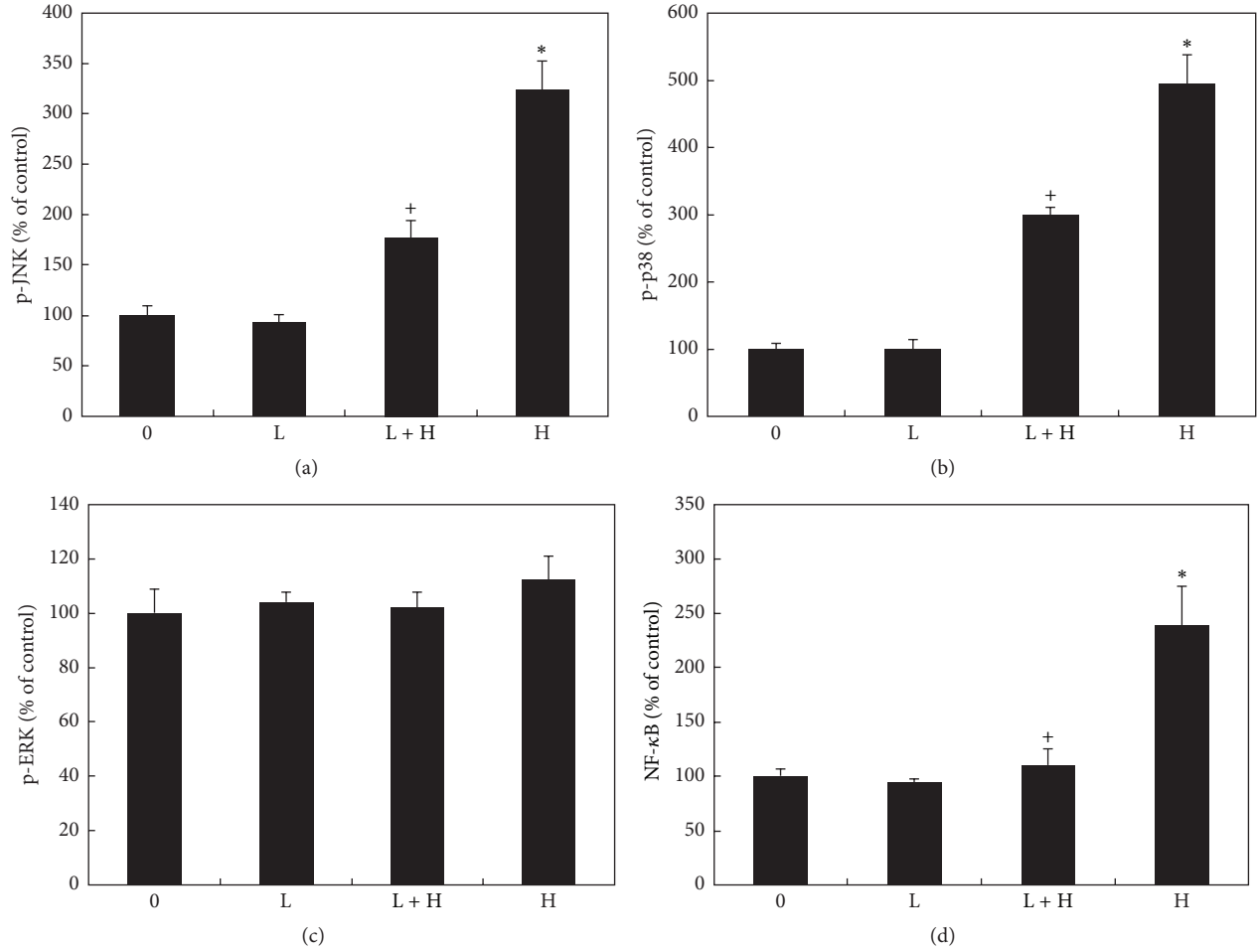

Figure 3: Effects of lutein and hyperosmotic medium on various signal pathways levels of cultured CE cells. Cells were cultured with isoosmotic medium (0) or hyperosmotic medium (H) at 450 mOsM with or without lutein at 10 μM (L). Cells were collected and the levels of phosphorylated- (p-) JNK (a), p-p38 (b), and p-ERK (c) in cell lysates and NF-κB in cell nuclear extracts (d) were measured using relevant ELISA kits, respectively. Hyperosmoticity caused a significant increase of p-p38, p-JNK, and NF-κB levels ($^{*}P < 0.05$) but not p-ERK levels. Lutein did not affect any pathways levels in cells cultured with isoosmotic medium. In cells cultured with hyperosmotic medium, lutein significantly reduced p-p38, p-JNK, and NF-κB levels ($^{+}P < 0.05$) but not p-ERK1/2 levels as compared to cells cultured with hyperosmotic medium but without lutein.

It has been reported that MAPK and NF-κB pathways play a role in proinflammatory factors-induced expression of IL-6 in different cells or tissues [16, 46–49]. In the present study, lutein significantly inhibited hyperosmoticity-induced elevation of IL-6 expression, and this effect was associated with the activation of p38 MAPK, JNK1/2, and NF-κB pathways, suggesting that p38 MAPK, JNK1/2, NF-κB are the upstream of the expression of IL-6. This is consistent with the fact that LPS stimulated IL-6 expression in macrophages and hyperosmoticity-induced expression of IL-6 in cultured CE cells through p38 MAPK, JNK1/2, and NF-κB pathways [8, 50].

Dry eye is a very common ocular disorder and characterized by deficiency of tear associated with inflammation and damage of ocular surface. There are numerous therapeutic procedures available for the treatment of dry eye, but all have their limitations [3–7]. The most common treatment for the dry eye is the use of topical tear substitutes. Tear substitutes provide palliative relief to eye irritation in dry eye patients. However, this effect is temporary and symptom-relief only [6]. Topical corticosteroids are effective in controlling inflammation and decreasing the signs and symptoms associated with dry eye and Sjögren syndrome. However, the possible complications associated with long-term usage, such as cataracts, glaucoma, and infection, limit their use to short-term or pulse therapy only [5, 6]. Topical cyclosporine (an immunosuppressive and anti-inflammatory drug) alleviates the signs and symptoms of dry eye. However, some patients experience bothersome adverse effects (e.g., burning or irritation) and not all of the patients with dry eye show a consistent therapeutic response to this treatment [3, 6]. Topical autologous serum also improves dry eye symptoms and signs. The limitations of using autologous serum include the nuisance of preparation, the need to refrigerate the drops, and the potential risk of infection if contamination of the solution occurs [5]. Therefore, novel efficient therapies with

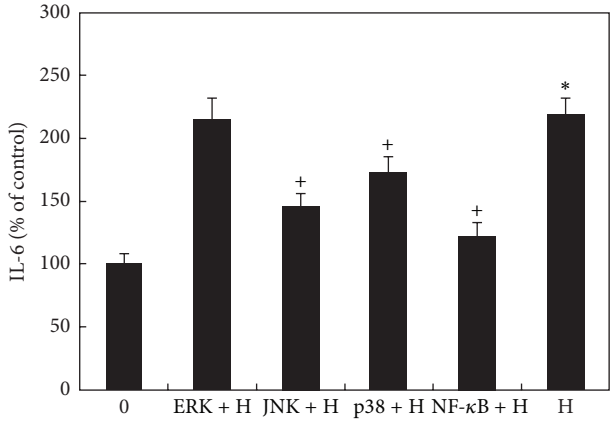

Figure 4: Effects of MAPK and NF-κB inhibitors on hyperosmoticity-induced secretion of IL-6 by CE cells. Cells were cultured with isoosmotic medium (0) or hyperosmotic medium (H) with or without various pathway inhibitors, including UO1026 (ERK inhibitor, ERK + H); SP600125 (JNK inhibitor, JNK + H); SB203580 (p38 inhibitor, p38 + H); and BAY11-7082 (NF-κB inhibitor, NF-κB + H). IL-6 levels in conditioned medium were measured by using IL-6 ELISA kit. Hyperosmotic medium significantly increased IL-6 levels ($^{*}P < 0.05$). JNK, p38, and NF-κB inhibitors significantly inhibited hyperosmoticity-induced increase of IL-6 by CE cells ($^{+}P < 0.05$). ERK inhibitors did not affect hyperosmoticity-induced increase of IL-6.

few or no side-effects for the management of dry eye patients are required. Lutein is a safe dietary supplement and has been used for the treatment of AMD and other ocular diseases for a long time [31–34]. The promising results of lutein on the inhibition of hyperosmoticity-induced elevation of IL-6 expression obtained from the present study support further investigation of the use of lutein in the treatment of dry eye.

In conclusion, this study suggested that lutein inhibited hyperosmoticity-induced elevation of secretion of IL-6 by cultured CE cells through the inhibition of p38 MAPK, JNK1/2, and NF-κB pathways. Lutein has been used for the treatment of various eye diseases without untoward effects. Therefore, lutein may be a promising agent to be explored for the treatment of dry eye.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Nonlethal Levels of Zeaxanthin Inhibit Cell Migration, Invasion, and Secretion of MMP-2 via NF-κB Pathway in Cultured Human Uveal Melanoma Cells

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Zeaxanthin at nonlethal dosages (3–10 μM) significantly inhibited the cell migration of cultured uveal melanoma cells (C918 cell line) as determined by wound healing assay and Boyden chamber assay. Matrigel invasion assay showed that cell invasion of uveal melanoma cells could be significantly inhibited by zeaxanthin. Secretion of MMP-2 by melanoma cells was significantly inhibited by zeaxanthin in a dose-dependent manner as measured by ELISA kit. Zeaxanthin also significantly inhibited the NF-κB levels in nuclear extracts of the UM cells, which is the upstream of the MMP-2 secretion. These results suggest that zeaxanthin might be a potentially therapeutic approach in the prevention of metastasis in uveal melanoma.

1. Introduction

Uveal melanoma (UM) is the most common intraocular malignant tumor in adults. UM has a high mortality rate due to a high incidence of metastasis that usually occurs in the liver [1–3].

Metastasis is the major cause of cancer-mediated death. Metastasis is a multistep process, which includes migration, adhesion, and invasion of cancer cells into the blood or lymphatic vessels that lead to the metastasis [4].

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes that play an important role in the degradation of extracellular matrix (ECM). MMP family members collectively can degrade all structural components of the ECM and lead to tumor cell migration, invasion, metastasis, and angiogenesis. The expression and activity of MMPs are increased in many types of human cancer, and this correlates with advanced tumor stage, increased invasion and metastasis, and shortened survival [4, 5]. MMP-2 is a member of the MMP family and can degrade matrix collagen and basement membrane. Overexpression of MMP-2 has been detected in various types of cancer. High levels of MMP-2 are associated with an increased of invasion and metastasis in several types of cancer [4–10].

Zeaxanthin, a natural bioactive, which belongs to the xanthophyll subclass of the carotenoid family, has been found to have specific cytotoxic effects on several types of cancer cells [11–18]. Our previous study revealed that zeaxanthin reduced the cell viability of UM cells whereas it did not affect the cell viability of normal ocular cells. Zeaxanthin induced apoptosis in human cultured UM cells through the activation of mitochondrial pathway [14]. However, to the best of our knowledge, the effects of zeaxanthin on the cell migration and invasion of UM cells have not been reported.

The purpose of the present study was to investigate the effects of nonlethal doses of zeaxanthin on the cell migration, invasion, and the secretion of MMP-2 by cultured human UM cells and its relevant signal pathways.

2. Material and Methods

2.1. UM Cell Lines. C918, a human choroidal melanoma cell line, was used in this study. C918 is an immortal UM cell line isolated from UM patients with metastasis by the University of Iowa. C918 was provided by Dr. Robert Folberg (University of Illinois, Chicago) and Dr. Xiaoliang Leon Xu (Memorial Sloan Kettering Cancer Center, New York). Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplements with 10% fetal bovine serum [14].

2.2. MTT Assay. The viability of cells was determined by MTT assay that has previously been described [14]. Briefly, UM cells (5×10^3/well) were seeded into 96-well plates and treated with zeaxanthin (0, 1, 3, 10, and 30 μM) for 24 h. Zeaxanthin was obtained from Dr. Dennis L. Gierhart (Chesterfield, MO, USA) and was prepared as a stock solution at 60 mM by dissolving into DMSO (Sigma, St. Louis, MO, USA). After washing with medium, MTT (50 μL of 1 mg/mL in DMEM) was used for the quantification of living cells. Mitochondrial dehydrogenases in living cells metabolize MTT to a purple formazan dye, which is measured photometrically at 540 nm by a microplate reader (Multiskan EX, Thermo, Ventana, Finland). Cell viability is proportional to the reading of absorbance and was expressed as the percentage of the reading from the control (cells cultured without zeaxanthin). All tests were performed in triplicate.

2.3. Wound Healing Assay. Cells were seeded in 12-well plates and were grown to nearly confluence. The cell monolayer was scratched with a 200 μL micropipette tip to create a wound. The cultures were washed twice in PBS to remove float cells and debris and replaced with fresh culture medium. Cultures were photographed at various time periods by using an Olympus IX70 inverted phase-contrast microscope (Olympus Inc., Shinjuku-ku, Tokyo, Japan) [19]. Cells migrating from the leading edge were counted in 4 random fields and expressed as mean ± SD. The leading edge at different time periods was adjusted based on the width of unclosed wound of the control (cells not cultured with zeaxanthin). All tests were performed in triplicate.

2.4. Boyden Chamber Assay. Migration assays for UM cells were performed by using a 48-well Boyden chamber and a cellulose nitrate membrane with 8 μm pore size (both from Neuro Probe, Inc., Gaithersburg, MD). The lower chamber was filled with DMEM with 10% serum. UM cells (2×10^4 cells/well) in serum-free DMEM solution with or without zeaxanthin (10 μM) were seeded into the upper chamber. After 8 h of incubation, cells on the upper surface of the membrane that had not migrated were gently scraped away with a cotton swab. The migrating cells on the lower side of the membrane were fixed with methanol and stained with hematoxylin. Ten fields were randomly selected and cells that had migrated to the lower surface of the membrane were counted under a light microscope at ×200 [20]. All tests were performed in triplicate.

2.5. Matrigel Invasion Assay. For cell invasion test, Corning Matrigel Invasion Chamber (8 μm pore size, coated with Matrigel; Discovery Labware Inc., Bedford, MA, USA) was placed into the wells of 24-well culture plates. DMEM with 10% serum was added into the lower chamber; 5×10^4 UM cells in serum-free DMEM with or without zeaxanthin (10 μM) were added to the upper chamber and cultured. After 16 h of incubation, cells in the upper surface of the filter membrane that had not migrated were gently scraped away with a cotton swab. The invading cells on the lower surface of the filter membrane were fixed, stained, and counted as described above [20]. All tests were performed in triplicate.

2.6. Cell Culture for MMP-2 Secretion Assay. UM cells were seeded into 12-well plates and were grown to nearly confluence. The cultured medium was withdrawn and replaced with serum-free medium after washing the cells. Cells were cultured with or without zeaxanthin at various levels. After being cultured for 24 h, conditioned medium was collected and centrifuged at 500 ×g for 10 min, and the supernatants were collected and stored in vials at −70°C until analysis. All experiments were performed in triplicate.

2.7. Measurement of MMP-2 Protein. The protein amount of MMP-2 in the conditioned media was determined using the Human MMP-2 Quantitation ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Optical density was read by using a microplate reader at 450 nm and corrected with 540 nm. The amount of MMP-2 (pg/mL) was calculated from a standard curve and expressed as percentages of the negative controls (cells cultured without zeaxanthin). The sensitivity of the MMP-2 kit was 0.082 ng/mL.

2.8. Cell Culture for NF-κB Assay. UM cells were plated into 6-well plates at a density of 1×10^6 cells per well. After 24 h of incubation, the medium was replaced and cells were cultured with or without zeaxanthin (10 μM) for 30 min. The culture medium was withdrawn and cultures were washed with cold PBS twice. Cells were scraped from the well and the nuclear fraction was extracted by using Nuclear Extraction Kit (BioSource, Camarillo, CA, USA). Cells were treated with Hypotonic Cell Lysis Buffer, incubated for 10 min at ice, treated with Detergent Solution, vortexed, and centrifuged (800 ×g, 6 min at 4°C). The pellet (nuclear fraction) was collected, washed with Nuclear Wash Buffer, and centrifuged (800 ×g, 6 min at 4°C). The pellet (nuclear fraction) was collected, treated with Complete Extraction Buffer, vortexed, incubated at ice, and centrifuged (14,000 ×g, 30 min at 4°C). The supernatants (nuclear extracts) were collected and stored at −70°C until analysis [21].

2.9. NF-κB in Nuclear Extracts of Cultured UM Cells Assay. The amount of nuclear factor-kappa B (NF-κB) in the nuclear extracts was measured by using NF-κB ELISA kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The levels of NF-κB were calculated using a standard curve and expressed as percentages of the negative

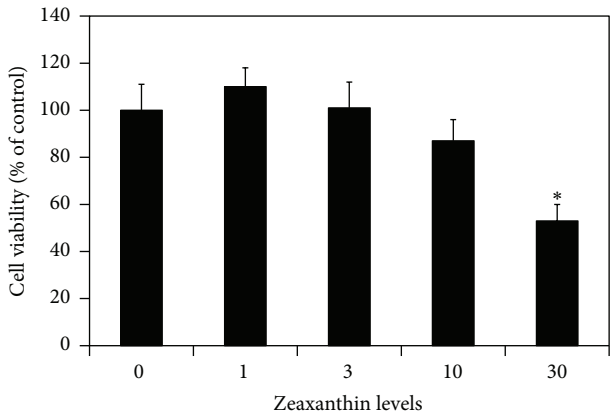

Figure 1: Effects of zeaxanthin on viability of uveal melanoma (UM) cells. Cultured human UM cells (C918) were seeded into 96-well plates and treated with zeaxanthin at different levels. MTT assay was used to determine the cell viability (see Material and Methods). Zeaxanthin only significantly affected the cell viability of UM cells (expressed as percentage of the controls) at 30 μM ($n = 3$, $P > 0.05$). $^{*}P < 0.05$, versus control (cells cultured without zeaxanthin).

controls (cells cultured without zeaxanthin). The sensitivity of this kit was <50 pg/mL. All tests were performed in triplicate [21].

2.10. Statistical Analysis. Data in each group were calculated and expressed as mean and standard deviation (mean ± SD). Statistical significances of difference of means throughout this study were calculated by Student's t-test in comparing data between two groups and ANOVA one-way test in comparing data from more than two groups. SPSS statistical software (SPSS Inc., Chicago, IL, USA) was used for the analysis of the data. A difference at $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Cell Viability Assay. In MTT assay, zeaxanthin at the final levels of 1.0 and 3.0 μM did not influence the cell viability of cultured human UM cells ($P > 0.05$, compared with cells not treated with zeaxanthin) (Figure 1). Cell viability in UM cells cultured with zeaxanthin at 10 μM was slightly lower than that of the controls and the difference was statistically nonsignificant ($P > 0.05$), whereas cell viability was significantly lowered in 30 μM zeaxanthin treated cells ($P < 0.05$). Dye exclusion staining showed that the number of nonvial cells was significantly increased in cells treated with 30 μM zeaxanthin, but not in 1–10 μM treated cells (data not shown). Therefore, level ranges 1–10 μM of zeaxanthin were chosen as nonlethal dosages for subsequent experiments.

3.2. Wound Healing Assay. UM cell cultures were scratched and cultured with 0, 1, 3, and 10 μM zeaxanthin. Photos taken at 0, 4, and 8 h after scratch (Figure 2) show that the migration of UM cells was dose-dependently inhibited by zeaxanthin. After incubation for 4 h, cells migrating from the leading edge at cultures treated with zeaxanthin at 0, 1, 3, and 10 μM were 152.5 ± 10.2, 142.0 ± 7.5, 124.0 ± 9.3, and 82.0 ± 6.6 (mean ± SD), respectively, and expressed as the percentages of the controls (without zeaxanthin) at 1.00 ± 0.07, 0.93 ± 0.05, 0.81 ± 0.06, and 0.54 ± 0.05 (mean ± SD), respectively (Figure 2(b)). Migrating cells in cells cultured with 1 μM zeaxanthin were not significantly different from those in the controls (cells cultured without zeaxanthin) ($P > 0.05$). Migrating cells at 3 and 10 μM zeaxanthin groups were significantly less than those in the controls and cultured with 1 μM zeaxanthin ($P < 0.05$). Migrating cells at 10 μM zeaxanthin group were significantly less than those cultured with 3 μM zeaxanthin ($P < 0.05$). After incubation for 8 h, cells migrating from the leading edge at cultures treated with zeaxanthin at 0, 1, 3, and 10 μM zeaxanthin were 196.8 ± 12.8, 178.5 ± 13.2, 134.5 ± 8.8, and 70.3 ± 6.8, respectively, and expressed as the percentage of the controls at 1.00 ± 0.07, 0.91 ± 0.07, 0.68 ± 0.04, and 0.36 ± 0.03, respectively (Figure 2(c)). Migrating cells in cells cultured with 1 μM zeaxanthin were not significantly different from those in the controls ($P > 0.05$). Migrating cells at 3 and 10 μM zeaxanthin groups were significantly less than those in the controls and with 1 μM zeaxanthin ($P < 0.05$). Migrating cells at 10 μM zeaxanthin group were significantly less than those cultured with 3 μM zeaxanthin ($P < 0.05$) (Figure 2).

3.3. Boyden Chamber Assay. The effects of zeaxanthin on UM cell migration were studied by Boyden chamber assay. Cells were treated with and without zeaxanthin (10 μM) and cultured for 8 h. The results are shown in Figures 3(a), 3(b), and 3(c). Numbers of migrating cells in cultures with and without zeaxanthin were 67.2 ± 6.16 and 107.3 ± 9.75 cells, respectively. Zeaxanthin significantly decreased the transmembrane migration of UM cells as compared with cells not treated with zeaxanthin ($P < 0.05$).

3.4. Matrigel Invasion Assay. The effects of zeaxanthin on cell invasion of UM cells were studied by Matrigel Invasion Chamber. Cells were treated with and without zeaxanthin (10 μM) and cultured for 16 h. The results are shown in Figures 4(a), 4(b), and 4(c). Numbers of invaded cells in cultures with and without zeaxanthin were 135.8 ± 12.2 and 231.9 ± 20.4 cells, respectively. Zeaxanthin significantly decreased the invasion of UM cells as compared with cells not treated with zeaxanthin ($P < 0.05$).

3.5. Secreted MMP-2 Protein Assay. One-way ANOVA analysis of the results on the MMP-2 assay revealed that zeaxanthin had a dose-dependent inhibitory effect on the secretion of MMP-2 protein by UM cells ($P < 0.05$) (Figure 5). Secretion of MMP-2 by UM cells treated with zeaxanthin at 3.0 and 10.0 μM was significantly less than that from the negative control (cells not treated with zeaxanthin, $P < 0.05$).

3.6. NF-κB in Nuclear Extracts of Cultured UM Cells Assay. Zeaxanthin (10.0 μM) treatment decreased NF-κB levels in nuclear extracts of the UM cells. The levels of NF-κB in nuclear extracts in cells cultured with zeaxanthin were only 42% of the control values (cells not treated with zeaxanthin).

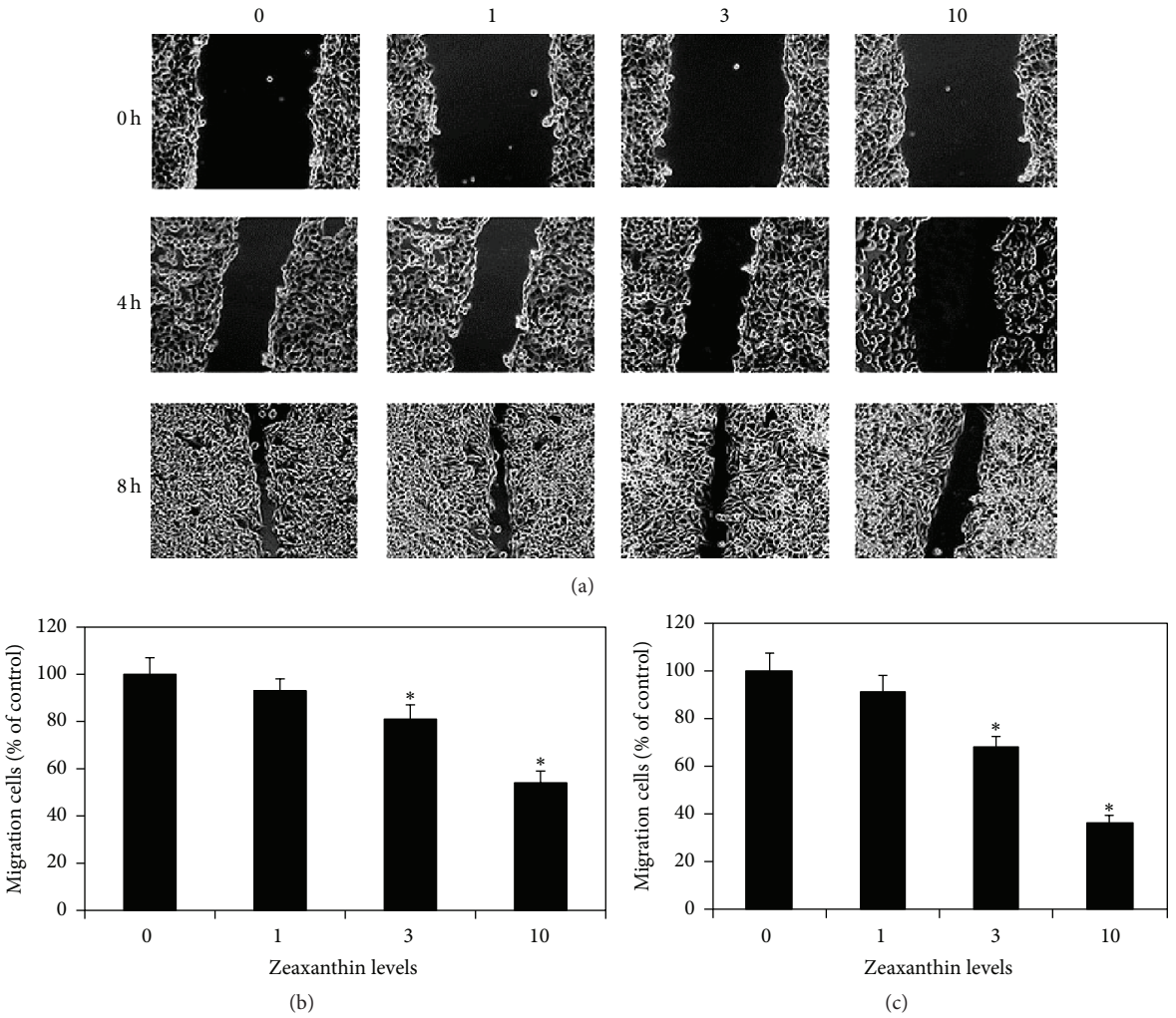

Figure 2: Zeaxanthin inhibits wound closure in cultured UM cells. Cultured human UM cells (C918) were seeded into 12-well plates, scratched, and treated with zeaxanthin (0, 1, 3, and 10 μM) for 0, 4, and 8 h. Phase-contrast pictures of the wounds were taken for the comparison of wound closing process between cells treated with different levels of zeaxanthin at different periods (a). Cells migrating from the leading edge were counted at 4 h (b) and 8 h (c) and expressed as the percentage of the controls (cells cultured without zeaxanthin). After incubation for 4 h and 8 h, migrating cells in cultures treated with zeaxanthin at 3 and 10 μM were significantly less than those from the controls (without zeaxanthin) ($P < 0.05$). Please see the text (Section 3.2) for the original data and the percentages of the controls (mean ± SD) of each group at different time periods.

The difference of NF-κB levels between cells treated with and without zeaxanthin was statistically significant ($P < 0.05$).

4. Discussion

In the present study, the nonlethal dosages of zeaxanthin significantly inhibited the cell migration of cultured human UM cells as demonstrated by the wound healing assay and the Boyden migration assay.

Matrigel is the extracellular matrix secreted by the Engelbrecht-Holm-Swarm mouse sarcoma cell line. It contains laminin, collagen IV, nidogen/entactin, and proteoglycans and resembles the basement membrane [4]. Cell invasion is usually tested by the use of Matrigel Invasion Chamber. In the present study, zeaxanthin significantly inhibited the invasion of UM cells through the Matrigel-coated membrane from the upper surface of the membrane to the lower side.

MMPs are a group of zinc-dependent proteinase capable of digesting virtually any component of the ECM to enhance the migration, invasion, and metastasis of cancer cells. The MMPs could be divided into collagenases, gelatinases, stromelysins, and matrilysins on the basis of their specificity for ECM components or be grouped according to their structure. MMP-2 (also called 72-kDa type IV collagenase, or gelatinase A) belongs to gelatinases (based on the substrate) or gelatin binding MMP group (based on the structure) [4]. MMP-2 can degrade denatured collagen (gelatin), native collagens IV, V, VI, and X, elastin, and fibronectin [4, 22]. It has been reported that MMP-2 also degrades native collagen I, the main component of mammal scleral protein [22].

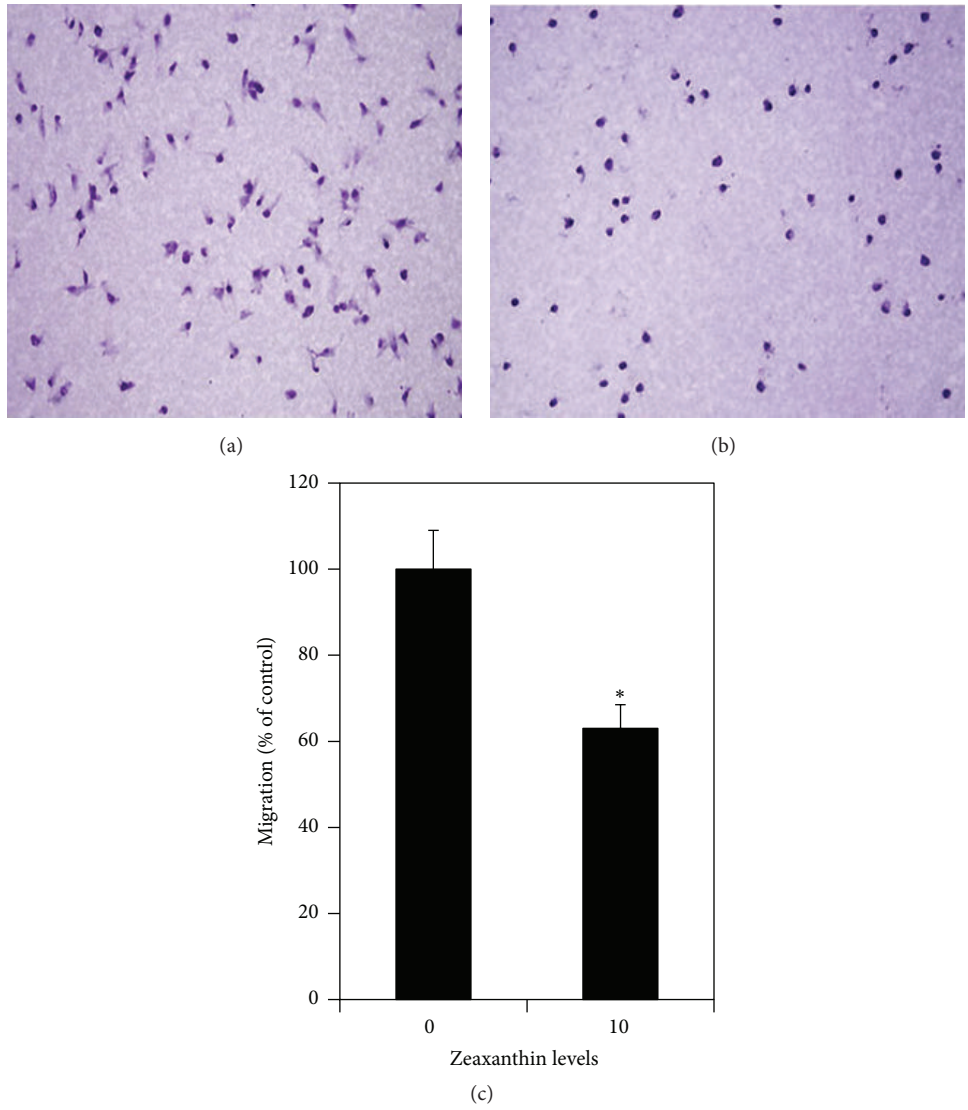

Figure 3: Zeaxanthin inhibits migration of UM cells by using Boyden chamber assay. Cells (C918) were seeded into the upper chamber and the lower chamber was filled with DMEM with 10% serum (see Material and Methods). After being cultured with (b) or without (a) zeaxanthin at 10 μM for 8 h, the migration of UM cells was measured by counting the migrating cells on the lower surface of the membrane at 10 fields. Photos were taken by a light microscope at ×200. Numbers of migrating cells in cultures without zeaxanthin and with zeaxanthin were 107.3 ± 9.75 and 67.2 ± 6.16 cells (mean ± SD), respectively, and expressed at the bar graph as 1.00 ± 0.09 and 0.63 ± 0.06 (percentages of the control), respectively. Zeaxanthin significantly inhibited the migration of UM cells ($n = 3$, $P < 0.05$) (c). $^{*}P < 0.05$, versus control (cells cultured without zeaxanthin).

MMP-2 has been detected in the UM pathologic specimens and cell lines [23–29]. UM cell lines from metastasis patients show a higher level of MMP-2 [29]. MMP-2 expression is associated with higher incidence of metastatic diseases and lower survival rate [23, 24]. It has been reported that MMP-2 may be used as a prognostic marker in UM [24].

In the present study, zeaxanthin dose-dependently inhibited the secretion of MMP-2 by UM cells. The inhibitory effects of zeaxanthin on the secretion of MMP-2 by UM cells might cause the inhibition of cell migration and invasion of UM cells by zeaxanthin. This is consistent with previous reports that various medications can inhibit the cell migration and invasion of various cancer cells through the inhibition of MMP-2 [19, 20, 30].

NF-κB is a major transcription factor that promotes the expression of many genes involved in a variety of cellular processes [31]. NF-κB is present in the cytoplasm in an inactive NF-κB complex which could be activated by various stimuli. Activated NF-κB translocates to the nucleus and binds to the promoter or enhancer regions of specific genes and then induces the expression of relevant genes, including various MMPs [31, 32].

NF-κB is constitutively activated in UM cells [32]. The expression of NF-κB in metastatic UM is higher than that

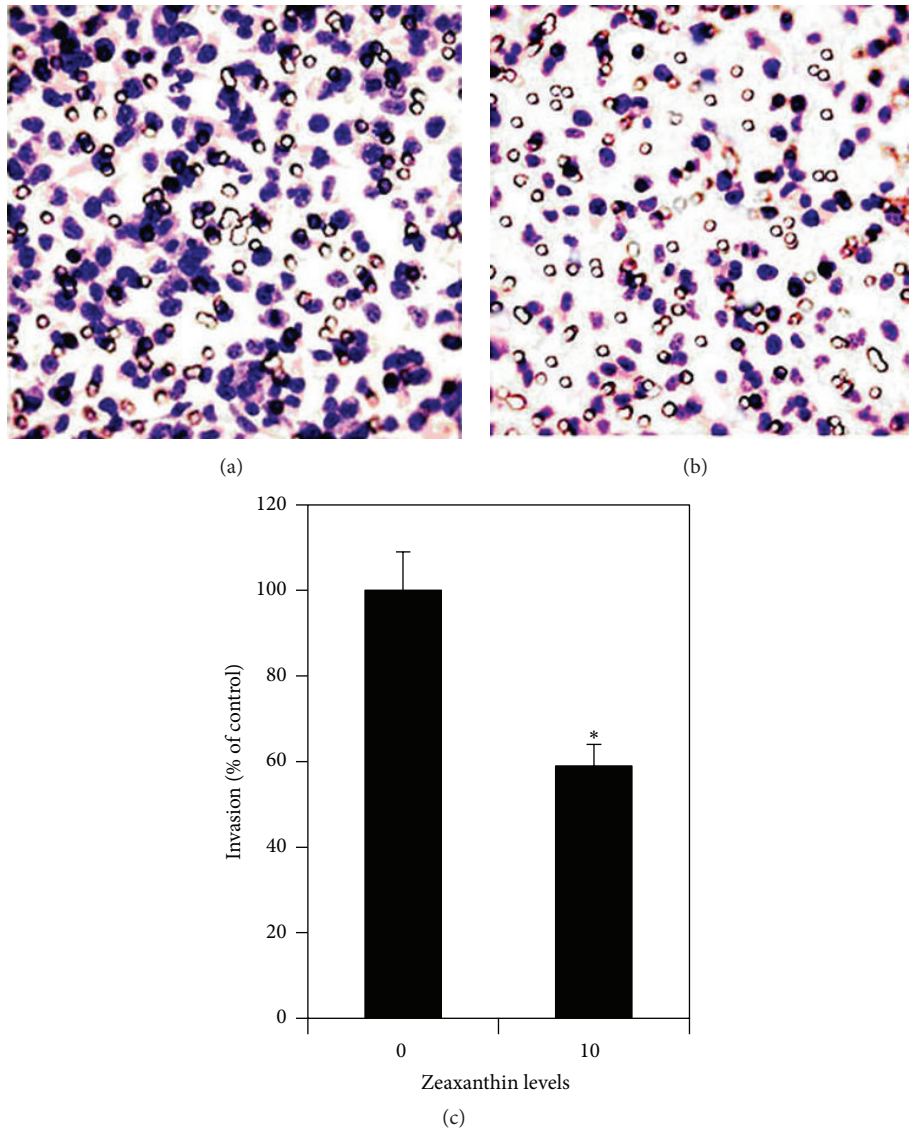

Figure 4: Zeaxanthin inhibits invasion of UM cells by using Matrigel invasion assay. Cells (C918) were seeded into the Matrigel Invasion Chamber and placed into a 24-well plate with DMEM supplemented with 10% serum. After being cultured with (b) or without (a) zeaxanthin at 10 μM for 16 h, the invasion of UM cells was measured by counting the invading cells on the lower surface of the filter membrane at 10 fields. Numbers of invading cells in cultures without zeaxanthin and with zeaxanthin were 231.9 ± 20.4 and 135.8 ± 12.2 cells (mean ± SD), respectively, and expressed at the bar graph as 1.00 ± 0.09 and 0.59 ± 0.05 (percentages of the control), respectively. Photos were taken by a light microscope at ×200. Zeaxanthin significantly inhibited the invasion of UM cells ($n = 3$, $P < 0.05$) (c). $^{*}P < 0.05$, versus control (cells cultured without zeaxanthin).

of the primary tumor [33, 34]. NF-κB inhibitor BAY11-7082 markedly decreased the nuclear translocation of NF-κB and inhibits the migration of human UM cells [32]. miR-9 is significantly reduced in highly invasive UM cell lines. miR-9 suppresses UM cell migration and invasion through downregulation of NF-κB signaling pathway [35].

In the present study, zeaxanthin inhibited the secretion of MMP-2 protein and decreased NF-κB levels in nuclear extracts of the UM cells, suggesting that zeaxanthin inhibits the secretion of MMP-2 via NF-κB signal pathway. This is consistent with the previous reports that NF-κB is the upstream of MMP-2 in various cancer cells [20, 32, 35–37].

Recently, Xu et al. published their studies regarding the effects of zeaxanthin on the growth and invasion of UM in nude mice eyes and revealed that zeaxanthin significantly inhibited the invasion of uveal melanoma [38]. This in vivo invasion inhibitory effect of zeaxanthin on UM was consistent with the result of our in vitro study. Furthermore, Wu et al. found that fibroblasts cultured with cutaneous melanoma conditioned medium showed an increase of

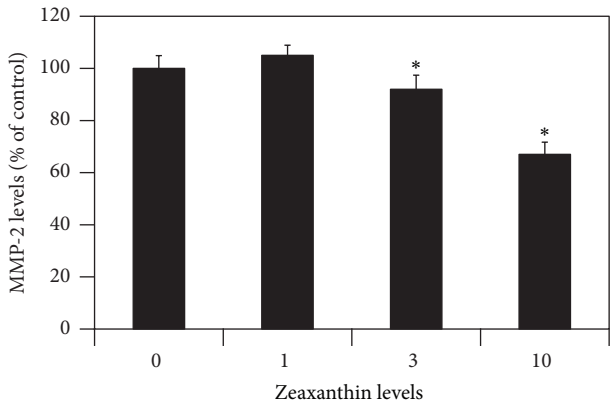

Figure 5: Zeaxanthin inhibits secretion of MMP-2 protein by cultured UM cells. UM cells were seeded into 12-well plates and were cultured with or without zeaxanthin (10 μM). After being cultured for 24 h, conditioned medium was collected and centrifuged and the supernatants were collected. The amount of MMP-2 in the supernatants was determined using the Human MMP-2 ELISA kit. Zeaxanthin at 3 μM and 10 μM significantly inhibited the secretion of MMP-2 ($n = 3$, $P < 0.05$). $^{*}P < 0.05$, versus control (cells cultured without zeaxanthin).

migration. Zeaxanthin inhibited melanoma-induced fibroblast migration [39]. This report indicated that zeaxanthin not only influenced the migration of UM as revealed by us but also inhibited factors secreted by cutaneous melanoma that stimulates the migration of fibroblast.

In conclusion, this study demonstrated that, in addition to the previously reported zeaxanthin-induced apoptosis effects on UM cells, zeaxanthin can also inhibit the cell migration and invasion of cultured human UM cells by the decrease of secretion of MMP-2. This effect is attributed to the inhibition of NF-κB pathway in UM cells by zeaxanthin. The results of this study further suggest that zeaxanthin might be a potentially therapeutic approach in the management of uveal melanoma.

Conflict of Interests

None of the authors have financial interests relevant to the contents of this paper.

Acknowledgments

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Review Article

Zeaxanthin: Review of Toxicological Data and Acceptable Daily Intake

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Zeaxanthin is a nutritional carotenoid with a considerable amount of safety data based on regulatory studies, which form the basis of its safety evaluation. Subchronic OECD guideline studies with mice and rats receiving beadlet formulations of high purity synthetic zeaxanthin in the diet at dosages up to 1000 mg/kg body weight (bw)/day, and in dogs at over 400 mg/kg bw/day, produced no adverse effects or histopathological changes. In developmental toxicity studies, there was no evidence of fetal toxicity or teratogenicity in rats or rabbits at dosages up to 1000 or 400 mg/kg bw/day, respectively. Formulated zeaxanthin was not mutagenic or clastogenic in a series of *in vitro* and *in vivo* tests for genotoxicity. A 52-week chronic oral study in Cynomolgus monkeys at doses of 0.2 and 20 mg/kg bw/day, mainly designed to assess accumulation and effects in primate eyes, showed no adverse effects. In a rat two-generation study, the NOAEL was 150 mg/kg bw/day. In 2012, this dosage was used by EFSA (NDA Panel), in association with a 200-fold safety factor, to propose an Acceptable Daily Intake equivalent to 53 mg/day for a 70 kg adult. The requested use level of 2 mg/day was ratified by the EU Commission.

1. Introduction

Zeaxanthin (3, 3′-dihydroxy-β-carotene, CAS number 144-68-3) is a nutritional carotenoid in a category referred to as xanthophylls. Zeaxanthin is structurally closely similar to lutein. The intake of both carotenoids in the human diet is regarded as healthy, with these components reflecting an adequate intake of fruit and vegetables.

Lutein as a human dietary supplement is often obtained as an extract from *Tagetes* (marigold) and the extract always contains some zeaxanthin. Zeaxanthin itself, on the other hand, tends to be produced from both biological sources and in a highly pure form synthetically. The predominant zeaxanthin stereoisomer in nature and consequently in the diet is the 3R, 3R′-stereoisomer, which is also the predominant stereoisomer of synthetic zeaxanthin (Figure 1).

In normal human food sources, lutein is more abundantly present than zeaxanthin, for example, in spinach, but there are other food sources with a relatively higher content of zeaxanthin, such as egg yolk, corn (maize), or orange pepper [1, 2]. The usual dietary ratio of lutein : zeaxanthin is approximately 5 : 1 (Table 1) [3, 4].

A closely related stereoisomer that is rarer than 3R, 3′R zeaxanthin isomer in nature is the 3R, 3′S stereoisomer, commonly referred to as meso-zeaxanthin. This stereoisomer, like lutein and zeaxanthin, is found in the human macula and its source has been determined in primates fed with a zeaxanthin-free diet to be derived from lutein [5, 6].

In addition to being generally healthy and acting as antioxidants, a specific protective activity exists in the eyes of primates. In the primate eye, in the center of the retina, an area known as the macula lutea is visible as a yellow spot due to the accumulation of the macular xanthophylls. The presence of the xanthophyll carotenoids in the human appears to be physiologically significant; the concentration of xanthophylls in the macula is the highest concentration found everywhere in the primate body. Furthermore, based on filtration of potentially damaging light and quenching of photochemically induced reactive oxygen species, it is believed [7, 8] that, via these mechanisms, lutein, zeaxanthin, and meso-zeaxanthin may contribute to reducing the risk of Age-Related Macular Degeneration (AMD), a leading cause of irreversible loss of vision observed in western countries.

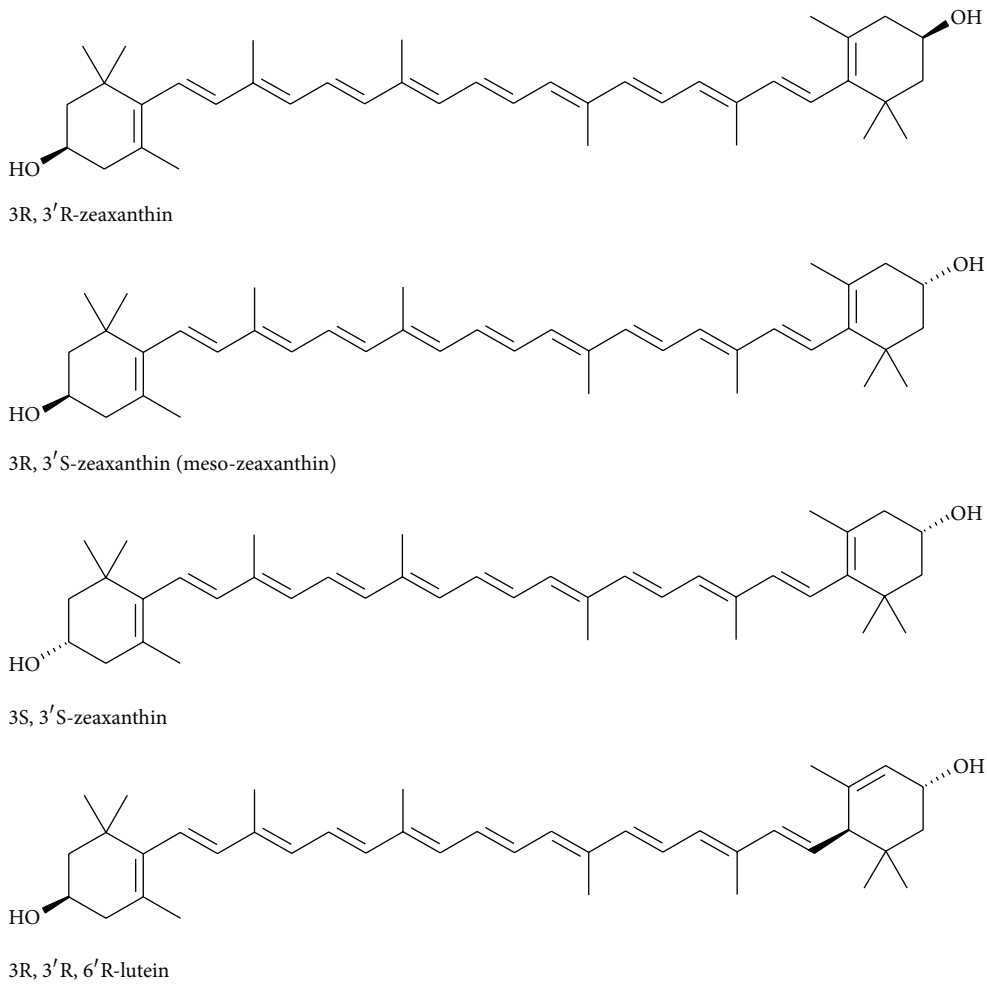

Figure 1: Structures for optical isomers of all-trans zeaxanthin and lutein.

Table 1: Average intake of lutein and zeaxanthin by age group (Mohamedshah et al., 1999) [3].

Age group	Lutein (μg/day)	Zeaxanthin (μg/day)	Lutein : Zeaxanthin ratio
20–29	745	178	4.2 : 1
30–39	896	174	5.1 : 1
40–49	920	187	4.9 : 1
50–59	1053	182	5.8 : 1
60–69	1056	170	6.2 : 1
70+	990	170	5.8 : 1

There is a considerable amount of safety data for zeaxanthin based mainly on routine regulatory studies with high purity synthetic zeaxanthin, manufactured by DSM Ltd. (previously manufactured by F. Hoffmann-La Roche Ltd.). A series of *in vitro* and *in vivo* tests for genotoxicity have been undertaken as well as subchronic safety studies (13 weeks in duration) by dietary exposure at high dosage levels in mice, rats and dogs. Developmental toxicity studies have been undertaken in rats and rabbits, a two-generation study was performed on the rat, and a chronic study of 52 weeks' duration was performed on Cynomolgus monkeys. ADME (absorption, distribution, metabolism, and excretion) studies have been undertaken. These studies with zeaxanthin are reviewed here.

Potentially, data for certain closely related substances may have relevance or should be taken into consideration, in the safety evaluation of zeaxanthin. The inclusion of a ferret study with the related xanthophyll, β-cryptoxanthin, on a read-across basis, to address the question if zeaxanthin consumption might have an adverse impact on cigarette smokers, is described. Reference is also made to known studies with lutein and meso-zeaxanthin.

Safety data from human intervention studies in which synthetic zeaxanthin has been supplemented, of which the AREDSII study is by far the largest, is also considered and the apparent safe level of intake from these studies compared with that derived from the animal studies.

The safety data for lutein have been evaluated by the European Food Safety Authority (EFSA). Due to the close similarity of lutein and zeaxanthin, it is probable that the toxicology for the pure substances is very similar, although it has to be remembered that within the eye a highly specific

Table 2: List of genotoxicity, repeat dose, and reproductive safety studies conducted with DSM-manufactured synthetic zeaxanthin based on international regulatory study designs.

Safety studies	Formulation nominal %	Concentration or dosage	Result
		Concentration	
Genotoxicity *in vitro*			
Ames, *S. typhimurium* mutation assay	Crystalline	0, 2.4–1500 μg/plate	Negative
Gene mutation in V79 cells	Crystalline	0, 1–16 μg/mL	Negative
Unscheduled DNA Synthesis (UDS) in rat hepatocytes	Crystalline	0.1–16 μg/mL	Negative
Human lymphocytes	Crystalline	0, 60, and 120 μg/mL	Negative
		Dose (mg/kg bw/day)	
Genotoxicity assays *in vivo*			
Mouse micronucleus	10% beadlet	0, 44.5, 89, and 178	Negative
Subchronic and chronic			
13-week oral (admix) in mice	10% beadlet	0, 0, 250, 500, and 1000	NOAEL, high dose
13-week oral (admix) in rats	10% beadlet	0, 0, 250, 500, and 1000	NOAEL, high dose
13-week oral (feed cubes) in dogs	10% beadlet	0, 123, 204, and 422 males : 0, 104, 238, and 442 females	NOAEL, high dose
1-year oral (gavage) in monkeys zeaxanthin or lutein	10% beadlet	0, 0.2, and 20 for zeaxanthin or lutein	NOAEL, high dose
Reproductive studies			
Teratology oral (admix) in rats	10% beadlet	0, 250, 500, and 1000	NOAEL, high dose
Teratology oral (gavage) in rabbits	Crystalline in oil	0, 100, 200, and 400	NOAEL, high dose
Two-generation (admix) in rats	10% beadlet	0, 0, 50, 150, and 500	NOAEL, inter. dose

biological stereoisomeric differentiation may occur. Many of the analytical methods used in the past did not differentiate zeaxanthin and lutein such that the information on the differential occurrence of lutein and zeaxanthin in fruits and vegetables for many years was incomplete. The Joint FAO (Food and Agriculture Organization of the United Nations)/WHO (World Health Organization) Expert Committee on Food Additives (JECFA) in 2006 [9] in their safety evaluation of lutein and zeaxanthin defined a "group" ADI (Acceptable Daily Intake) for lutein and zeaxanthin of 0–2 mg/kg bw/day, covering both substances.

The toxicity of compounds can often be strongly influenced by their purity. If coming from a natural source, the other natural components or contaminants (e.g., pesticides) need to be taken into consideration. If coming from chemical synthesis, a representative batch of typical purity containing the synthetic by-products that might be present needs to be tested. In the regulatory studies for zeaxanthin presented here coming from DSM Nutritional Products Ltd., the zeaxanthin tested was in accord with the manufacturer's purity specification of at least 98% zeaxanthin (>96% all-trans, <2% cis). This high purity substance is marketed in a formulation designed to provide stability against oxidation and enhance bioavailability.

The DSM studies reported here have been undertaken by a number of toxicologists and safety specialists over a number of years and are described mainly in summary form. The individual study reports describe the results in detail. In the regulatory processes to obtain approval for human use, the detailed reports are supplied to the respective regulatory authority.

2. Methods: Regulatory Animal Safety Studies with Synthetic Zeaxanthin

An extensive array of conventional toxicology studies has been undertaken with DSM synthetic zeaxanthin (Ro 01-9509). The studies undertaken by DSM were for the purpose of assessing safety from oral intake, or for worker safety purposes, and are listed in Table 2.

The toxicology studies undertaken by DSM were predominantly undertaken using recognized international regulatory guidelines and, in particular, respective Organization for Economic Cooperation and Development (OECD) guidelines. The OECD guideline stipulates for each study design how the study should be performed with a detailed range of study design requirements, such as numbers of replicates or animals, the concentrations or dosages that are appropriate to ensure sufficiently stringent testing, details of the endpoints that should be investigated, and guidance on the evaluation of the data obtained.

In the case of the two-generation rat study, the key regulatory study for the overall safety assessment, the study was designed to meet the known requirements of the OECD 416 guidelines (22 January, 2001) and the US FDA Center for Food Safety and Applied Nutrition, Redbook 2000, Toxicological Principles for the Safety of Food Ingredients, IV.C.9.a. Guidelines for Reproduction Studies (20 July, 2000).

Additionally, the studies were undertaken following the principles of Good Laboratory Practice (GLP). In the case of the two-generation rat study, the study was conducted in accordance with the OCD GLP guideline and UK GLP guidelines, as the study was conducted in UK. Analyses undertaken to confirm the stability and concentrations of zeaxanthin in the treated diet as well as analyses undertaken to assess the concentration of zeaxanthin in plasma and liver samples were performed at the DSM test site in Switzerland and performed in accordance with Swiss ordinance on GLP.

Additionally, *in vivo* studies were undertaken following the local national requirements on animal housing and animal welfare requirements such as in the UK, the Home Office "code of practice for the housing and care of animals used in scientific procedures."

Importantly, the evaluation of zeaxanthin involved a special study on Cynomolgus monkeys, which included a range of specific endpoints to investigate safety to the primate and human eye. The Cynomolgus monkey has been shown to be an excellent model to investigate the induction and dose dependency of canthaxanthin crystal formation in the retina [10–13]. Using similar procedures as described in these publications, the study included indirect ophthalmoscopic examinations performed using the Bonnoskop and direct ophthalmoscope and a contact lens biomicroscope. Additional evaluations were performed using the ophthalmic slit lamp biomicroscope in combination with wide-field corneal contact fundus lenses. Electroretinography (ERG) was undertaken at intervals during the study. Terminal eye pathology involved evaluation of whole-mounts of retinas from the right eyes by microscopic investigation with light or confocal microscopy. Maculas were investigated under the polarization microscope and semiquantitative analysis of inclusions was performed. Routine histopathological investigation of paraffin sections from retinal periphery was performed and zeaxanthin and lutein in the retina and lens were measured analytically by High-Performance Liquid Chromatography (HPLC).

3. Results

3.1. Genotoxicity Studies

3.1.1. S. typhimurium Mutagenicity (Ames) Test, OECD 471. Crystalline zeaxanthin was evaluated for mutagenic activity in the Ames assay using the plate incorporation and the preincubation method. Seven *Salmonella typhimurium* standard tester strains were employed (TA1535, TA1537, TA1538, TA97, TA98, TA100, and TA102) with and without an exogenous metabolic activating enzyme system (S9-mix) derived from livers of phenobarbital/β-naphthoflavone treated male rats. Due to the strong precipitation of the test compound in the aqueous medium, 1500 μg/plate was chosen as the highest dose level. There was no increase of the numbers of mutants in any of the tester strains, while the positive controls verified the sensitivity of the strains and the activity of the S9-mix [14].

In one very early laboratory batch of pure crystalline zeaxanthin, a positive result was found in the Ames test. It was determined that pure zeaxanthin is not mutagenic; however, degradation products formed during exposure of crystalline zeaxanthin to air and light were considered responsible for the mutagenic activity [15]. In addition, it was determined that components in the beadlet formulation scavenged the mutagenic activity of degraded crystalline zeaxanthin, thus further protecting against the occurrence of mutagenic activity. The beadlet formulation for the marketed product includes the antioxidants (ascorbyl palmitate, sodium ascorbate, and *dl*-α-tocopherol), which prevent degradation.

Crystalline zeaxanthin that had been kept in storage beyond the maximum shelf life was subsequently assessed in a non-GLP Ames test [16]. The purpose of this study was to confirm the absence of mutagenic activity of crystalline zeaxanthin. Five *Salmonella typhimurium* tester strains (TA1535, TA97, TA98, TA100, and TA102) were employed with and without metabolic activation (S9-mix). No relevant increase in the number of revertant colonies was apparent and it was concluded that neither zeaxanthin nor any of the metabolites formed by the metabolic activation system was mutagenic in the Ames test.

3.1.2. Gene Mutation Assay in V79/HGPRT Cells, OECD 476. In the gene mutation assay in cultured mammalian cells, zeaxanthin was tested for its ability to induce gene mutations at the HGPRT (Hypoxanthine Guanine Phosphoribosyl Transferase) locus in the established cell line V79, derived from Chinese hamster lung cells. Treatment with 1 μg to 16 μg/mL (0.002–0.03 mmol/L) did not induce mutations to 6-thioguanine resistance in V79 cells *in vitro*, neither in the absence nor in the presence of a rat liver activation system [17].

3.1.3. Unscheduled DNA Synthesis (UDS) Assay, OECD 482. The ability of zeaxanthin to induce DNA damage was tested by the Unscheduled DNA Synthesis assay (UDS test) as measured by the incorporation of radiolabeled nucleotides into nonreplicated DNA of freshly isolated rat hepatocytes. A 20-hour exposure to 1 μg to 16 μg zeaxanthin per mL did not induce DNA repair synthesis in primary cultures of rat hepatocytes [18].

3.1.4. Chromosome Analysis of Human Peripheral Lymphocytes, OECD 473. The potential *in vitro* clastogenic activity of zeaxanthin was assessed using human peripheral blood lymphocytes as target cells in the presence and absence of rat liver activating enzyme system (S9-mix). Under the experimental conditions described, neither zeaxanthin nor any of its metabolites induced chromosomal aberrations in human peripheral blood lymphocytes [19].

3.1.5. Mouse Micronucleus Assay, OECD 474. Zeaxanthin was tested in the *in vivo* micronucleus assay in mice. Zeaxanthin, 10% beadlet formulation, was administered orally at dose levels of 44.5, 89.0, and 178 mg/kg of zeaxanthin 30 and 6 hours prior to sacrifice. There was no increase of micronuclei; thus, it was concluded that under the conditions of the study zeaxanthin did not induce chromosome breaks or mitotic nondisjunctions in mouse bone marrow cells at doses up to 178 mg/kg of zeaxanthin [20].

3.2. Short-Term Toxicity Studies

3.2.1. Acute Safety Studies, Pre-OECD Guideline, Similarities to Guideline OECD 401. Acute studies with zeaxanthin were performed in rats and mice. Zeaxanthin has a low order of acute toxicity. All mice and rats survived a single oral dose of up to 4000 mg/kg in rats and 8000 mg/kg in mice. The LD_{50} values in rats and mice, therefore, were greater than 4000 and 8000 mg/kg body weight, respectively [21].

3.2.2. Guinea Pig Skin Sensitization Test, OECD 406. An optimization test (according to Maurer) was performed with zeaxanthin in albino guinea pigs of both sexes. No signs of skin irritation or sensitization were observed [22]. A subsequent maximization test in albino guinea pigs, based on OECD guideline 406, was also negative [23].

3.2.3. Rabbit Irritation Test, OECD 405. The primary eye irritation potential of zeaxanthin was studied in young adult rabbits [24]. The risk that an accidental or occasional ocular exposure to zeaxanthin could cause injury to the eye in man was considered to be low.

3.3. General Toxicology Studies

3.3.1. Subchronic Safety Studies. 13-week subchronic toxicity studies have been performed with synthetic zeaxanthin in three species, mouse, rat, and dog. Preliminary studies (5- and 10-day studies) were conducted beforehand to ensure appropriate selection of dosages for the main studies.

3.3.2. 13-Week Study in Mice, Similar to OECD 408. A 13-week oral safety study was performed in mice with a 9.3% beadlet formulation of zeaxanthin, administered as a feed admixture. Groups of 10 male and 10 female mice were treated with 0, 250, 500, and 1000 mg/kg body weight/day (mg/kg bw/day) of zeaxanthin. The placebo beadlets were added to the diet so that all 4 groups received similar amounts of beadlets. There was no treatment-related hematology or clinical chemistry findings. No discoloration of adipose tissue or other findings were observed at necropsy and there were no histopathological effects attributable to zeaxanthin or the beadlet formulations. The No Observed Adverse Effect Level (NOAEL) of zeaxanthin was >1000 mg/kg bw/day in mice [25].

In line with the respective OECD guideline procedures for mice, the study did not include ophthalmoscopy, although histopathology of the eyes was undertaken.

3.3.3. 13-Week Study in Rats, OECD 408. An original 13-week oral safety study was conducted in rats with a 9.3% beadlet formulation of zeaxanthin administered as a feed admixture. Groups of 16 male and 16 female rats were treated with 0, 250, 500, and 1000 mg/bw/day of zeaxanthin. The NOAEL for zeaxanthin was >1000 mg/kg bw/day in rats [26].

Due to a change in manufacturing process, a second 13-week oral safety study in rats was performed with a 10% beadlet formulation of zeaxanthin from an updated process. Groups of 16 male and 16 female rats were treated with doses of 0, 250, 500, and 1000 mg/kg bw/day of zeaxanthin as a dietary admixture [27]. All groups received similar amounts of beadlets, by adjusting the diet with control beadlets. There was no effect of treatment on food intake and body weight. Yellow-orange discoloration of the feces was seen in all zeaxanthin-treated rats, especially at the high dose. No treatment-related changes in hematological and clinical chemistry parameters were observed. Urine pH values were slightly decreased in male rats of all dose groups. In line with the respective OECD guideline, the study included ophthalmoscopic evaluations. About 20–30 minutes prior to examination, a mydriatic was instilled into each eye of control and high-dose animals. The examinations were made using a "KEELER" Fison binocular ophthalmoscope. There were no treatment-related changes.

At necropsy, a slight orange discoloration of the adipose tissue was reported in all treated animals; however, this was not considered an adverse effect but due to color of the test compound. There were no treatment-related changes in organ weights or histopathological findings. Under the conditions of this study, the NOAEL in this second rat study was again >1000 mg/kg bw/day.

3.3.4. 13-Week Dog Study, Similar to OECD 409. A 13-week safety study in dogs was conducted with a 9.4% beadlet formulation of zeaxanthin. Zeaxanthin beadlets were incorporated into feed pellets and fed to groups of 3 male and 3 female beagle dogs to achieve a dose of zeaxanthin of 0, 123, 204, and 422 mg/kg bw/day (males) and 0, 104, 238, and 442 mg/kg bw/day (females). This corresponds to test article concentrations in feed of 0, 4, 8, and 16%, respectively. Control beadlets were added so that the amount of beadlets present in the feed cubes was similar for all groups.

No treatment-related toxicity was observed throughout the study. The test article was found to strongly discolor and to slightly soften the feces, particularly in the high-dose group. Ophthalmoscopic evaluations were undertaken at day and at the end of week 13. Following induction of mydriasis, eyes including cornea, chambers, lens, and retina of all dogs were examined using a fundus-camera KOWA RC-2. The central parts of the retina (generally including the optic disc) were recorded on an Ektachrome-X film. No treatment-related findings were reported. Urinalysis as well as hematological and serum clinical chemistry investigations showed no treatment-related effects. At necropsy, male dogs from the mid- and high-dose groups showed slight to moderate discoloration (yellow to reddish) in the adipose tissue, which was considered not an adverse effect and probably reflected presence of zeaxanthin. There were no treatment-related histopathological findings. The NOAEL in this study was >422 mg/kg bw/day [28].

3.4. Reproductive Safety Studies

3.4.1. Developmental Toxicity Study in Rats, OECD 414. In a developmental toxicity study in rats, zeaxanthin (10% beadlet formulation) was administered at doses of 0, 250, 500, and 1000 mg/kg bw/day orally as a feed admixture from day 7 through day 16 of gestation [29]. A subgroup was

Table 3: Effects on reproduction data in the zeaxanthin two-generation study in rats [31].

Nominal dosage (mg/kg bw/day)	0	0 (placebo)	50	150	500
F_1 generation					
Adults, mating index %	100.0	100.0	96.0	96.0	79.3 $^{*}F$
Mean number of pups:					
born	10.2	10.9	10.7	10.7	9.7
alive day 4 *postpartum* (before culling)	10.0	10.9	10.5	10.5	9.7
P generation					
% pup weight gain					
Days 4–7 *postpartum*	62.3	62.0	64.2	61.6	59.3
Days 1–21 *postpartum*	774.7	753.3	780.9	755.0	725.3 $^{**}J$
F_1 generation					
% pup weight gain					
Days 4–7 *postpartum*	64.4	62.8	60.5	61.3	58.1 $^{*}J$
Days 1–21 *postpartum*	738.9	745.1	713.5	724.9	695.0

F = Cochran-Armitage and Fisher's exact test.
J = dose response test, Kruskal-Wallis, Terpstra-Jonckheere, and Wilcoxon.
$^{*}p < 0.05$; $^{**}p < 0.01$.

Caesarian-sectioned on day 21 of gestation and a rearing subgroup was allowed to deliver naturally and was observed up to day 23 of lactation. There was no indication of any embryotoxic or teratogenic action of zeaxanthin in any of the treated groups. The rearing subgroup showed no indication of any functional abnormalities in the treated groups. It was concluded that, under the conditions of this study, zeaxanthin was neither embryotoxic nor teratogenic in rats at doses up to 1000 mg/kg bw/day.

3.4.2. Developmental Toxicity Study in Rabbits, OECD 414. In a developmental toxicity study in rabbits, zeaxanthin was administered at doses of 0, 100, 200, and 400 mg/kg bw/day orally in rapeseed oil from day 7 through day 19 of gestation. Rabbits were Caesarian-sectioned on day 30 of gestation [30]. No deaths or signs of maternal toxicity were observed in the treated groups. There was no indication of any embryotoxic or teratogenic action of zeaxanthin in the treated groups. There were some isolated malformations among the groups, including controls, but there was no evidence of any treatment-related effect. It was concluded that, under the conditions of this study, zeaxanthin was neither embryotoxic nor teratogenic in rabbits at doses up to 400 mg/kg bw/day.

3.4.3. Two-Generation Study in Rats, OECD 416. A two-generation study was performed with synthetic zeaxanthin in rats [31]. Multigeneration studies involve exposure to test compounds beginning before mating, continuing during mating, and throughout gestation and lactation, until weaning, and cover all reproductive life phases over two generations. There was a range of developmental and behavioral testing in addition to reproduction endpoints. Ophthalmologic examination is not part of the OECD 416 guideline study requirements but testing on the young rats included confirmation of the pupillary reflex response and corneal tactile response.

In the two-generation study, DSM-manufactured zeaxanthin was administered in the diet at nominal doses of 0 (control diet), 0 (placebo beadlet control), 50, 150, and 500 mg/kg bw/day active ingredient, by admixture of 10% WS beadlets to the feed. Although zeaxanthin in a 13-week toxicity study in the rat was well tolerated at 1000 mg/kg bw/day, to achieve a dose level of 1000 mg/kg/day, the beadlet concentration in the diet approached 20%. The high-dose level 500 mg/kg bw/day was selected on the basis of avoiding potential nutritional imbalance due to the high beadlet content in the diet over the duration of a two-generation study. Two control groups received either the control diet only or placebo beadlets incorporated in the diet at the same concentrations as the zeaxanthin beadlets in the high-dose group. The parental (P) generation females were allowed to litter and rear their offspring to weaning. Young were randomly selected from each group to form the filial (F_1) generation.

Administration of 500 mg/kg bw/day zeaxanthin active ingredient to rats for two successive generations produced marginal adult toxicity in terms of slightly lower food intake during the lactation period of the P generation, a slightly lower body weight gain during the gestation period of the F_1 generation, and a possible, slight, adverse effect on fertility of the F_1 generation with a lower mating index (mating index is the number of females with determined copulations/number of oestrous cycles required for their insemination × 100) and slightly fewer pups were born (Table 3). At this dosage in both generations, percentage of pup growth during lactation was also slightly lower than in the control groups (Table 3).

Samples of plasma and liver from the P and F_1 generation adults and pups were analysed for zeaxanthin exposure monitoring. The analysis results showed that exposure was

Table 4: Plasma and liver concentration of zeaxanthin in adults and weanlings in the two-generation study in rats [31].

Zeaxanthin concentration (μ/L or μ/kg) at nominal dosage (mg/kg bw/day)					
Dosage	0	0	50	150	500
P generation					
Plasma, μ/L					
Adults					
Male	—	—	24	47	111
Female	—	—	19	29	71
Weanlings	—	—	61	127	353
Liver, μ/kg					
Adults					
Male	14	—	599	1121	2581
Female	4	5	1147	1992	3159
Weanlings	—	—	4015	10892	23836
F_1 generation					
Plasma, μ/L					
Adults					
Male	—	—	22	42	109
Female	—	—	20	42	87
Weanlings	—	—	52	85	213
Liver, μ/kg					
Adults					
Male	—	—	114	645	1689
Female	11	—	1077	1382	3785
Weanlings	—	11	4313	7904	21611

essentially similar in the P and F_1 generations. Higher plasma concentrations and notably higher liver concentrations were observed in the weanling pups at day 21 postpartum compared to the adults of the corresponding treatment group (Table 4). Exposure increased with increasing dose although it was not proportional to dose. The higher exposure in the 21-day-old pups probably reflects intake through the maternal milk and that they had already started eating the treated diet provided to the mothers. The relative concentrations in the liver in comparison to concentrations in the plasma were notably higher, in both adults and weanlings (Table 4), suggesting these are accumulation in the liver. However, the high accumulation in the liver of weanlings (3 weeks of age) clearly diminished during subsequent rearing and as adults of the F_1 generation showed similar tissue concentrations to the P generation, which was exposed from 6 weeks of age.

In conclusion, the NOAEL was defined as the nominal dosage of 150 mg zeaxanthin/kg bw/day.

3.5. Chronic Rodent Carcinogenicity Studies. No carcinogenicity studies are available for zeaxanthin, or for lutein. Genotoxicity studies were negative and histological examinations of tissues from repeat dose toxicity studies have not shown any preneoplastic effects or indeed no indication of histological effects at all.

There is data for two carotenoids (canthaxanthin and astaxanthin) showing that chronic administration over two years in rats, but not mice, induces liver toxicity. These two carotenoids induce liver enzyme including cytochrome P450 enzyme CYP1A in the rat [32, 33], although not in the mouse [34] and not in vitro in human hepatocytes [35]. However, lutein did not affect phase-I or phase-II liver enzyme activities in the rat [32]. Due to its close isomeric relationship to lutein, it is considered unlikely that zeaxanthin is a liver enzyme inducer.

In 2010, EFSA applied an additional safety factor of 2 in the ADI calculations for lutein [36] and in 2012 for zeaxanthin [37] to take account of the absence of chronic rodent studies.

3.6. ADME (Absorption, Distribution, Metabolism, and Excretion) Studies. Metabolism studies, or ADME studies, are a useful component of safety testing as the provided information on the metabolism and kinetics of a substance and, when human data is available, on human relevance.

3.6.1. Balance Study in Rats. A distribution study with (^{14}C)-zeaxanthin was performed in male rats, after a pretreatment feeding with zeaxanthin-poor or zeaxanthin-enriched diet (0.001% in feed) and subsequent single dose administration of (^{14}C)-zeaxanthin in a liposomal preparation. One day after dosing, approximately two-thirds of the administered radioactivity was excreted in feces and urine and approximately 1/3 of the administered radioactivity was present in the body and GI-tract. The pattern of distribution in the tissues and excretion was similar for rats prefed with zeaxanthin-poor and those fed with zeaxanthin-enriched diet. After 1 week, less than 1% of the administered radioactivity was in the body and the digestive tract. The amount of radioactivity absorbed and excreted in the urine tended to be lower for animals fed with the zeaxanthin-poor diet. It was concluded that the radioactivity from (^{14}C)-zeaxanthin is rapidly depleted from the body and the GI-tract of rats [38].

3.6.2. Distribution Study in Rats. A study was performed to investigate zeaxanthin distribution in rats fed with a zeaxanthin 5% beadlet formulation-enriched diet. Male rats received a diet containing 10 mg or 100 mg zeaxanthin/kg feed (approximately 0.8 mg or 8 mg/kg bw/day) for five weeks. A dose-dependent accumulation of zeaxanthin was found in various tissues with the highest concentrations in the small intestine and spleen, followed by liver, fat, and adrenal glands. The thyroid gland and the eye levels were below the levels of detection. There was a marked decrease of zeaxanthin concentration during a subsequent 5-week reversibility period [39].

3.6.3. Radioactivity in Expired Air, Mass Balance Study. In balance studies with a liposomal preparation of (^{14}C)-zeaxanthin in male rats, about 1% of the administered dose, that is, about 4% to 7% of the absorbed dose, was measured in the expired air during the first 24 hours after administration. Contribution of respiration in the excretion of radioactivity was considerably higher in the case of zeaxanthin when

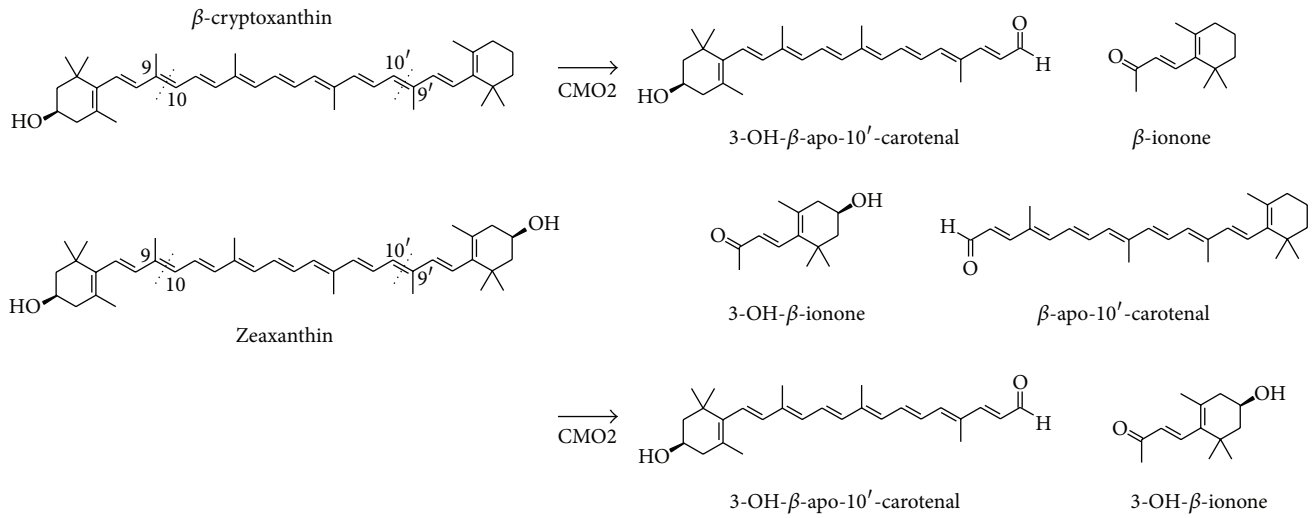

Figure 2: Xanthophyll carotenoids β-cryptoxanthin and zeaxanthin, and the metabolites from CMO2 cleavage, in ferrets (adapted from Mein et al., 2011) [42].

compared to previous studies with astaxanthin and canthaxanthin. Absorption (biliary excretion not considered) varied from around 10% to around 20% [38, 40].

3.6.4. Metabolite Studies. It is known that β-carotene is metabolised by central cleavage by the enzyme β, β-carotene-15, 15′-monooxygenase (CMO1). On the other hand, the nonprovitamin A xanthophylls, lutein, and zeaxanthin are metabolised preferentially by eccentric cleavage by carotene-9, 10-monooxygenase (CMO2), alternatively known as β-carotene oxygenase 2 (BCO2). β-Cryptoxanthin is also metabolised eccentrically through CMO2, which has been identified in humans, mice, and ferrets [41].

The metabolite pathways of lutein, zeaxanthin, and β-cryptoxanthin have been published in 2011 [42]. In this publication, the production of apocarotenoids from CMO2 metabolism in ferrets was shown for all of these three xanthophylls. All three are cleaved at the 9, 10 position as well as at 9′, 10′. This gives rise to four metabolites for both lutein and β-cryptoxanthin. Zeaxanthin however is symmetrical such that there are only two metabolites, 3-OH-β-apo-10′-carotenal and 3-OH-β-ionone. Both of these metabolites are derived from eccentric cleavage of lutein and β-cryptoxanthin (Figure 2). As zeaxanthin has the same ring structure at each end of the molecule, the same two metabolites are produced irrespective of whether cleavage occurs at the 9, 10 position or the 9′, 10′ position.

In addition to cleavage reaction products, there is evidence of a common metabolite from both lutein and zeaxanthin from noncleavage metabolism. In a human study with zeaxanthin, the metabolite all-E-3′-dehydro-lutein was formed; under normal dietary conditions, all-E-3′-dehydro-lutein is predominantly formed from other sources, most likely from lutein, rather than from dietary zeaxanthin [43].

Further, using chiral-phase HPLC, two diastereoisomers, (3R, 6′R)-3′-dehydro-lutein and (3R, 6′S)-3′-dehydro-lutein, were identified and shown to be common metabolites of lutein and zeaxanthin in rhesus monkeys [5].

3.7. Special Toxicological Studies

3.7.1. One-Year Chronic Study in Monkeys. Safety studies are not normally undertaken in monkeys, at least not for nutritional substances. However, a known profile of human response that has been observed in the past in humans, with high intake of the carotenoid canthaxanthin, is accumulation in the eye and for the so-called "canthaxanthin retinopathy" [10]. Therefore, a chronic study was undertaken in monkeys with the purpose to assess the chronic safety of zeaxanthin and lutein in primates and to determine the potential for crystal formation in the retina. There are morphological differences in the structure of the eye between rodents and primates and further investigation in primates was considered important. There is no specific OECD guideline for the study design undertaken, which was designed taking into account general requirements for primate safety studies.

The chronic study performed in Cynomolgus monkeys was 52 weeks in duration and was an investigation for zeaxanthin and also for lutein (each with separate groups). 10% beadlet formulations of both substances were used. Oral doses of 0.2 and 20 mg/kg bw/day of zeaxanthin or lutein were given respectively by gavage to groups of 2 male and 2 female monkeys. For both 20 mg/kg bw/day groups, one additional male and female were sacrificed after 6 months of treatment. Normal toxicological endpoints were included as well as specific endpoints for the eyes.

All monkeys survived the treatment period. There were no clinical signs of toxicity and there was no effect of treatment on overall mean body weight gain or group mean food intake. At the high dose of zeaxanthin, orange-yellow coloration of the feces was observed during the treatment period and, at necropsy, yellow discoloration of adipose tissue was observed. These were considered as coloration

changes from the presence of the test compound and were not considered an adverse effect. There were no changes in ECG or blood pressure data, considered to be related to zeaxanthin treatment. There were no treatment-related changes in urine, hematological, and serum clinical chemistry parameters. At necropsy, there were no abnormal gross findings or organ weight change. There were no treatment-related histopathological findings.

A comprehensive examination of the eyes of treated monkeys was performed which included ophthalmoscopy and biomicroscopy examinations, fundus photography, and electroretinography (ERG). Postmortem examinations of the retina of the right eye included macroscopic inspection, microscopic pathology under polarized and bright light, for peripheral retina and macula, confocal microscopy of macula, and histopathological examination of the peripheral retina. A determination of lutein and zeaxanthin in retina and lens of the left eye was performed by HPLC. These procedures and results are described in more detail.

Ophthalmic Examinations. Ophthalmic examinations were performed on the monkeys by two independent examiners. Indirect ophthalmoscopic examinations were performed using the Bonnoskop and direct ophthalmoscope and a contact lens biomicroscope. Overall, based on the ophthalmic examination findings, it was concluded that there were no adverse findings that were considered to be related to treatment and there was no evidence for crystalline deposits in the retina of treated monkeys [44, 45].

Additional evaluations were performed using the ophthalmic slit lamp biomicroscope in combination with wide-field corneal contact fundus lenses. The results of these examinations showed that there were no crystalline deposits or inclusions similar to those that have been seen in humans or in Cynomolgus monkeys ingesting high dosages of the carotenoid, canthaxanthin. There were some retinal findings often seen in the human- and nonhuman-primate retina; however, none of these were considered to be related to treatment [44, 45]. The polarising structures that were observed were found not only in the zeaxanthin- and lutein-treated monkeys, but also in the control monkeys. The implication of the special eye examinations included into the 52-week monkey study is considered to be that even for high intake zeaxanthin or lutein consumers; there is no indication that crystalline deposits could develop in the retina, as was seen in man and monkeys with high-dose canthaxanthin supplementation.

Electroretinography (ERG). ERG was performed in all animals once predose and during weeks 25 to 26, weeks 38 to 39, and weeks 51 to 52 of treatment. There were no treatment-related effects in electroretinograms, which is considered a sensitive procedure to detect early signs of generalized retinal degeneration [46].

Eye Pathology. Whole-mounts of retinas from the right eyes were used for microscopic investigations with light or confocal microscopy. Maculas were punched out with a 7 mm trephine before mounting them on slides and the peripheral remaining parts of the retinas were flat-mounted and investigated under the polarization microscope separately. Semiquantitative analysis of inclusions was performed by screening the flat-mounted retinas of the right eyes under polarized light using a Zeiss Axioplan. In addition, all maculas were investigated using a confocal microscopic system. Routine histopathology of paraffin sections from retinal periphery was performed [44, 45, 47]. The routine histopathological investigation of paraffin sections from retinal periphery did not show any differences between treated or control animals.

It was concluded that there were no treatment-related adverse changes in the eyes noted under the conditions of this study. Polarizing inclusions were observed in the macula of monkeys, which were not related to zeaxanthin nor lutein treatment. The incidence and grade of the inclusions in the maculas of the monkeys were not treatment or dose related. The inclusions clearly differed from crystals observed after long-term treatment at high doses of canthaxanthin. In the case of canthaxanthin, crystals were strongly dose-dependent, occurred predominantly in the peripheral retina, and exhibited crystalloid morphology and larger size [12]. In contrast, inclusions in the current study were restricted to the fovea, were very small, and showed no typical crystalline morphology. The nature of the observed polarizing structures remains unknown. Since they were also observed in control animals with a naturally yellow macula, a physiological function may be hypothesized [48].

Zeaxanthin and Lutein Determinations in Retina and Lens by HPLC. Determination of lutein and zeaxanthin in retina and lens was made using HPLC. Treatment with lutein resulted in a dose-related increase of lutein in central retina, peripheral retina, and lens. In addition, after treatment with lutein at both dose levels, elevated amounts of zeaxanthin were observed in the central retina. This finding may be due to the residual zeaxanthin content in the lutein test article. Zeaxanthin levels in peripheral retina and lens were similar to those observed in the placebo group [49].

Treatment with zeaxanthin resulted in a dose-related increase of zeaxanthin in the peripheral retina. In central retina and lens, zeaxanthin content was markedly increased in animals of the high-dose group. Levels in the low dose group were comparable to those determined in the placebo group. In animals treated with zeaxanthin, lutein content was in the same order of magnitude as in the placebo group [49].

Variability of individual animal lutein and zeaxanthin content was considerable in all tissues investigated for both sexes and at all dose levels including the placebo group. Considering the variability, there was no significant difference between sexes. In addition, no relevant difference was observed in animals sacrificed in week 26 and animals sacrificed at the end of the treatment period. This suggests that steady state conditions were reached before week 26 in all eye segments investigated.

Overall, there were no clinical and no morphological evidence for treatment-related adverse changes in the eyes of Cynomolgus monkeys during or after 52 weeks of treatment with zeaxanthin or lutein, both as a 10% beadlet formulation. Specifically, there was no evidence for crystal formation in

the eyes of treated monkeys. The NOAEL for lutein and for zeaxanthin was the highest dosage, 20 mg/kg bw/day.

3.7.2. Other Monkey Studies. There is a published study with female rhesus macaques (5/group) exposed to 10 mg/kg bw/day of lutein supplements providing 9.34 mg lutein and 0.66 mg zeaxanthin, 10 mg/kg bw/day of zeaxanthin supplements, or supplements of a combination of lutein and zeaxanthin (each at 0.5 mg/kg bw/day) for 12 months [50]. After 12 months, one control animal, two lutein-treated animals, two zeaxanthin-treated animals, and all lutein and zeaxanthin combined-treated animals were killed. The other animals were kept under observation for six additional months without receiving further supplementation and were then killed. Plasma and ocular carotenoid analyses, fundus photography, and retina histopathology were performed on the animals.

Supplementation of female rhesus macaques with 9.34 mg lutein/kg bw/day or 10 mg zeaxanthin/kg bw/day for 12 months resulted in 3.2-fold and 3.7-fold increases in the mean concentrations of lutein and 4.0-fold and 4.3-fold increases in the mean concentrations of zeaxanthin, in plasma and retina, respectively. Supplementation of monkeys with lutein or zeaxanthin for one year at a dose of approximately 10 mg/kg bw/day did not cause ocular toxicity and had no effect on biomarkers associated with nephrotoxicity.

3.8. Inhalation Study in Ferrets. No carcinogenic hazard is expected from direct intake of zeaxanthin or lutein. There has been a question as to whether these xanthophylls might exacerbate the risk of lung tumors in heavy smokers as was indicated to occur in two human intervention studies with high dosages of β-carotene [51, 52]. It has been established that this exacerbating influence of β-carotene could be mimicked in the ferret [53], a species selected on the basis of metabolic considerations and certain similarities to man. Ferrets show a weak central (CMO1) cleavage of β-carotene in a similar way to humans. In contrast, rats show a much stronger CMO1 activity and a greater propensity to centrally split beta-carotene, which raised doubts about the relevance of the rat as a suitable human model.

This concern of a possible adverse influence in combination with smoking can potentially be addressed for zeaxanthin using a published study in ferrets treated with β-cryptoxanthin and exposed to cigarette smoke [54]. Zeaxanthin itself has not been tested in the ferret model. Structurally, zeaxanthin is closely related to β-cryptoxanthin. As described previously, the metabolites of zeaxanthin central cleavage, 3-OH-β-apo-10$'$-carotenal and 3-OH-β-ionone (Figure 2), are also metabolites of β-cryptoxanthin central cleavage. From this overlap of CMO2 metabolites and as CMO1 in the lung of man and the ferret is not the predominant cleavage enzyme, data from the β-cryptoxanthin study can contribute to zeaxanthin evaluation on a "read-across" basis.

In this β-cryptoxanthin study, both the low and high dose lowered the incidence of cigarette smoke-induced lung squamous metaplasia. The reduction was significant for the high dose ($p = 0.015$, 1/6 ferrets affected) and was marginally significant for the low dose ($p = 0.06$, 2/6 ferrets affected), compared to the control (6/6 ferrets affected). Further, the expression of proinflammatory markers TNFα (expression of which was tremendously increased in smoke exposed ferret lungs) and of NF-κB was lowered by β-cryptoxanthin administration, with stronger beneficial effects for high-dose β-cryptoxanthin than for the low-dose β-cryptoxanthin.

However, the usefulness of this read-across approach was limited by the dose selection in the β-cryptoxanthin ferret study. The dosages of β-cryptoxanthin used (7.5 μg/kg and 37.5 μg/kg bw/day) were based on equivalence to an average American intake of 104 μg/day (approximately 1.5 μg/kg bw/day for a 70 kg person) increased by a factor of 5 and 25, and not by a factor of at least 100, as is usual in toxicological safety testing. Also from a read-across perspective, only half of the CMO2 metabolites formed from β-cryptoxanthin would be theoretically common to those from zeaxanthin. So ignoring any possible kinetic differences, 37.5 μg/kg bw/day possibly only corresponds to 18.75 μg/kg bw/day in terms of zeaxanthin dosage, or 1.3 mg/day for a 70 kg adult. The relative "internal" human dose could be even lower if systemic carotenoid absorption in the ferret is lower than in man, as indicated by the authors [54].

So from this study with β-cryptoxanthin in ferrets, it is considered that zeaxanthin supplementation at low intakes is unlikely to exacerbate the occurrence of lung cancer and might even have a protective effect against the occurrence of squamous metaplasia. However, due to the low dosages of β-cryptoxanthin used, the extent to which the dosage-related influences might extend to higher intakes of β-cryptoxanthin or intakes of zeaxanthin above 1.3 mg/day is unclear.

4. Summary and Discussion

A series of well-conducted safety studies are available and provide a good basis for a safety assessment of zeaxanthin. Acute studies in rats and mice show a low order of acute toxicity with LD_{50} values greater than 4000 and 8000 mg/kg, respectively. Subchronic safety studies demonstrated that repeated intakes of high oral doses up to 1000 mg/kg bw/day in rat and mouse and 400 mg/kg bw/day in the dog are well tolerated systemically. The macroscopic observation of yellow discoloration of the adipose tissue, which can be attributed to the presence of the zeaxanthin, indicates that there was systemic exposure in these studies and this has been analytically confirmed by analysis of plasma and liver samples in the two-generation rat study. Despite the systemic exposure and high dosages, no target organ toxicity was identified in the subchronic studies during the in-life phase or by pathological/histopathological evaluation.

In developmental toxicity studies, there was no evidence of maternal toxicity, fetal toxicity, or teratogenicity in treated rats or rabbits at doses up to 1000 mg/kg/day and 400 mg/kg/day, respectively.

For zeaxanthin, there is no chronic study in rodents, as is also the case for lutein, but there is a two-generation study. This study design involves exposure to test compounds beginning before mating, continuing during mating and

throughout gestation and lactation, until weaning, and covers all reproductive life phases over two generations. The new version of the study design finalized in 2001 introduced a range of additional end-points focused on detection of fine disturbances of reproductive function and fertility. Such studies can sometimes give a lower NOAEL than respective subchronic toxicity studies, in a similar way that the NOAELs from chronic studies in general are lower than in corresponding subchronic studies. Indeed, on the basis of the dosages for which data was available, the NOAEL from the rat two-generation study with zeaxanthin (150 mg/kg bw/day) was a factor of 6.7 down from the NOAEL in the subchronic rat study.

Potentially, the high systemic exposure observed in young animals during the lactation phase of the two-generation study, from a combination of intake from maternal milk and direct feeding, may have contributed to an effect of treatment occurring at a lower nominal dosage than in the other safety studies. Irrespective of the reason, the lowest dosage from repeat dose toxicity studies was the intermediate dosage of 150 mg zeaxanthin/kg bw/day.

ADME studies in the rat showed that zeaxanthin was rapidly but incompletely absorbed after oral administration following a single dose of ^{14}C-zeaxanthin and there is a wide bodily distribution with clear deposition in fatty tissues reflecting the lipophilic nature of zeaxanthin. In addition to specific ADME studies, important information on the potential to bioaccumulate can be obtained from samples taken during the course of the toxicology studies. As referred to in the two-generation study, there is evidence of accumulation in the liver in comparison to concentrations in the plasma.

Metabolite studies have shown there is eccentric CMO2 cleavage of zeaxanthin and other xanthophylls but for zeaxanthin, being symmetric, only two rather than four metabolites are expected. Both CMO2 cleavage metabolites of zeaxanthin occur as cleavage metabolites of lutein and β-cryptoxanthin.

Genotoxicity studies are important studies to indicate if there is interaction with DNA. When unformulated pure crystalline zeaxanthin is exposed to air and light, there may be a potential for mutagenic breakdown products to occur. However, DSM formulated zeaxanthin contains antioxidants that prevent the degradation of zeaxanthin. No mutagenicity was observed in the Ames test with zeaxanthin, or crystalline zeaxanthin retained beyond the shelf life, or in cultures of V79 at the HGPRT locus. No evidence of Unscheduled DNA Synthesis was detected in rat hepatocytes up to the highest dose tested. There was no evidence of clastogenic potential with or without metabolic activation from tests with peripheral blood lymphocytes at doses. In the *in vivo* mouse micronucleus test, there was no evidence for mutagenicity or clastogenicity. It is concluded that there is no evidence for mutagenicity or clastogenicity with formulated zeaxanthin under appropriate conditions of use.

Based on the wide range of genotoxicity studies with no indication of DNA damage and the absence of any indication of preneoplastic organ changes in repeat dose toxicity studies, and the absence of clear liver enzyme induction effect for lutein, no carcinogenic hazard is expected from direct intake of zeaxanthin.

The question as to whether these xanthophylls might exacerbate the incidence of lung tumors in heavy smokers, as was demonstrated to occur for high dosages of β-carotene in two human intervention studies [51, 52], has only been partly addressed by using published studies in ferrets for this related xanthophyll. The ferret model with exposure to cigarette smoke has been positively validated for β-carotene [53] and the metabolite overlap has enabled theoretical use of a study with β-cryptoxanthin to support the safety of zeaxanthin, on a read-across basis. Although the study showed a protective effect of β-cryptoxanthin against pulmonary squamous metaplasia, the potential applicability of this data for zeaxanthin intake was considered to be limited, due to the low β-cryptoxanthin dosages that were used in the study.

Besides this study in ferrets, a pooled analysis of seven cohort studies demonstrated that the association between intake of xanthophylls (lutein or lutein plus zeaxanthin) and the risk of lung cancer was negative in smokers and nonsmoking subjects [55].

In general, the structural similarity of the xanthophyll compounds might be considered sufficient to enable the principles of read-across, where there are safety data gaps, as has been done with the β-cryptoxanthin data in ferrets. However, it appears to be the case that, in the human and primate eye, there is a notable specificity in biological differentiation between the xanthophyll isomers [56]. With this being the case, there is the need for caution in carrying across information from one of these related substances to another, at least in respect to the primate eye.

The animal safety data for lutein and meso-zeaxanthin is notably less than what is available for zeaxanthin. EFSA has reviewed the available data for lutein [36]. For meso-zeaxanthin, there is published safety information [57]. In genotoxicity studies reported by Xu et al., there was no evidence of genotoxicity, which is consistent with the data for zeaxanthin. The NOAEL from their 13-week rat toxicity study was 300 mg/kg bw/day with clear adverse effects in the liver being reported at the higher dosages of 600 and 1200 mg/kg bw/day. This is in contrast to the subchronic safety data for synthetic zeaxanthin, where higher NOAELs were obtained with no indication of liver toxicity. No published regulatory 13-week rat study with β-cryptoxanthin could be located although there is ADME data in the rat following chronic oral intake [58].

There is a publication reporting toxicology studies for a lutein and zeaxanthin concentrate from marigold flowers (*Tagetes erecta L.*), with a minimum 80% carotenoid content [59]. In the subchronic study, Wistar rats were administered the concentrate at dose levels of 0, 4, 40, and 400 mg/kg bw/day (gavage) for 13 weeks with no toxicologically significant treatment-related changes. The dosage in terms of zeaxanthin, at the high dose, can be calculated to be 21.6 mg/kg bw/day (taking 7.5% of the carotenoid content to be zeaxanthin).

In a recent publication of safety studies of the zeaxanthin concentrate OmniXan, RR-zeaxanthin 65% enriched product obtained from paprika [60], there was no indication of

genotoxicity. In the 13-week rat toxicity study, the highest dosage in terms of concentrate was 400 mg/kg bw/day and this was considered the NOAEL.

A known profile of human response that has been observed in the past in humans, with a high intake of the carotenoid canthaxanthin, is accumulation in the eye and for so-called "canthaxanthin retinopathy." The accumulation in the eyes, however, was not found to be functionally harmful and gradually reversible following discontinuation of consumption. Nevertheless, this accumulation is regarded as undesirable and has been evaluated as an adverse effect by EFSA [61].

A chronic study with synthetic zeaxanthin in Cynomolgus monkeys, an animal model used to investigate the induction and dose dependency of canthaxanthin crystal formation in the retina, has been undertaken involving a comprehensive battery of ocular testing as well as usual toxicological endpoints. Overall, there were no clinical and no morphological evidence for treatment-related adverse changes in the eyes and specifically no evidence for crystal formation in the eyes of treated monkeys.

In the safety evaluation of dietary substances, including nutritional substances being consumed at higher intakes than traditionally occurs, human data needs to be kept in mind as it becomes available. A number of human intervention studies have been undertaken or are in progress with respect to investigating the protective function for zeaxanthin and lutein in the eye. These studies indicate good systemic tolerance of zeaxanthin. At the upper end of the dosage range were a study with a dose of up to 20 mg/day for up to 6 months [62] and a study with 8 mg/day for a year, both without evidence of adverse effects. A further study has been more recently reported in which 24 subjects were supplemented with 20 mg/day of zeaxanthin over 4 months, without any adverse effects [63, 64].

4.1. ADI (Acceptable Daily Intake). In the 13-week subchronic toxicity studies, the NOAEL in all cases was the highest dosage investigated, namely, 1000 mg/kg bw/day in the mouse and rat and at least 422 mg/kg bw/day in the dog. A traditional approach of a 100-fold safety factor in conjunction with the lowest relevant NOAEL from the safety studies would be used to derive the ADI.

For zeaxanthin, the lowest NOAEL from a standard regulatory study was 150 mg zeaxanthin/kg bw/day in the two-generation study in rats. This NOAEL is at least a factor of 6.7 lower than the NOAEL in the 13-week rat study (>1000 mg/kg bw/day). In their evaluation of the safety of synthetic zeaxanthin as a Novel Food, the EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA) [37] used the 150 mg/kg bw/day NOAEL with a 200-fold safety factor to define an ADI of 0.75 mg/kg bw/day, or 53 mg/day for a 70 kg adult (70 kg is the new default human weight used by EFSA). Use of the lowest NOAEL for ADI calculations, as was done by the NDA Scientific Panel, is the traditional precautionary approach used in safety evaluation. The NDA Scientific Panel stated that a daily intake of 53 mg for a person with a body weight of 70 kg does not raise safety concerns and that the use level of 2 mg/day requested by the applicant was confirmed as safe.

In the case of lutein, the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) in their reevaluation [36] introduced an additional safety factor of 2 (making a total 200-fold safety factor), due to the absence of chronic studies or a multigeneration reproductive toxicity study. Additional factors taken into account were that the other data (reproductive studies and genotoxicity data) did not indicate a cause for concern and that lutein is a normal constituent of the diet. The highest dose tested for lutein in a comprehensive 13-week rat toxicity study was 200 mg/kg bw/day and this was the NOAEL. The EFSA ANS Panel applied a 200-fold factor to this NOAEL giving an ADI of 1 mg/kg bw/day or 60 mg lutein/day for an adult.

As a passing comment, application of the 200-fold factor to the rat zeaxanthin subchronic data would give a 5-fold higher ADI than for lutein, due to the higher dosages used and the higher NOAELs that were established for zeaxanthin.

In the United States, synthetic zeaxanthin is marketed since 2002 under the Generally Regarded as Safe legislation, based on DSM safety studies available at the time, with use level in foods and beverages of 0.25 mg/serving.

The use level of zeaxanthin of 2 mg/day proposed by the applicant was ratified by the European Union (EU) Commission in 2013 [65]. However, this upper use level is much lower than the safe level (53 mg/day) defined for zeaxanthin by the NDA Scientific Panel. Potentially, therefore, the currently approved level for synthetic zeaxanthin in Europe could be set as a higher level. Probably, this could be closer to the ADI calculated by the NDA Panel. This ADI (53 mg/day) is similar to the ADI of 60 mg/day currently defined by EFSA for lutein [36].

5. Conclusion

Zeaxanthin was negative for mutagenic and clastogenic activity in a comprehensive battery of *in vitro* and *in vivo* tests for genotoxicity. Based on these studies, it is concluded that there is no evidence for mutagenicity or clastogenicity with formulated zeaxanthin under appropriate conditions of use.

In repeat dose toxicity studies in the rat, mouse, and dog, synthetic zeaxanthin was well tolerated at high dosages with no indication of target organ toxicity or preneoplastic organ changes. Taken together, these data indicate that no carcinogenic hazard is expected from direct intake of zeaxanthin. A study in primate did not indicate any evidence of ocular toxicity or excessive accumulation. A published study in ferret provides limited support for the absence of any stimulating effect of zeaxanthin consumption on the incidence of lung cancer in heavy smokers.

The regulatory study that gave rise to the lowest overall NOAEL of 150 mg zeaxanthin/kg bw/day was a comprehensive two-generation study in the rat. In their evaluation of the safety of synthetic zeaxanthin as a Novel Food, the EFSA NDA Scientific Panel [37] applied a 200-fold safety factor to this NOAEL to define an ADI of 0.75 mg/kg bw/day, or 53 mg/day for a 70 kg adult. The EU in 2013 [65] formally approved upper use levels of 2 mg/day (equivalent to

0.03 mg/kg bw/day) as this was the use level proposed by the applicant.

Information from human intervention studies also supports that an intake higher than 2 mg/day is safe, and an intake level of 20 mg/day for up to 6 months was without adverse effect.

Disclosure

The studies referred to in this publication are principally the work of many dedicated toxicologists and safety specialists over a number of years and are not the work of the author.

Conflict of Interests

The author is an employee of DSM Nutritional Products Ltd., which manufactures synthetic zeaxanthin.

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Review Article

Lutein, Zeaxanthin, and *meso*-Zeaxanthin in the Clinical Management of Eye Disease

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Lutein, zeaxanthin, and *meso*-zeaxanthin are xanthophyll carotenoids found within the retina and throughout the visual system. The retina is one of the most metabolically active tissues in the body. The highest concentration of xanthophylls is found within the retina, and this selective presence has generated many theories regarding their role in supporting retinal function. Subsequently, the effect of xanthophylls in the prevention and treatment of various eye diseases has been examined through epidemiological studies, animal studies, and clinical trials. This paper attempts to review the epidemiological studies and clinical trials investigating the effects of xanthophylls on the incidence and progression of various eye diseases. Observational studies have reported that increased dietary intake and higher serum levels of lutein and zeaxanthin are associated with lower risk of age-related macular degeneration (AMD), especially late AMD. Randomized, placebo-controlled clinical trials have demonstrated that xanthophyll supplementation increases macular pigment levels, improves visual function, and decreases the risk of progression to late AMD, especially neovascular AMD. Current publications on the preventive and therapeutic effects of lutein and zeaxanthin on cataracts, diabetic retinopathy, and retinopathy of prematurity have reported encouraging results.

1. Introduction

Macular pigments are xanthophyll carotenoids that provide the macula lutea with its yellow appearance. Lutein (L), zeaxanthin (Z), and *meso*-zeaxanthin (MZ) are the three major xanthophylls found in the eye. L and Z cannot be synthesized *de novo* and must be acquired from the diet. MZ is a metabolite of L but also can be absorbed from the diet [1]. The highest dietary concentration of L and Z are found in green leafy vegetables, egg yolk, corn, citrus, and other fruits [2]. With the exception of the cornea, vitreous, and sclera, these xanthophylls are found throughout the visual system [3]. The highest concentration of L and Z is in the retina [4]. Macular pigments account for 20–30% of total carotenoids in the human serum, but 80–90% of carotenoids in the human retina [5]. The concentration of L, Z, and MZ in the macula is much higher than concentrations in the serum and liver. This suggests a specific uptake and storage mechanism for L, Z, and MZ in the retina and emphasizes their essential role in retinal function [6]. The aim of this review is to briefly describe the role of these xanthophylls in maintaining visual function. In addition, it provides an overview of current clinical investigations studying the role of macular pigments on visual function and preventing the development and progression of age-related macular degeneration (AMD), retinopathy of prematurity (ROP), diabetic retinopathy (DR), and cataract.

2. Lutein, Zeaxanthin, and *meso*-Zeaxanthin in the Retina

Macular pigments have a unique distribution within the retina. Concentrations of L, Z, and MZ are highest in the macula, especially in the center of the macula (the fovea). While zeaxanthin has a peak concentration in the central fovea, lutein predominates in the periphery [7, 8]. The ratio

of L to Z in the fovea is approximately 1 : 2.4. Moving eccentrically from the fovea to the periphery, zeaxanthin concentrations decline rapidly while lutein levels slowly rise. Therefore, in the periphery the ratio of L to Z reverses, exceeding 2 : 1 [9]. Bone et al. demonstrated that in the fovea zeaxanthin coexists with its isomer MZ [7]. They proposed that L, MZ, and Z are actually found in equal quantities in the central macula (in an area with 3 mm diameter of the macula). MZ, unlike L and Z, was previously thought to be undetectable in the human liver or serum. Therefore, it was theorized that MZ was a specific metabolite of lutein found only in the retina [10]. The 3 : 1 ratio of L to Z in serum and the 2 : 1 ratio in the fovea support the theory of the conversion of L to MZ in the macula. However, more recently MZ has been detected in the serum, and supplementation trials have demonstrated a significant increase in macular pigments levels after oral supplement with MZ, suggesting that MZ can be absorbed after oral administration and transported to the macula [11]. Supplementation trials involving L, MZ, and Z suggest that MZ may be absorbed and converted in the retina, as supplementation with high dosages of MZ (10 mg MZ, 10 mg L, and 2 mg Z or 17 mg MZ, 3 mg L, and 2 mg Z) resulted in higher macular pigment levels and higher MZ serum levels than supplementing without MZ (20 mg L and 0.86 mg Z) [12]. The results of this trial will be discussed in greater detail below.

Macular pigments are found in their highest concentration in the outer plexiform layer and inner plexiform layer [13]. L and Z have a peak absorbance near 460 nm. In the inner retina they serve as a filter for high energy, short wavelength blue light [13]. This protects the outer retina from photochemical injury easily induced by these high energy wavelengths [14]. They also enhance visual performance by decreasing chromatic aberration and enhancing contrast sensitivity [15–17].

Blue light filtration is one of the many functions of macular pigment [4]. L and Z are also found in the rod and presumably cone outer segments. In the outer retina, macular pigments serve as antioxidants. Photoreceptor outer segments contain chromophores that act as photosensitizers susceptible to oxidative damage. Macular pigments are capable of quenching reactive oxygen species produced from chromophore irradiation, which protects the retina from the deleterious effects of lipid peroxidation [9, 18]. Polyunsaturated fatty acids, especially docosahexaenoic acid (DHA), have high concentrations in the rod outer segments [19]. DHA is highly susceptible to lipid peroxidation and a subsequent cascade of cellular damage. L can return singlet oxygen to the ground state and remove resultant energy as heat, preventing lipid peroxidation. Lutein autoregenerates in the process and is not consumed [20]. This makes L a more efficient quencher of singlet oxygen than other antioxidants such as alpha tocopherol (vitamin E) [21]. Macular pigments are very effective antioxidants, capable of quenching singlet oxygen and triplet state photosensitizers, inhibiting peroxidation of membrane phospholipids, scavenging reactive oxygen species, and reducing lipofuscin formation [22–28].

Although L and Z differ only by the placement of a single double bond, this small alteration in configuration has a great impact on the function of these two carotenoids [29]. Compared to L, Z is a much more effective antioxidant [30]. MZ also has a greater capability of quenching oxygen radicals than L [10]. The functional differences of these carotenoids correlate with the spatial distribution of L, MZ, and Z. The ratio of L to Z varies linearly with the ratio of rods to cones in the fovea. MZ and Z predominate where cone density is highest and risk of oxidative damage is greatest [30, 31]. The macular pigments also differ in other aspects. For example, L has a greater filtering efficacy, and Z is superior in preventing lipid peroxidation induced by UV light [32, 33].

These essential functions of macular pigment decrease oxidative stress in the retina and enhance vision in both normal and diseased retinas.

3. Lutein and Zeaxanthin and Visual Function

Macular pigments enhance visual function in a variety of ways. The filtration of blue light reduces chromatic aberration which can enhance visual acuity and contrast sensitivity. L and Z also reduce discomfort associated with glare and improve visual acuity, photostress recovery time, macular function, and neural processing speed.

Discomfort glare is a term used to describe photophobia and discomfort experienced when intense light enters the eye. When testing photosensitivity, subjects are more sensitive to shorter wavelengths of light, which are capable of inducing retinal damage with less energy compared to other wavelengths. Despite increased sensitivity to shorter wavelengths, Stringham et al. found a minimum sensitivity was observed at macular pigment peak absorbance (460 nm). They proposed that photosensitivity serves as a protective function to prevent damage to the eye, and macular pigments could attenuate this visual discomfort by absorbing the high energy wavelengths before they reach the photoreceptor layer [34, 35]. In analyzing the photophobic response produced by glare, subjects with higher macular pigment levels tolerated light better [35]. They also noted that a small increase in macular pigment provided significant improvement in photophobia thresholds and lessened visual discomfort. Similarly, Wenzel et al. also showed a direct correlation between macular pigment levels and photophobia thresholds [36]. This evidence suggests that macular pigment supplementation has a role in reducing discomfort associated with glare.

Disability glare is a term used to describe decreased visual acuity resulting from scattered light, another phenomenon that results from bright light settings. Stringham and Hammond Jr. demonstrated that subjects with higher macular pigment levels maintained acuity better than subjects with lower levels when exposed to both bright white light and short wavelength (blue) light [37]. The response was more exaggerated with the white light, suggesting that macular pigment has a filtering effect integrated across all wavelengths and can reduce disability glare under broad illumination [38, 39]. When patients were supplemented with L and Z, glare disability was improved [40].

Photostress recovery is another parameter of visual performance affected by macular pigments. Photostress recovery is a term used to describe the time necessary to recover

vision following exposure to a bright light source. Physiologically, this describes the time necessary for photopigments bleached by a bright light source to regenerate. Stringham and Hammond Jr. demonstrated that subjects with higher macular pigment levels had shorter photostress recovery time when tested with intense short wavelength and bright white light sources [37]. They proposed that macular pigment reduces photostress recovery time by reducing photoreceptor exposure to short wavelength light in the foveal and parafoveal regions. Recovery time for the subject with the lowest macular pigment levels was twice as long as subjects with the highest macular pigment levels [38]. After supplementing patients with L and Z, photostress recovery time was significantly decreased [40]. Hammond et al. reported that daily supplementation with L (10 mg/d) and Z (2 mg/d) for 3 months resulted in significant increase in serum levels of L and Z and MPOD and improvements in chromatic contrast and recovery from photostress in 57 young and healthy subjects as compared with 58 controls [41]. Correlation of MPOD and visual performance also has been studied in patients suffering from eye diseases [17, 42–47], which will be described later.

Nolan et al. performed a randomized, placebo-controlled clinical trial supplementing young, healthy subjects with lutein for 12 months. Their goal was to identify if visual performance could be improved with supplementation in a population with relatively high macular pigment levels considered to be at peak visual performance. They were not able to show a significant change in visual performance in supplemented patients despite doubling serum L levels and significantly increasing central macular pigment levels. They did demonstrate, however, that there was a significant difference in visual performance in subjects in the lowest versus the highest tertile groups [48]. These findings suggest subjects with a sufficient baseline macular pigment level and good visual performance may not benefit from supplementation.

In addition to enhancing visual performance, macular pigments have also been implicated in benefiting neurophysiological health by affecting the complex relationships between optical, neurological, and physiological mechanisms underlying vision. Higher macular pigment levels are attributed to better critical flicker fusion frequency [49], transparency of the crystalline lens [50–52], higher concentrations in the visual cortex [53], and improvements in ERG [54, 55].

Macular pigments are present throughout the visual system, including the brain [53, 56, 57]. Animal models have shown macular pigment optical density (MPOD), a method for quantitating macular pigment levels *in vivo*, is a good proxy for the quantity of xanthophylls in the brain [58]. MPOD correlates with processing speed and cognitive performance in healthy elderly subjects as well as those with mild cognitive impairment [10, 59–61]. Bovier et al. found moderate but statistically significant improvements in both MPOD and cognitive function when supplementing young, healthy individuals considered to be at peak cognitive efficiency [62]. These studies suggest that both young, healthy adults and the elderly population can gain cognitive benefits from L and Z supplementation. Proposed mechanisms for improvement are based in cellular connectivity, as carotenoids may influence the production of connexin proteins that improve intracellular communication [63–65]. This data suggests that patients suffering from poor visual or cognitive performance may experience an improvement in symptoms with increased dietary intake or supplementation of L and Z.

4. Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the most common cause of irreversible blindness in people over the age of 50 in the developed world [66]. Although the pathogenesis of AMD is poorly understood, oxidative stress has been implicated as a major contributing factor. As L and Z are powerful antioxidants selectively absorbed and maintained in the retina, their role in AMD has been studied extensively.

4.1. Observational Studies (Dietary Intake of L and Z). Initial studies focused on the relationship between dietary intake of L and Z and the risk for AMD. While the results of these studies were variable, most suggested that high dietary intake of L and Z is associated with a decreased risk of AMD.

Ma et al. published a systematic review and meta-analysis on this subject [67]. They analyzed six longitudinal cohort studies [68–72] and found that early and late AMD have different relationship with the intake of L and Z. In the late AMD, the pooled relative risk (RR) was 0.74 with 95% confidence intervals (CI) at 0.57–0.97, which indicated that increase in the intake of L/Z was significantly associated with a 26% risk reduction for late AMD. Furthermore, a significant inverse association was observed between L/Z intake and neovascular AMD risk (RR 0.68; 95% CI 0.51–0.92), but not with geographic atrophy. The meta-analysis found that dietary intake of L/Z was not significantly associated with a reduced risk of early AMD.

In addition to the six papers analyzed by Ma et al., there were several other important observational studies published. The Eye Disease Case-Control Study reported that subjects with the highest quintile of carotenoid intake had a 43% reduced risk of AMD compared with subjects in the lowest quintile [73]. Vitamin A, vitamin C, or vitamin E consumption did not provide a similar risk reduction. Similarly, the Blue Mountain Eye Study reported a 65% reduced risk of neovascular AMD between subjects with the highest and lowest intake of L/Z. Subjects above the median carotenoid intake also had a reduced risk of indistinct soft or reticular drusen [69].

In the Age-Related Eye Disease Study (AREDS) Report 12, the relationship between dietary intake of L/Z and late AMD was studied in 4,519 AMD patients. Dietary L/Z intake was inversely associated with neovascular AMD (odds ratio (OR), 0.65; 95% CI: 0.45–0.93), geographic atrophy (OR, 0.45; 95% CI: 0.24–0.86), and large or extensive intermediate drusen (OR, 0.73; 95% CI, 0.56–0.96), comparing the highest versus lowest quintiles of intake, after adjustment for total energy intake and nonnutrient-based covariates. Other nutrients (β-carotene, vitamin C, vitamin E, lycopene, etc.) were not independently related to AMD [74].

Furthermore, participants from the Rotterdam Study were enrolled into a case-control study investigating whether dietary nutrients could reduce the genetic risk of early AMD. A total of 2,167 participants from the population-based Rotterdam Study at risk of AMD were followed up for a mean of 8.6 years. They reported that high dietary intake of nutrients with antioxidant properties such as L and Z, β-carotene, omega-3 fatty acids, and zinc reduced the risk of early AMD in those at high genetic risk [75]. This is the first report to evaluate both genetic and environmental risk factors for AMD.

4.2. Observational Studies (Serum Levels of L/Z). Several studies evaluated the serum levels of L/Z. The Beaver Dam Eye Study found that L/Z serum levels did not correlate with AMD [76]. Gale et al. examined the relationship between AMD and plasma L/Z levels in 380 AMD patients and found that the risk of AMD (early or late) was significantly higher in individuals with lower plasma Z levels. Subjects with the lowest third Z levels had double the risk of AMD compared to those with the highest third. Risk of AMD was also associated with plasma L levels; however, the relationship between L and AMD was not significant [77].

4.3. Studies on In Vivo Macular Pigment Levels. A case-control study of human donor eyes by Bone et al. demonstrated that donors with AMD had significantly lower levels of macular pigment (MP) compared to eyes without; and donors with the highest quartile of L/Z had an 82% lower risk of having AMD compared to donors in the lowest quartile [78]. This was the first study to report decreased retinal levels of MP in patients with AMD, which correlated with previous studies analyzing serum carotenoid levels. The authors did note that decreased MP could at least in part be attributable to the disease process [79].

Subsequently concentrations of L and Z in the retina have been studied extensively. Macular pigment levels within the retina are easily measured *in vivo* as macular pigment optical density (MPOD) with heterochromatic flicker photometry and retinal reflectometry [80, 81]. MPOD correlates with dietary intake of carotenoid-rich foods [52] and circulating serum L and Z levels [82]. MPOD in healthy subjects shows an age-related decline, and healthy eyes at risk for AMD have significantly lower MPOD than healthy eyes not at risk [83]. The CAREDS study, a prospective cohort analysis of nearly two thousand postmenopausal women, did not find a correlation between MPOD and AMD [84]. However, other studies have reported a correlation of lower MPOD in eyes with AMD, and several supplementation trials studying subjects with AMD reported a decreasing MPOD in their placebo group over the course of the trial [42, 85, 86]. Lower levels of macular pigment have also been associated with other risk factors for the disease, including a positive family history of AMD, tobacco use, and obesity [87].

4.4. Xanthophyll Supplementation Trials. After the established correlation between the risk of AMD and low serum and retinal concentrations of L and Z, supplementation trials were initiated. These trials have shown extremely consistent results as compared to any other single nutrient supplementation trial.

The first supplementation trial reported was the Veterans Lutein Antioxidant Supplementation Trial (LAST). This was a double-masked, placebo-controlled trial that investigated lutein supplementation alone compared to combined supplementation (lutein, other carotenoids, antioxidants, vitamins, and minerals) in 90 patients with dry AMD and geographic atrophy. Both groups demonstrated a significantly increased level of MP, improved visual acuity (VA) at near, and improved contrast sensitivity (CS). The disease progression was halted with supplementation over the course of the 12-month study. While the duration of the study was short and study group numbers were small, few studies have monitored the effects of MP supplementation alone compared to combined supplementation [17].

The Age-Related Eye Disease Study (AREDS) was one of the largest and earliest supplementation trials which demonstrated that subjects with extensive intermediate-sized drusen, at least one large druse, noncentral geographic atrophy, or advanced AMD in one eye had 25% reduced risk of severe vision loss at 5 years if supplemented with vitamin C (500 mg), vitamin E (400 IU), b-carotene (15 mg) with or without zinc (80 mg), and copper (2 mg cupric oxide) [88]. The treatment effect appeared to persist following 5 additional years of follow-up after the trial ended [89]. However, the effects of L and Z were not evaluated in this study.

Weigert et al. evaluated the role of lutein supplementation in MPOD, visual acuity, and macular function (assessed with microperimetry) in intermediate to advanced AMD. A total of 126 patients were randomized to L (20 mg daily for 3 months and then 10 mg daily for 3 months) or placebo for a period of 6 months. Supplementation significantly increased MPOD. There was a trend toward increased macular function and visual acuity that was not statistically significant [47].

Ma et al. evaluated the role of macular pigment supplementation in early AMD over 48 weeks. A total of 107 subjects were randomized to a placebo, L (10 mg/day), L (20 mg/day), or L (10 mg/day) and Z (10 mg/day). They reported a significant increase in MPOD in all study groups with the exception of the 10 mg lutein group. There was no change in the placebo group. Subjects with the lowest baseline MPOD had the greatest increase in MPOD regardless of supplementation. Visual acuity (VA) improved in all treatment groups, but not significantly. Contrast sensitivity (CS) was significantly different at 48 weeks in all treatment groups. The authors noted that MPOD was significantly increased at 24 weeks, while VA and CS did not show improvement until 48 weeks, suggesting that visual function cannot be improved until MPOD levels reach and maintain high levels [44].

The CARMIS study reported a significant improvement in CS and NEI visual function questionnaire at 12 and 24 months in AMD patients supplemented with vitamin C (180 mg), vitamin E (30 mg), zinc (22.5 mg), copper (1 mg), L (10 mg), Z (1 mg), and astaxanthin (4 mg) compared to controls. VA was not significantly improved until 24 months [46], consistent with other supplementation trials.

The LUTEGA study evaluated the long term effects of L, Z, and omega-3 fatty acid supplementation on MPOD in

145 dry AMD patients randomized to placebo, daily or twice daily dosage of supplement. The supplement provided was L (10 mg), Z (1 mg), and omega-3 fatty acid (100 mg DHA, 30 mg EPA). After 12 months, MPOD increased significantly in supplementation groups and decreased significantly in controls. VA also improved compared to placebo. There was no significant difference in accumulation of MPOD between the two dosage groups. No progression was noted in any of the participants [43].

The CLEAR study evaluated the effects of L (10 mg) supplementation on early AMD subjects over a 12-month period. This group reported a significant increase in mean MPOD after 8 months of supplementation, with no change in the control group. VA improved in the study group and declined slightly in the placebo group. There was also an increase in serum L levels in the study group, increasing anywhere from 1.8 to 7.6 times the baseline values. Those with lower baseline serum levels tended to have greater improvements, but the response to supplementation varied markedly between individuals [45].

The CARMA study investigated the role of L and Z with other antioxidant vitamins and minerals in subjects determined to be at highest risk of progression to advanced AMD. A total of 433 subjects were randomized to the placebo or supplementation group. Patients were supplemented with Ocuvite twice daily (L 12 mg, Z 0.6 mg, vitamin E 15 mg, vitamin C 150 mg, zinc oxide 20 mg, and copper gluconate 0.4 mg). VA improved after 12 months of supplementation but was not significant until 24 months. CS was also improved, but not significantly. Fewer eyes in the active group progressed compared to controls (41.7% versus 47.4%, resp.). Macular pigment values in the study group demonstrated a small increase over time, while the placebo group steadily declined. Serum concentrations of all antioxidants were increased after six months of supplementation. The increases in these serum levels did not correlate with improvements in VA. However, an increase in serum L levels was associated with slower progression of AMD. A similar pattern was seen with serum Z levels but did not achieve statistical significance [42].

Liu et al. performed a meta-analysis which compared the results of the above-mentioned seven randomized, double-blind, placebo-controlled trials, including the LAST, Weigert et al., Ma et al., CARMIS, LUTEGA, CLEAR, and CARMA studies [17, 42–47]. Four of the seven studies demonstrated an increase in VA with supplementation. A stronger effect was noted for studies using higher doses of supplements. The analysis demonstrated that supplementation is associated with significant improvements in VA and CS in a dose-response relationship. A linear association of MPOD and an increase in VA and CS was also noted. Compared with early AMD patients, late AMD patients tended to have a less significant improvement in VA. This was attributed to the loss of macular photoreceptors in the late stage of the disease [90].

After the release of several smaller supplementation trials mentioned above, the Age-Related Eye Disease 2 Study (AREDS2) was published. AREDS2 was a multicenter, randomized, double-masked, placebo-controlled clinical trial following 4,203 participants with intermediate AMD or large drusen in 1 eye and advanced AMD in the fellow eye for approximately 5 years. Participants were assigned to one of four groups: placebo, L (10 mg) and Z (2 mg), omega-3 fatty acids (DHA 350 mg and EPA 650 mg), or a combination of L, Z, and omega-3 fatty acids. In addition they were given either the original AREDS formulation or some modification of the original formulation (eliminating β-carotene, lowering zinc dose, or a combination of the two). The original analysis id not find significant effects from xanthophyll supplementation. However, a secondary analysis (2014) of the effects of L/Z on AMD progression in AREDS2 revealed definitively positive results [91]. The authors reanalyzed the results of AREDS2 by analyzing L/Z versus no L/Z and comparing L/Z and β-carotene. In the analysis of L/Z versus no L/Z, the development to the late AMD was significantly decreased in patients treated with L/Z; the risk ratio (RR) of late AMD was 0.90 (95% CI, 0.82–0.99; $P = 0.04$). Analyses of the comparison of L/Z versus β-carotene also showed significant decrease of risk of development of late AMD and neovascular AMD in L/Z group but did not appear to influence development of geographic atrophy. In analyses restricted to eyes with bilateral large drusen at baseline, the comparison of L/Z versus β-carotene showed even better effects, RR of 0.76 for progression to late AMD, and RR of 0.65 for neovascular AMD. The totality of evidence regarding beneficial and adverse effects of β-carotene in AREDS2 and other studies suggests that L/Z is more appropriate than β-carotene for the new AREDS2 formulation.

These studies established that structural changes in the retina can be achieved with supplementation, and over time supplementation appears to affect visual acuity. More recent studies have evaluated functional changes in carotenoid supplementation with the multifocal electroretinogram (MfERG). As a secondary analysis to their initial study, Ma et al. compared 107 subjects with early AMD randomly assigned to one of four treatment groups (placebo, L 10 mg/day, L 20 mg/day, or L 10 mg/day and Z 10 mg/day) comparing MfERG responses at baseline, 24, and 48 weeks. They demonstrated that early functional abnormalities in the central retina of subjects with early AMD at baseline could be improved with supplementation of L and Z. They attributed these improvements to the significant increase in MPOD seen at both 24 and 48 weeks [55]. Berrow et al. reported a similar study with smaller sample size randomizing 14 subjects with AMD to placebo or supplementation with Ocuvite Duo for 40 weeks (L 12 mg, Z 0.6 mg, omega-3 fatty acids consisting of EPA 240 mg and DHA 840 mg, vitamin E 15 mg, vitamin C 150 mg, zinc oxide 20 mg, and copper gluconate 0.4 mg). MfERG was performed at 20, 40, and 60 weeks (20 weeks after supplement withdrawal). There was no significant difference in MfERG results during the course of the trial. However, subjects in the treatment group had significant improvement in MfERG results compared to baseline that regressed at the final visit 20 weeks after the supplement was removed [54].

These trials suggest that with long term supplementation of antioxidants in patients with AMD increase in macular pigment in the retina allows for improved macular function, visual acuity, and contrast sensitivity. Evidence

suggests with supplementation serum levels increase quickly, macular pigment increases over a period of several months, and a minimum of one to two years is necessary before improvements in visual function reach statistical significance. Recent studies show that macular pigment levels continue to increase with long term supplementation [12, 92]. Subjects with lower baseline macular pigment levels often show the greatest response to supplementation. The supplementation of L and Z also can retard the progress of intermediate AMD to late AMD, especially in regard to neovascular AMD.

Previous studies mainly investigated the preventive and therapeutic effects of L and Z; very little was known on the effects of MZ on the AMD. Recent reports investigating the ratio of L, Z, and MZ supplementation suggest supplementing with a higher proportion of MZ leads to higher MPOD values and an improvement in CS [12, 92] indicating that including MZ in a supplement may confer benefits for the treatment of early AMD.

While the last two decades of research have provided many insights into the role of macular pigments and other antioxidants in AMD, future research studies investigating the optimal antioxidant supplement, the role of early supplementation, the relationship of MPOD as a risk factor for disease onset and progression, and the impact of genetic risk factors are necessary to better understand the disease process and provide more therapeutic options to patients with AMD.

5. Other Retinopathies

The role of carotenoids in age-related macular degeneration has been studied extensively. The encouraging results have led to subsequent investigations into the role of antioxidants in other diseases, including diabetic retinopathy and retinopathy of prematurity. The retinal ischemia in these conditions can lead to neovascularization, hemorrhage, and blindness. Oxidative stress plays a role in the pathogenesis of both conditions, and early evidence suggests antioxidant supplementation may prevent disease progression [93].

5.1. Retinopathy of Prematurity. In retinopathy of prematurity, premature infants are exposed to higher oxygen tensions compared to conditions *in utero*, which downregulates VEGF generation and the development of normal retinal vasculature. The relatively avascular retina then becomes hypoxic with increasing metabolic demand, which initiates expression of proangiogenic factors. This stimulates aberrant angiogenesis, leading to intravitreal neovascularization [94–97]. The ischemic retina in ROP also has an imbalance between the generation and sequestration of reactive oxygen species (ROS). The developing retina in premature infants is particularly susceptible to oxidative damage for several reasons. The high proportion of long chain polyunsaturated fatty acids (PUFA) [98, 99] leaves the retina susceptible to lipid peroxidation which can damage retinal tissues. In addition, preterm infants have reduced levels of antioxidants compared to full term infants, as they are often produced or accumulated later in gestation [100]. Hence, in preterm infants the endogenous antioxidant system is overwhelmed, leading to a prooxidative state capable of causing irreversible damage to various cell structures. Biomarkers of retinal stress, such as lipofuscin, show rapid increase in RPE cells during the first few years of life. This suggests even infants without ROP are at risk [101, 102]. Antioxidants can protect retinal cells from oxidative damage and have inhibited microvascular degeneration in animal models of diabetic retinopathy and oxygen-induced retinopathy [103, 104]. The relative deficit of antioxidants in preterm infants and the growing evidence from animal studies suggest a possible role for antioxidant supplementation in the prevention of ROP progression.

During fetal development L is the dominant retinal carotenoid [10]. Z and MZ slowly accumulate with time. The presence of L in umbilical cords at birth indicates there is placental transfer to the fetus, with concentrations peaking in the third trimester [105]. A randomized controlled trial of 150 newborns demonstrated that neonatal supplementation of L in the first hours of life increased biological antioxidant potential and reduced levels of total hydroperoxide [106]. Subsequently, four randomized controlled trials investigated the relationship between xanthophylls and ROP [107–110]. L was the primary xanthophyll used in the supplementation trials due to its predominance in the infant retina.

Two multicenter placebo-controlled randomized clinical trials studying ROP prevention supplemented preterm infants (<33 weeks of gestational age) with 0.5 mL daily dosage of 0.14 mg L and 0.0006 mg Z via oral feeds of maternal milk, donor human milk, or preterm formula [107, 108]. The supplemented groups showed reduced incidence of ROP compared to control groups (6.2% versus 10.3% and 19% versus 27%, resp.). In addition, while not statistically significant, supplemented subjects with ROP showed a 50% decrease progression from early to threshold and higher ROP stages compared to controls.

A third clinical trial investigated the effect of weight-based dosages, as AMD trials have suggested better outcomes with higher carotenoid doses. This trial did not show a difference in ROP incidence with weight-based doses, but the study was limited by small sample size [109].

The fourth multicenter randomized controlled trial compared carotenoid levels in preterm infants fed formula with and without L, lycopene, and β-carotene to carotenoid levels in full term infants fed human milk. A secondary outcome was visual complication. ROP incidence was similar between the premature formula fed groups, but the supplemented group had less progression to severe ROP versus the control group (8% versus 28%). The supplemented group also had similar plasma L levels compared to full term infants fed human milk. The study also compared L levels with photoreceptor activity and found that normal plasma lutein levels at 50 weeks of age correlated with a saturated response amplitude in rod photoreceptors and rod photoreceptor sensitivity [110]. The authors suggest that L may play a role in photoreceptor maturation and visual acuity in the developing retina.

To date no clinical trials have specifically tested the hypothesis that L affects ROP outcomes. While future supplementation trials monitoring long term outcomes in ROP would be beneficial, current evidence suggests a role for carotenoid supplementation in the prevention of ROP and normal photoreceptor development in preterm infants.

5.2. Diabetic Retinopathy. In diabetic retinopathy, prolonged hyperglycemia causes oxidative stress via several different pathways [111–116]. Evidence from animal models suggests L and Z can block the pathways leading to oxidative stress by quenching oxygen radicals and therefore preserving retinal function [117–121]. Animal studies have found that the neuroprotective activities of L prevent neuronal loss in the diabetic retina [120, 121].

While a number of studies have examined the role of carotenoids in the development of diabetes mellitus (DM), there are a limited number of studies examining their role in the development of diabetic retinopathy. A serum analysis of patients with Type II DM demonstrated that patients with a higher concentration of serum L, Z, and lycopene compared to serum alpha-carotene, β-carotene, and β-cryptoxanthin had a 66% reduction in the risk of diabetic retinopathy after adjusting for confounding variables [122]. Studies of MPOD have shown subjects with Type II DM have lower MPOD compared to age-matched normals. In comparing the diabetic subjects, those with retinopathy had lower MPOD than subjects without, and MPOD levels correlated with glycosylated hemoglobin levels [123]. While there are not currently any supplementation trials that evaluate the role of L and Z in the prevention or treatment of DR, one study demonstrated that daily supplementation of nonproliferative diabetic subjects with 6 mg L and 0.5 mg Z increased MPOD, improved VA and CS, and increased foveal thickness compared to controls [124].

Evidence supporting the role of macular pigments in the prevention and treatment of retinopathies is currently limited, but animal models and early human supplementation trials suggest there is a role for lutein and zeaxanthin in reducing oxidative damage and possibly preventing disease progression.

6. Cataracts

Age-related cataracts are another leading cause of blindness in the United States and worldwide. Treatments that can delay the progression of lens opacities have been studied extensively as this would reduce the burden of disease and reduce healthcare costs. Numerous studies have investigated the role of dietary nutrients in the development of cataracts or need for cataract surgery [125–130]. Specifically, antioxidants are of interest for their potential role in reducing oxidative damage leading to cataract formation. L and Z are the only carotenoids found within the human lens, although in significantly lower concentrations compared to the retina [131]. Approximately 74% of L and Z are located in the epithelium and cortex, where the lens is exposed to oxygen in the surrounding aqueous humor [132]. Proposed functions include preventing oxidative stress and lipid peroxidation in the epithelial cells.

The first trial to suggest a relationship between vitamins and minerals and cataractogenesis was a trial in Linxian, China, aimed at reducing the risk of esophageal and gastric cancer in a nutritionally deprived population. The initial trial compared multivitamin/mineral supplement and placebo, and the second trial compared 4 different supplements (retinol/zinc, riboflavin/niacin, ascorbic acid/molybdenum, and selenium/vitamin e/β-carotene). The authors found the risk of nuclear cataract progression over 5 to 6 years was decreased by at least 36% when supplementing with multivitamins [133]. However, the AREDS clinical trial found no effect of nutrients supplementation on the development of lens opacity. There was an equal proportion of subjects that underwent cataract surgery in treatment and control groups [134]. Similar results were reported for the Physicians' Health Study and the Women's Health Study. None of these studies have investigated the effects of L and Z.

While the trials mentioned above were underway, Hammond et al. demonstrated that higher levels of MPOD correlated with a more transparent lens. They hypothesized that higher concentrations of xanthophylls in the retina correlate with higher concentrations in the lens, impacting the rate of cataract progression [52]. Two early epidemiologic studies support these findings. Both demonstrated subjects with the highest quintile of L and Z had a 20% reduced risk of developing cataract compared to subjects in the lowest quintile [51, 135]. The Beaver Dam Eye Study reported similar results regarding L intake and nuclear cataract. They found that increased L intake at baseline decreased the risk of nuclear opacities among subjects younger than 65 by 50% compared to those with the lowest L intake. There was no significant influence in older subjects [136].

A retrospective study by Gale et al. demonstrated a 50% reduced rate of posterior subcapsular cataract in subjects with higher plasma L concentrations. High plasma vitamin C, vitamin E, and Z were not associated with a decreased risk [137]. Berendschot el al. examined serum antioxidant levels and MPOD and found an association between higher MPOD and a lower incidence and progression of cataracts [138]. Vu et al. studied 3,271 subjects in Australia and reported a 36% reduced rate of nuclear cataract in those with the top quintile of lutein and zeaxanthin intake combined. There was no correlation with cortical or posterior subcapsular cataracts [139].

Another population-based study (Pathologies Oculaires Liees a l'Age (POLA) study) investigating plasma L and Z levels of 899 subjects found those with the highest quintile of plasma Z had a significantly reduced risk of AMD, nuclear cataract, or any cataract [140]. There was no association between serum L or serum L and Z combined. While the numerous observational studies provide varied results on the impact of carotenoid supplementation on nuclear and posterior subcapsular cataracts, the general trend suggests there is a role for L and Z in prevention of cataract progression.

In a ten-year prospective study examining serum carotenoid levels in 35,551 female subjects, Christen et al. demonstrated that women in the highest quintile of L and Z intake had an 18% lower risk of developing cataract compared to those in the lowest quintile [141].

Subsequently a few prospective supplementation trials have investigated the role of carotenoids in the prevention of cataract formation. Omedilla et al. studied the visual effects of L supplementation on subjects with cataracts in a double-blind placebo-controlled study. Visual acuity and glare sensitivity were improved after 2 years of supplementation with L 15 mg. However, sample sizes of the treatment and study

groups were small ($n = 5$ and $n = 6$, resp.). They did not evaluate cataract progression [142]. AREDS2 evaluated cataract formation as a secondary outcome and is currently the largest clinical trial investigating carotenoid supplementation and cataract progression. They reported that L and Z supplementation had no statistically significant overall effect on rates of cataract surgery or vision loss related to cataract progression. While the epidemiologic studies provide encouraging data, there are a limited number of randomized controlled trials to support the role of L and Z supplementation in the prevention of cataractogenesis.

7. Conclusions

Three xanthophylls (L, Z, and MZ) are found selectively within retina, concentrated in the macula, and have been appropriately referred to as macular pigments. Epidemiological studies have revealed that low macular pigment levels are associated with higher risk of AMD. Several large observational studies demonstrated that high dietary intake and higher serum levels of L and Z are associated with a lower risk of AMD, especially late AMD. Randomized controlled clinical trials have revealed that supplementation of L and Z increases macular pigment density, improves visual function, and decreases the risk of progression of intermediate AMD to late AMD, especially neovascular AMD. Future studies may include additional assessments of the relationship between macular pigment and different genotypic and phenotypic forms of AMD, the optimum dosages of L, MZ, and Z, and the possible synergistic effects associated with supplementing with other nutrients. Current studies on preventive and therapeutic effects of L and Z on ROP, DR, and cataract have yielded varied results. Further investigations are necessary to fully understand the role of macular pigment in the prevention and treatment of eye diseases such as AMD, ROP, DR, and cataract.

Disclosure

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Photobiology of Lutein and Zeaxanthin in the Eye

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Lutein and zeaxanthin are antioxidants found in the human retina and macula. Recent clinical trials have determined that age- and diet-related loss of lutein and zeaxanthin enhances phototoxic damage to the human eye and that supplementation of these carotenoids has a protective effect against photoinduced damage to the lens and the retina. Two of the major mechanisms of protection offered by lutein and zeaxanthin against age-related blue light damage are the quenching of singlet oxygen and other reactive oxygen species and the absorption of blue light. Determining the specific reactive intermediate(s) produced by a particular phototoxic ocular chromophore not only defines the mechanism of toxicity but can also later be used as a tool to prevent damage.

1. Introduction

Lutein and zeaxanthin are antioxidants that accumulate in the lens and retina of the human eye [1–4]. These antioxidants protect ocular tissues against singlet oxygen and lipid peroxide damage [5]. Unfortunately, beginning with middle age, antioxidant protection is depleted and this leads to the formation of age-related cataracts and macular degeneration [6].

Increasing the intake of fruits and vegetables high in lutein and zeaxanthin [7–10] has been found to retard age-related cataracts and macular degeneration [11]. In addition, supplementation with lutein and zeaxanthin has been very effective at restoring these important ocular antioxidants [12, 13]. The level and distribution of these carotenoids can be directly and noninvasively measured in the human eye [14–16]. Increasing these carotenoids has been found not only to lower the risk for irreversible blindness [12, 17–20] but also to potentially improve cognitive function in the elderly [21–23].

Determining the specific reactive intermediate(s) produced by a particular phototoxic ocular chromophore not only defines the mechanism of toxicity but can also later be used as a tool to prevent damage. For instance, lutein and zeaxanthin prevent singlet oxygen damage [5], whereas N-acetyl cysteine has been shown to be particularly effective in quenching UV phototoxic damage and inflammation [24, 25]. In this review, we describe the underlying photobiological mechanisms involved in the induction of light-induced damage to the eye and the appropriate and inappropriate antioxidants to protect against such damage.

2. Ambient Radiation Ocular Damage

The primary factors that determine whether ambient radiation will injure the human eye are the wavelengths emitted from sunlight or a specific lamp [26] and received by ocular tissues; the intensity of the light; and the age of the recipient.

2.1. Wavelength Emitted from Source. Radiation from the sun emits varying amounts of UV-C (220–280 nm), UV-B (280–320 nm), UV-A (320–400 nm), and visible light (400–700 nm) [27]. Most of the UV-C and some short wavelengths of UV-B are filtered by the ozone layer [28]. Artificial light sources emit differing wavelengths of light depending on their spectral distribution [29]. UV radiation contains wavelengths shorter than visible light; the shorter the wavelength, the greater the energy and the greater the potential for biological damage. However, although the longer wavelengths are less energetic, they penetrate the eye more deeply [30].

2.2. Wavelength Transmission of Light through the Human Eye. In order for a photochemical reaction to occur in the eye, the light must be absorbed in a particular ocular tissue. The primate/human eye has unique filtering characteristics that determine in which area of the eye each wavelength of

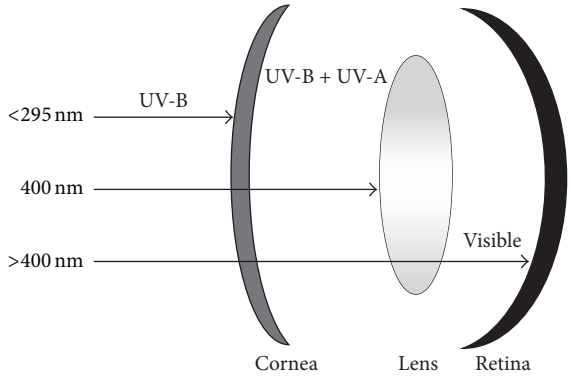

Figure 1: Wavelength transmission of the adult human eye.

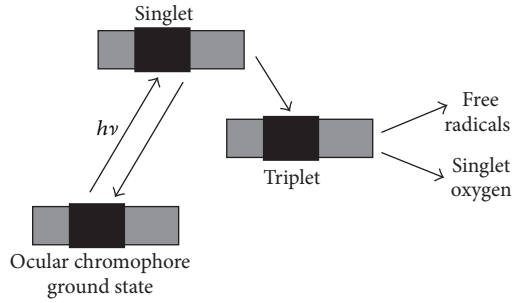

Figure 2: Photooxidation.

light will be absorbed [30]. All UV radiation of wavelengths shorter than 295 nm is filtered by the human cornea. This means that the shortest, most energetic wavelengths of light (all UV-C and some UV-B) are filtered out before they reach the human lens. Most UV light is absorbed by the adult lens, but the exact wavelength absorbed depends upon age [31] as shown in Figure 1. The very young human lens transmits UV radiation to the retina, while the elderly lens filters out much of the short blue visible light (400–500 nm) [32] before it reaches the retina. In adults, the lens absorbs UV-B and all the UV-A (295–400 nm); therefore only visible light (>400 nm) reaches the retina. Transmission also differs with species; the lenses of mammals other than primates transmit ultraviolet light longer than 295 nm to the retina [33]. Aphakia (removal of the lens) and implanted Intraocular Lenses (IOLs) after cataract surgery will also change the wavelength characteristics of light reaching the retina [34–37].

2.3. Intensity and Mechanism. Ocular damage from light can occur through either an inflammatory response or a photooxidation reaction. Acute exposure to intense radiation, for example, exposure to sunlight reflected from snow (snow blindness), or from staring at the sun during an eclipse [37] or directly staring at an artificial light source that emits UV-A or UV-B [38, 39] causes a burn in the eye similar to sunburn. This induces an inflammatory response in the eye. The initial insult to the tissue provokes a cascade of events that eventually results in wider damage to the cornea, lens, and/or retina [24, 40, 41].

Chronic exposure to less intense radiation damages the eye through a photooxidation reaction. In photooxidation reactions, a chromophore in the eye absorbs light and produces reactive oxygen species such as singlet oxygen and superoxide that damage ocular tissues as shown in Figure 2. The chromophore may be endogenous (natural) or exogenous (drug, herbal medication, or nanoparticle that has accumulated in the eye) [27]. If an ocular pigment is excited by ambient radiation to the excited state (singlet) but very quickly (in picoseconds) goes back to the ground state, it will safely dissipate the energy received [42].

3. Age and Endogenous Singlet Oxygen Chromophores

As the eye ages, chromophores which were once protective of the eye are modified and become phototoxic. The potential to produce singlet oxygen is measured as a quantum yield. Quantum yield measures the amount of an excited state produced by an amount of light energy used. The higher the number is, the more efficient the chromophore is at making a specific reactive oxygen species. For instance, a chromophore with a Quantum Yield for Singlet Oxygen of 0.10 is a very strong oxidant, while a chromophore with a Quantum Yield for Singlet Oxygen of 0.002 produces negligible amounts of singlet oxygen.

3.1. Lens. The primary function of the human lens is to focus light undistorted onto the retina. Although the transmission properties of most of the components of the eye are stable, the transmission properties of the lens change throughout life. The lens is clear for the first 3 years of life and then gradually develops yellow chromophores (3-hydroxy kynurenine and its glucoside). These are endogenous protective agents which absorb UV radiation and safely dissipate its energy [42].

As long as these chromophores are present, neither UV-A nor UV-B radiation reaches the retina, and in this way, the adult human retina is protected against normal levels of UV radiation [43]. However, children are at particular risk for UV damage to the retina because UV is directly transmitted to their retinas [33].

After middle age the protective chromophores 3-hydroxykynurenine and its glucoside are enzymatically converted into the phototoxic chromophores xanthurenic acid and xanthurenic glucoside [44, 45]. These xanthurenic derivatives absorb UV radiation, form triplet states, and produce singlet oxygen [46, 47] with a quantum yield of 0.170. These endogenous singlet oxygen photosensitizers cross-link lens protein [44] and induce apoptosis in lens epithelial cells [45]. There is also an increase in N-formylkynurenine [48, 49] in the lens; it is also an endogenous singlet oxygen photosensitizer. These quantum yields are seen in Table 1.

All of these phototoxic tryptophan derivatives are responsible for UV-A-induced damage to certain target genes [50]. With aging there is also a decrease in the production of antioxidants and antioxidant enzymes in the lens, which would normally quench these reactive oxygen species and

Table 1: Quantum yields for singlet oxygen for lenticular chromophores.

	Xanthurenic	NFK
Singlet oxygen	0.17	0.17
	3-OH Kyn	Kynurenine
Singlet oxygen	None	0.006

prevent damage to the lens. As a result of the increase in phototoxic chromophores concomitant with the loss of antioxidant protection, both the lens epithelial cells and lens proteins are injured, which results in the eventual clouding of the lens, commonly known as a cataract [44].

Phototoxic reactions, whether they are caused by endogenous or exogenous singlet oxygen photosensitizers, can cause a modification of certain amino acids (histidine, tryptophan, and cysteine) [51] and/or a covalent attachment of a sensitizer to cytosol lens proteins. In either case, the physical properties of the protein are changed, leading to aggregation and finally opacification (cataractogenesis). The covalently bound chromophore may now act as an endogenous sensitizer of singlet oxygen, producing prolonged sensitivity to light. Since there is little turnover of lens proteins this damage is cumulative. Any modification in the clarity of the lens impairs both vision and circadian function [52] and has a dramatic effect on retinal function.

3.2. Retina. The young retina is at particular risk for damage from UV exposure because the young lens has not as yet synthesized the yellow chromophores that prevent UV transmission to the retina [42, 43]; UV damage to the eye is cumulative and may increase the possibility of developing eye disorders (macular degeneration) later in life [26].

In addition to UV damage, short-wavelength blue visible light (430 nm) damages the retinas of those over 50 years of age through a photooxidation reaction with an accumulated chromophore, lipofuscin [30, 53–56].

Lipofuscin is a heterogeneous material composed of a mixture of lipids, proteins, and various fluorescent compounds. It is mainly derived from the chemically modified residues of incompletely digested photoreceptor outer segments [57]. Photoreceptor cells (rods and cones) shed their outer segments (disc shedding) daily to be finally phagocytosed (digested) by RPE cells. This RPE phagocytosis [58, 59] releases lipofuscin. With age, the rates of lipofuscin formation and disposal become unbalanced [60, 61], resulting in lipofuscin accumulation in the RPE [62, 63].

In response to short blue visible light (430 nm), lipofuscin efficiently produces singlet oxygen and lipid peroxy radicals; there is also some production of superoxide and hydroxyl radicals [64–67]. Lipofuscin is autofluorescent, and in previous studies [68] it was hypothesized that the main phototoxic component of lipofuscin was A2E [*N-retinylidene-N-retinylethanolamine*]. This is a pyridinium bisretinoid produced by the condensation of phosphatidylethanolamine with two moles of all-*trans*-RAL [*trans*-retinal]. However, current studies have proven that, rather than being a photooxidative agent, A2E forms the basis of a natural protective mechanism that removes the strong singlet oxygen photosensitizer all-*trans*- RAL [69] and keeps it from damaging the RPE cells by forming the very weak singlet oxygen inducer A2E [27, 30, 56, 70, 71]. While the quantum yield for lipofuscin [$\Phi = 0.09$] is relatively high, the quantum efficiency for the generation of singlet oxygen by A2E is very low ($\Phi = 0.0003$) [67, 72]. Table 2 gives the quantum yields of these retinal chromophores.

Table 2: Quantum yields for singlet oxygen for retinal chromophores.

	Lipofuscin	*trans*-Retinal	A2E
Singlet oxygen	0.09	0.24	.004

Further *in vivo* mouse studies [55] and human studies using matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI IMS) and FT-ICR tandem mass spectrometry confirm that although A2E accumulation in the retina may be hazardous, the damage done is not through a photooxidative mechanism [73–75]. Another mechanism for A2E toxicity to the retina may be the inhibition of phagolysosomal degradation of photoreceptor phospholipids [76], which would increase the production of lipofuscin [60, 77], a blue light singlet oxygen photosensitizer [66, 67], leading to damage to RPE cells. Because the rods and cones survival is dependent on healthy RPE, these primary vision cells will eventually die, resulting in a loss of (central) vision (macular degeneration) and other retinopathies. Another potential toxic mechanism of A2E that does not involve light is the activation of microglial phagocytosis of photoreceptor cells [78, 79].

4. Prevention of Damage by Lutein and Zeaxanthin

Lutein and zeaxanthin are ocular antioxidants of dietary origin [80]. These carotenoids are found in the human lens, [81], retinal pigment epithelium/choroid (RPE/choroid), the macula, the iris, and the ciliary body [2]. Recent clinical trials have determined that age- and diet-related loss of lutein and zeaxanthin enhances phototoxic damage to the human eye, while supplementation of these carotenoids has a protective effect against photoinduced damage to the lens and the retina. The use of improper carotenoids as an antioxidant (β-carotene) for quenching light damage to the eye as was used in the AREDS 1 clinical trial is not only ineffective because it does not pass blood ocular barriers but may be hazardous to human health [82, 83].

4.1. Structure of Carotenoids in relation to Their Function and Location in the Eye. Lutein and zeaxanthin have a 40-carbon basal structure, which include a system of conjugated double bonds (alternating double and single bonds) as shown in Figure 3. Chemical structures with extensive conjugated bonds absorb light in the visible range; lutein and zeaxanthin absorb blue visible light (400–500 nm).

Carotenoids that are substituted with hydroxyl (-OH) functional groups are known as xanthophylls. Lutein and

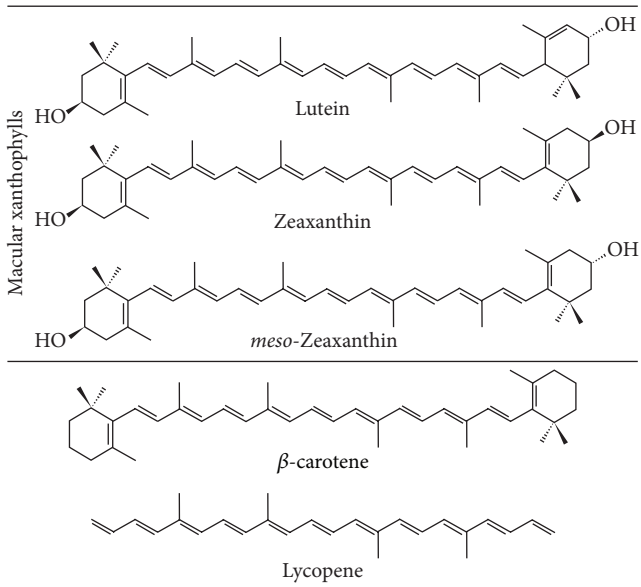

Figure 3: Structures of lutein, zeaxanthin, B-carotene, and lycopene.

zeaxanthin are xanthophylls, and their hydroxyl functional groups permit both lutein and zeaxanthin and their structural isomers to cross both blood-ocular and blood-brain barriers. Other carotenoids (β-carotene and lycopene) contain only carbon and hydrogen atoms and do not cross the blood-brain or ocular barriers [84].

4.2. Photochemical Mechanism of Protection. Ocular exposure to sunlight, UV, and short blue light-emitting lamps directed at the human eye can lead to the induction of cataracts and retinal degeneration. This process is particularly hazardous after the age of 40 because there is a decrease in naturally protective antioxidant systems and an increase in UV and visible light-absorbing endogenous phototoxic chromophores that efficiently produce singlet oxygen and other reactive oxygen species. The primary mechanism of damage is through a photooxidation reaction. In photooxidation reactions, phototoxic chromophores in the eye absorb light, are excited to a singlet and then a triplet state, and from the triplet produce free radicals and reactive oxygen species which in turn damage the ocular tissues [83, 85]. The phototoxic reactions damage can be prevented by the appropriate antioxidant quenchers as shown in Figure 4.

Lutein and zeaxanthin are naturally accumulating ocular antioxidants that efficiently quench both singlet oxygen and lipid peroxy radicals [86]. Zeaxanthin, with 11 conjugated double bonds, has a higher ability to quench singlet oxygen than lutein (10 conjugated double bonds) as shown in Figure 3 [87].

The synergistic action of several ocular antioxidants not only mimics the natural antioxidant protection of the eye (xanthophylls, vitamin E, vitamin C, and glutathione) but also has been found to be most effective. The highly successful synergistic action of zeaxanthin and vitamin E or vitamin C indicates the importance of the antioxidant interaction in efficient protection of cell membranes against oxidative

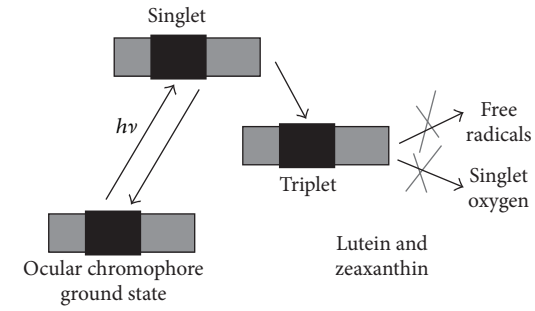

Figure 4: Photochemical mechanism of protection.

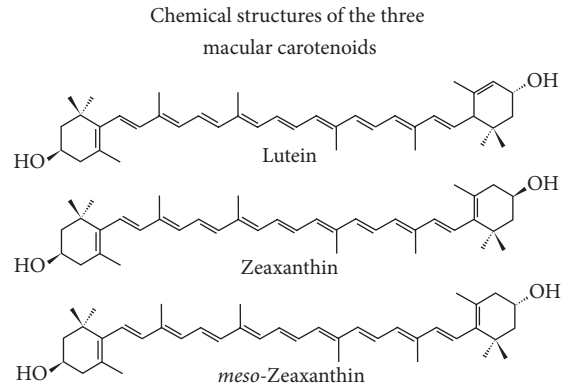

Figure 5: The structures of xanthophyll isomers.

damage induced by photosensitized reactions [88]. Increased levels of both lutein and zeaxanthin were found to reduce age-related nuclear cataracts [89, 90]. Clinical trials with a combination of lutein, zeaxanthin, and its isomer *meso*-zeaxanthin were found to be more protective of the retina than lutein or zeaxanthin alone [12, 91]. This is not surprising as the order of efficiency of quenching singlet oxygen is lutein < zeaxanthin < *meso*-zeaxanthin < all three combined [86, 92]. The structures of these xanthophylls are shown in Figure 5.

4.3. Photochemical Mechanism of Prooxidation and Damage by Antioxidants. Both lutein and zeaxanthin are very effective quenchers of singlet molecular oxygen ($^1O_2$) and lipid peroxy radicals. However, in the process, these carotenoids are oxidized to their corresponding radical cations. These cations must be reduced to regenerate the original carotenoid, allowing their reuse as an antioxidant. Vitamin E (α-tocopherol) is an antioxidant that can reduce oxidized carotenoids, but in turn, this leaves the tocopherol oxidized [93]. However, the oxidized vitamin E can be reduced and regenerated by vitamin C (ascorbic acid). Vitamin C can then be further reduced by copper and zinc [94, 95]. Without this appropriate combination of oxidizing and reducing agents, antioxidants become prooxidants and can potentially damage the retina and other organs as was found in the AREDS 1 clinical trial [82, 96].

Summary. It is essential to determine the specific reactive intermediate(s) produced by a particular endogenous or

exogenous photosensitizing agent in each compartment of the eye. This information not only defines the mechanism of toxicity but can also later be used as a tool to prevent damage. For instance, singlet oxygen that forms with the photooxidation of lipofuscin in the aged retina may be quenched by dietary or supplemental lutein and zeaxanthin, thereby preventing damage to the human retina. Using the proper sunglasses to block wavelengths that excite endogenous and exogenous ocular photosensitizers has been shown to limit the singlet oxygen damage to the eye. In the future, gene therapy for retinal dystrophies will be initiated. Ocular imaging techniques using confocal imaging or with adaptive optics are now available. These techniques will allow for direct verification of the physical and metabolic state of the human eye and accurate and digitalized monitoring of any therapeutic benefit of all new treatments against blindness including antioxidant supplements such as lutein and zeaxanthin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effects of Zeaxanthin on Growth and Invasion of Human Uveal Melanoma in Nude Mouse Model

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Uveal melanoma cells were inoculated into the choroid of nude mice and treated with or without intraocular injection of zeaxanthin. After 21 days, mice were sacrificed and the eyes enucleated. Histopathological analysis was performed in hematoxylin and eosin stained frozen sections. Melanoma developed rapidly in the control group (without treatment of zeaxanthin). Tumor-bearing eye mass and tumor mass in the control group were significantly greater than those in zeaxanthin treated group. Melanoma in the controlled eyes occupied a large part of the eye, was epithelioid in morphology, and was with numerous mitotic figures. Scleral perforation and extraocular extension were observed in half of the eyes. Melanomas in zeaxanthin treated eyes were significantly smaller with many necrosis and apoptosis areas and no extraocular extension could be found. Quantitative image analysis revealed that the tumor size was reduced by 56% in eyes treated with low dosages of zeaxanthin and 92% in eyes treatment with high dosages of zeaxanthin, as compared to the controls. This study demonstrated that zeaxanthin significantly inhibits the growth and invasion of human uveal melanoma in nude mice, suggesting that zeaxanthin may be a promising agent to be explored for the prevention and treatment of uveal melanoma.

1. Introduction

Uveal melanoma is the most common malignant intraocular tumor in adults. It accounts for 80% of all noncutaneous melanomas. Up to 50% of uveal melanoma patients die from metastatic disease within 10 years of initial diagnosis and it accounts for 13% of all deaths caused by melanoma [1, 2]. Chemotherapy has had little or no success in both primary and metastatic uveal melanoma [3]. Therefore, it is an urgent necessity to develop more efficient and novel therapeutic agents for improving the survival of uveal melanoma patients.

Zeaxanthin is a nontoxic xanthophyll present in fruits and leafy green vegetables. Zeaxanthin is an antioxidant and can absorb blue light like a yellow filter. It has been used as a nutrition supplement for patients with various ocular diseases [4–9]. In addition to these effects, zeaxanthin may influence the viability and function of cells through various signal pathways or transcription factors [7]. It has been reported that higher intake and higher blood levels of zeaxanthin appear to be associated with a lower risk of occurrence of various cancers [10].

Our previous study demonstrated that zeaxanthin inhibits the proliferation and induces apoptosis of human uveal melanoma cells through intrinsic apoptosis pathway [10]. To our best knowledge, the effects of zeaxanthin on uveal melanoma in experimental animal models have not been reported previously. In the present study, we examined the effects of zeaxanthin on the growth and invasion of human uveal melanoma in an immune-nude mouse model.

2. Materials and Methods

2.1. Experimental Animals. Athymic nude mice were purchased from the Charles River (Kinston, NY) and were incorporated into experiments at 6 weeks of age. This study was approved by the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center. The study complied with the principles of Laboratory Animal Care (NIH publication number 85-23, released in 1985) and also conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Uveal Melanoma Cell Line. Melanoma cell line C918 used in this animal study was isolated from a choroidal melanoma patient with liver metastasis at the University of Iowa. This cell line was provided by Dr. Robert Folberg (University of Illinois, Chicago) [10, 11]. C918 cell line is a highly invasive, metastatic, and aggressive melanoma cell line. Melanoma cells in this cell line are epithelioid cells in morphology with round nuclei and prominent nucleoli [10, 11]. Cells were cultured in RPMI 1640 Medium with 10% fetal bovine serum and 1% penicillin/streptomycin (all from Gibco; Grand Island, NY, USA). Cells were trypsinized and resuspended in the above medium and held on ice until inoculation.

2.3. Inoculation of Melanoma Cells and Zeaxanthin Treatment. Mice were randomly divided into three groups, zeaxanthin high dose group (14 eyes) zeaxanthin low dose group (14 eyes), and the control group (not treated with Zeaxanthin, 14 eyes). The methods for inoculation of tumor cells into the posterior segments of the eye have been described previously [12, 13]. Briefly, nude mice were anesthetized by intraperitoneal injection of a ketamine (final concentration; 10 mg/mL) and xylazine (final concentration; 1 mg/mL) mixture (0.01 mL/g mouse weight) and with Alcaine (proparacaine HCL) ocular surface anesthesia. Under a surgical microscope, a 30-gauge sharp needle was used to make two holes through the sclera, one into the intravitreal space to reduce intraocular pressure and one tangentially through the sclera into the subretinal space for injection. Uveal melanoma cells (1×10^6 cells) were injected through the second hole into the choroid and subretinal space using a 1.5 cm, 33-gauge blunt end microinjection needle (7803-05, Hamilton, Reno, NV). After the injection, eyes were covered with ophthalmic bacitracin ointment and buprenorphine was administrated for controlling of pain [12, 13]. Zeaxanthin (supplied by ZeaVision LLC; Chesterfield, MO, USA), solved with DMSO and diluted by PBS, was coinjected with the cellular suspension. The dosages were 114 μg in the low dose group and 570 μg in the high dose group. DMSO at the same levels as zeaxanthin treated group was injected into the eyes in the control group. The mice were kept under sterile conditions in laminar air-flow clean benches at room temperature (25–28°C) and a relative humidity of 55%. Sterile food pellets and water were given. Mice were examined by dissecting microscopy. One week after inoculation of melanoma cells, mice were treated by intravitreous injection of zeaxanthin. Mice were anaesthetized by isoflurane inhalation. Zeaxanthin was solved with DMSO at 50 mM and 57 μg of zeaxanthin was injected into vitreous of mice eyes with 31 G needle in zeaxanthin low group and 114 μg of zeaxanthin in high group. Control groups were injected with 2 μL of DMSO. After 21 days, mice were sacrificed by CO_2 asphyxiation and the eyes enucleated.

Table 1: Comparison of tumor-bearing eye mass in different groups.

Eye mass	Control	ZL	ZH
Mean (mg, mean ± SD)	21.3 ± 3.5	16.1 ± 3.4	12.4 ± 3.2
Percentage	100%	76%	58%

Control: mice not treated with zeaxanthin; ZL: zeaxanthin low group; ZH: zeaxanthin high group; one-way ANOVA, $p < 0.001$; ZL: control, $p < 0.001$; ZH: control, $p < 0.001$; ZL: ZH, $p < 0.05$.

2.4. Gross Examination and Measurement of Tumor Mass. Enucleated eyeballs were examined grossly. Extraocular tissue was removed and tumor-bearing eye mass determined. Tumor mass was calculated by the mass of the eye minus the average mass of control uninjected eyes.

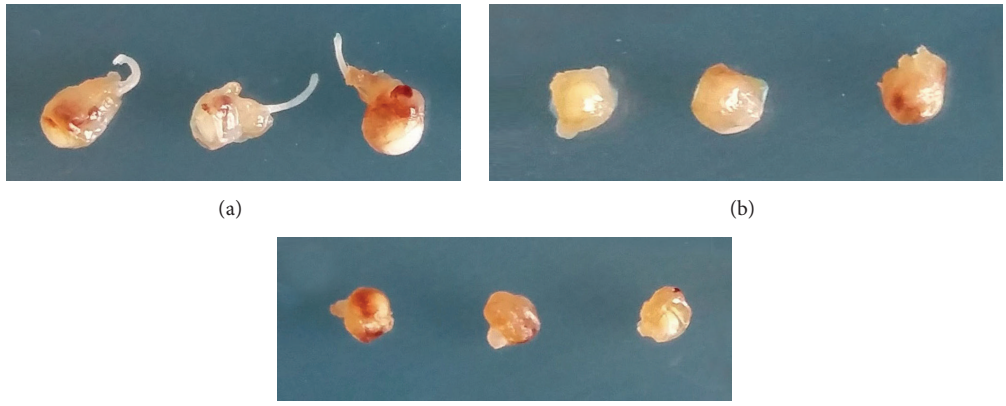
2.5. Microscopic Examination and Measurement of Tumor Size. The methods for the fixation of the eye have been reported previously [13]. Briefly, the tumor-bearing eyes were fixed overnight at 4°C in 4% paraformaldehyde in PBS (PFA/PBS), incubated in 30% sucrose/PBS overnight at 4°C, embedded in one-part 30% sucrose/PBS and two-part optimal cutting temperature compound (OCT; Miles Laboratories, Elkhart, IN), frozen, and sectioned at 5 to 7 μm [13]. Slides were fixed with Rapid Fixative (Poly Scientific R&D Corp., Bay Shore, NY). Hematoxylin and eosin (HE) staining was carried out using Leica HE Stainer (Leica Biosystems, Buffalo Grove, IL). HE stained sections were examined by a senior ophthalmic pathologist (CI) and a senior uveal melanoma researcher (DNH) to determine the presence and the extent of melanoma. Microscopic photography of eye section was taken using Olympus BX 41 light microscope (Shinjuku, Tokyo, Japan). Tumor size was determined by using of Adobe Photoshop CS6 [14, 15].

3. Results

3.1. Gross Examination and Tumor Mass. Gross examination revealed that the eyeballs were enlarged in controlled eyes (Figure 1(a)). Half of the eyes had visible extraocular extension of melanoma under stereomicroscope. Most of the zeaxanthin treated eyes were normal in size and were without extraocular extension of melanoma (Figures 1(b) and 1(c)).

Both the eye mass and tumor mass in the eyes of control group were significantly greater than those in eyes of zeaxanthin treated groups (Tables 1 and 2). Furthermore, the eye mass and tumor mass in the eyes of zeaxanthin low group were significantly higher than those in the eyes in zeaxanthin high group (Tables 1 and 2).

3.2. Microscopic Examination. Melanoma grew rapidly in the control eyes (melanoma cells inoculated without zeaxanthin treatment). Microscopic examination confirmed the presence



(a) (b) (c)

Figure 1: Photographs of enucleated mouse eyes inoculated with human uveal melanoma cells with or without zeaxanthin treatment. Eyes not treated with zeaxanthin (a) show enlargement of the eyeball and with visible extraocular extension of melanoma in some eyes. Eyes treated with zeaxanthin at low dosages (b) or high dosages (c) do not have extraocular extension of melanoma and most of eyeballs are normal in size.

of large melanoma xenografts filling the eyes of most control mice (Figure 2(a)). Half of the eyeballs had definitely scleral perforation and extraocular extension of melanoma cells (Figure 2(a)). Tumor cells in the mouse eye were mostly epithelioid in morphology with few spindle cells. Large nuclei and prominent nucleoli were observed in the tumor cells. Mitoses were common (Figure 3(a)).

Tumors in zeaxanthin low group were smaller than those of the control group (Figure 2(b)). The tumor cells were epithelioid or spindle in morphology with large nuclei and prominent nucleoli. Mitoses were observed occasionally. Necrotic or apoptotic tumor cells were present in part of the eyes. Scleral invasion and extraocular extension of melanoma have not been found in this group.

Tumors in zeaxanthin high group were much smaller than those of the zeaxanthin low group and control group (Figure 2(c)). Patches of definite melanoma cells could be found only in approximately two-thirds of eyes. No miotic figures were present. Necrotic or apoptotic tumor cells could be found in most eyes (Figure 3(b)) and no scleral invasion and extraocular extension of melanoma were present in this group.

3.3. Tumor Size. Tumor size was $1.69 \pm 0.95\,mm^2$ (mean ± standard deviation), $0.74 \pm 0.55\,mm^2$, and $0.13 \pm 0.13\,mm^2$ in the control group, zeaxanthin low group, and zeaxanthin high group, respectively. The difference of tumor size between these three groups was statistically significant ($p < 0.001$). The tumor sizes in the control group were significantly greater than those in both zeaxanthin high and low groups (both $p < 0.0001$), whereas the tumor size in eyes treated with high dosage of zeaxanthin was significantly smaller than that in mice treated with low dose of zeaxanthin ($p < 0.05$). Using the tumor size of control group as 100%, the tumor sizes in zeaxanthin low group and zeaxanthin high group were 43.9% and 7.7%, respectively (Table 3).

Table 2: Comparison of tumor mass in different groups.

Eye mass	Control	ZL	ZH
Mean (mg, mean ± SD)	12.3 ± 3.5	7.1 ± 3.4	3.4 ± 3.2
Percentage	100%	58%	32%

Control: mice not treated with zeaxanthin; ZL: zeaxanthin low group; ZH: zeaxanthin high group; one-way ANOVA, $p < 0.001$; ZL: control, $p < 0.001$; ZH: control, $p < 0.001$; ZL: ZH, $p < 0.05$.

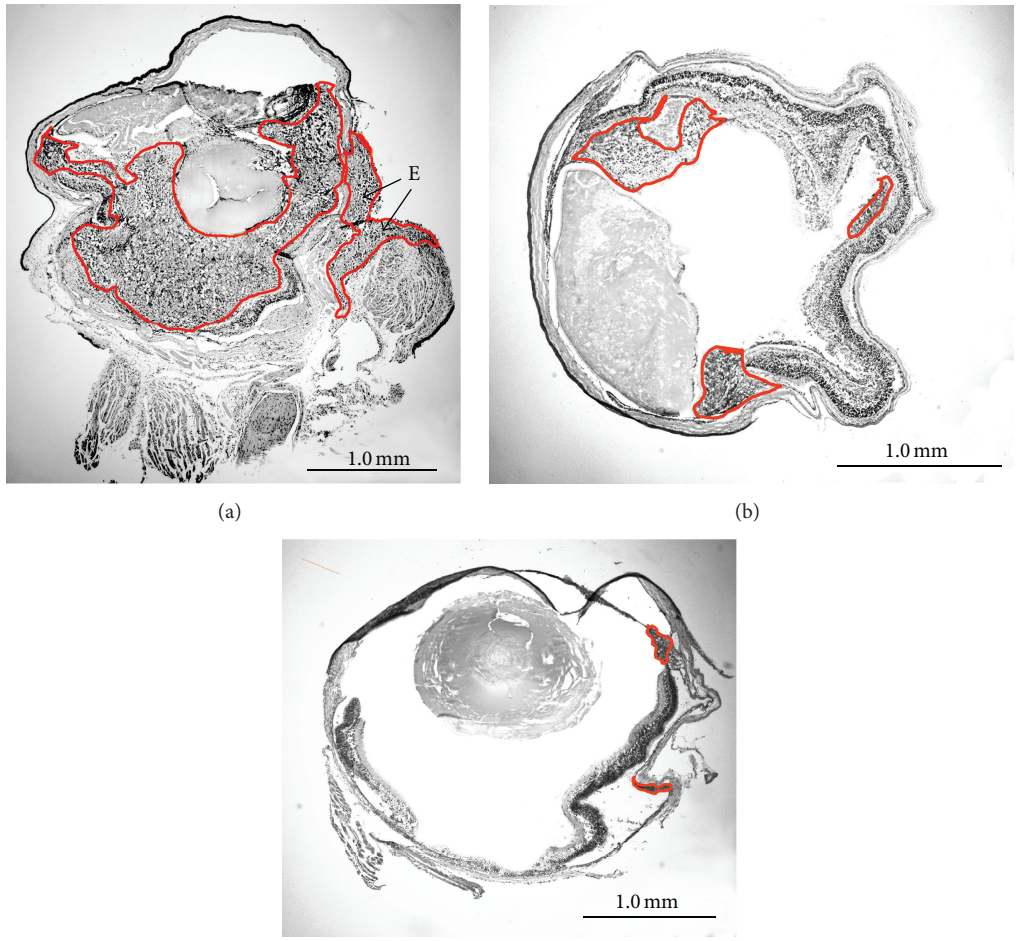
Table 3: Comparison of tumor size in different groups.

Tumor size	Control	ZL	ZH
Mean (mm^2, mean ± SD)	1.70 ± 0.95	0.74 ± 0.55	0.13 ± 0.12
Percentage	100%	44%	7.7%

Control: mice not treated with zeaxanthin; ZL: zeaxanthin low group; ZH: zeaxanthin high group; one-way ANOVA, $p < 0.001$; ZL: control, $p < 0.001$; ZH: control, $p < 0.001$; ZL: ZH, $p < 0.05$.

4. Discussion

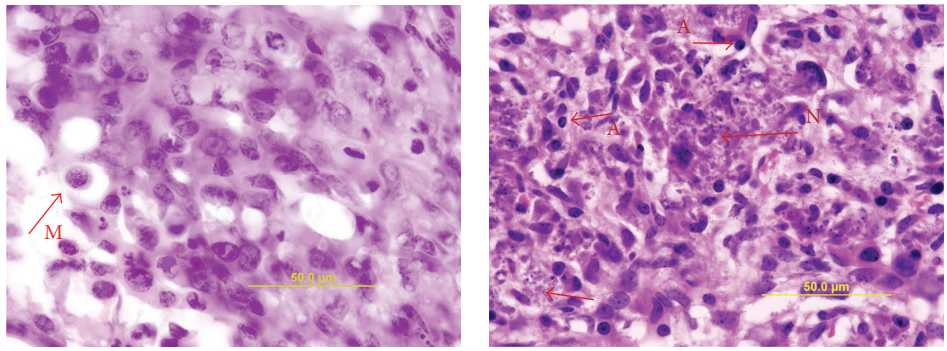
Our previous study demonstrated that zeaxanthin significantly inhibits the growth and induces apoptosis of human uveal melanoma cells *in vitro* [10]. However, the results of *in vitro* study may or may not accurately predicate the results obtained from *in vivo* study. For example, it has been reported that interleukin-1 (IL-1) may play a role in promoting uveal melanoma progression. However, inhibiting IL-1 with IL-1ra (an antagonist of IL-1) slows tumor growth only *in vivo* but not *in vitro* [16]. *In vitro* studies test only the direct effects of a medication on the tumor cells. *In vivo* studies test the effects of the medication on the production of various bioactive factors produced by tumor cells or neighbor cells, which in turn may affect the growth and invasion of tumor *in vivo* (paracrine effect), in addition to its direct effects. For example, angiogenesis plays an important role in the growth and progress of uveal melanoma. VEGF is a potent stimulator for angiogenesis. The results of several previous studies suggested that zeaxanthin inhibits the production



(a) (b)

(c)

Figure 2: Microscopic photographs of mouse eyes inoculated with human uveal melanoma cells with or without zeaxanthin treatment (original magnification ×4). Eyes were enucleated and stained with hematoxylin-eosin in frozen sections. Tumor was marked by red outlines. In the eye not treated with zeaxanthin (control eye), tumor fills large part of the eyeball (a) with scleral perforation and extraocular extension of melanoma (arrow E). Tumor in eye treated with low dosage of zeaxanthin (b) is smaller than that of the control eye. Tumors in eye treated with high dosage of zeaxanthin (c) are much smaller than that of eye treated with low dosage of zeaxanthin and control eye.



(a) (b)

Figure 3: Microscopic photographs of mouse eyes inoculated with human uveal melanoma cells with or without zeaxanthin treatment (original magnification ×100). Eyes were stained with hematoxylin-eosin in frozen section and observed under oil lens. Tumor cells in the eye not treated with zeaxanthin are mostly epithelioid in morphology with few spindle cells. Large nuclei and prominent nucleoli were observed in the tumor cells (a). Mitoses are common (arrow M). In eyes treated with high dosage of zeaxanthin, necrotic (arrow N) or apoptotic tumor cells (arrow A) can be observed. No mitotic figures are present (b).

of VEGF by various ocular cells or inflammatory cells [17–21]. This may reduce the angiogenesis and results in the inhibition of the growth of uveal melanoma *in vivo*. *In vivo* studies are an important component of preclinical evaluation of any therapeutic approach to the clinical management of patients with uveal melanoma. For this reason, we designed and carried out the current study for testing the effects of zeaxanthin on the growth and invasion of human uveal melanoma *in vivo* using a nude mouse model.

Numerous animal models have been developed and used in the *in vivo* study of uveal melanoma. The melanoma cells used could be experimental animal melanoma cells (Greene hamster or B16 mouse melanoma cell lines) [22] or human melanoma cells [22–44]. Use of human melanoma cells has the advantage of avoiding the species difference and may more accurately reflect the biological behavior of uveal melanoma in the patients. Tumor cells are antigenic and can induce immune rejection of inoculated tumor graft, especially in transplantation of human tumor cells into experiment animals (xenografts). It has been reported that immune privilege is present in the anterior chamber of the eye, permitting melanoma xenografts to survive if inoculated into the rabbit's anterior chamber [22]. However, since this privilege is incomplete, therefore, in order to grow human melanoma cells in an experiment animal model, it is necessary to use animals incapable of mounting immune rejection to xenograft tumor cells [23–44]. This can be achieved by using immune inhibitory drugs [41–44] or inoculate tumor cells into immune incompetent nude mice [23–40]. The nude mouse is a hairless mutant born without a thymus, which causes a severe defect in cellular immunity, that is, in the transformation process of T lymphocyte precursors to functional T cells. Nude mice have the ability to accept human melanoma cells while preserving many human uveal melanoma characteristics [32]. Therefore, uveal melanoma xenografts in nude mice are a widely used model for studying melanoma growth and response to therapeutic interventions [23–40].

Melanoma cells can be inoculated intraocularly (orthotopic model) [22–31] or subcutaneously (heterotopic model) [32–38]. Tumors transplanted to heterotopic sites may not display biological behavior consistent with the original tumor. The difference of biological behaviors between orthotopic and heterotopic transplantations may be related to the influence of local organ-specific factors. Therefore, the importance of orthotopic, rather than heterotopic, transplantation cannot be overemphasized [24].

The site for intraocular inoculation of melanoma cells could be the anterior part (anterior chamber) [23–25, 29–31, 44] or the posterior part of the eye (vitreous, choroid, subretinal, or suprachoroidal space) [26–28, 30, 45, 46]. Uveal melanoma may arise clinically in the iris (anterior part) or in the ciliary body/choroid (posterior part). Most iris melanomas are relatively benign and only account for approximately 5% of uveal melanoma, which is different from the relatively poor prognosis for patients with melanoma of the ciliary body or choroid [45]. Therefore, we selected the inoculation of melanoma cells into the posterior segment. We ideally inoculated the cells into the choroid; however, in such tiny eyes it is virtually impossible to direct the cells only into the choroid; some cells may enter the suprachoroidal space, subretinal space, or the vitreous [45].

Human uveal melanoma cells used in the present study are the C918 melanoma cell line, which was isolated from a choroidal melanoma patient with liver metastasis. Melanoma cells in this cell line are epithelioid in morphology with round nuclei and prominent nucleoli [10, 11]. The morphologic phenotype of a uveal melanoma provides an important indication of malignancy. The Challender classification scheme categorizes uveal melanoma cellular components as either spindle A, spindle B, or epithelioid. A uveal melanoma predominance of epithelioid components carries significantly greater malignant potential and a shorter patient survival time than melanomas comprised largely spindle cellular elements [47]. C918 cell line is a highly invasive, metastatic, and aggressive melanoma cell line *in vitro* and has been used previously in several animal studies of uveal melanoma [28, 32, 33]. In the present study, melanoma developed rapidly and had potent invasive capacity in mice inoculated with C918 cells and these cells also showed the epithelioid morphology, indicating that this melanoma model reflects the biological behavior of uveal melanoma *in vitro* and in patients with uveal melanoma quite well.

In the present study, melanoma developed in mice without the treatment of zeaxanthin. Melanoma grew rapidly to occupy a large part of the eye and extraocular extension occurred in one-half of the eyes. In zeaxanthin treated groups, zeaxanthin was injected to the posterior part of the eye twice with a total dosage of 171 μg (zeaxanthin low group) or 684 μg (zeaxanthin high group). Zeaxanthin treatment significantly inhibited the growth and invasion of melanoma in nude mice eyes, especially in zeaxanthin high group. Gross examination and histopathological examination found that the tumor mass and size in zeaxanthin treated eyes were significantly less than those in the controls and the extraocular extension only occurred in eyes without the treatment of zeaxanthin. Numerous necrotic or apoptotic tumor cells could be found in eyes treated with zeaxanthin. Quantitative histopathological study demonstrated that the tumor size was reduced by 56% in zeaxanthin low group and 92% in zeaxanthin high group as compared to the control group. All of these results are consistent with those in our previous *in vitro* study which demonstrated the growth inhibition and apoptosis induced effects of zeaxanthin on cultured human uveal melanoma cells.

The dosages used in the animal study have been calculated and compared to the dosages used in the *in vitro* study. In the low dosage group of the animal study, 1×10^6 cells were injected into the eye, and the dosage of zeaxanthin used was 114 μg (first injection) added to 57 μg (second injection); therefore, the total dosage used was 171 μg of zeaxanthin per 1×10^6 cells. The tumor mass in eyes treated with this dosage was 58% of the control (reduced by 42%). In the high dosage group, the total dosages used were 570 μg (first injection) added to 114 μg (second injection); therefore, the total dosage used was 684 μg of zeaxanthin per 1×10^6 cells. The tumor mass in eyes treated with this dosage was 32%

of the control (reduced by 68%). In the *in vitro* study, the ID50 dosage of zeaxanthin in C918 cells was 28.7 μM [10]. In that study, 5×10^3 cells were tested in 96 wells with 200 μL of culture medium containing 28.7 μM zeaxanthin, which equals 3.26 μg of zeaxanthin [10]. Therefore, the dosage of zeaxanthin that can reduce the cell viability to 50% of the control was 3.26 μg zeaxanthin/5×10^3 cells, which equals 652 μg of zeaxanthin per 1×10^6 cells, slightly lower than that used in the high dosage group but greater than in the low dosage group in the animal study. Therefore, the dosages in the animal study are consistent with the dosages used in the *in vitro* study.

It has been reported that zeaxanthin can inhibit the growth and/or induced apoptosis in lymphoma, breast cancer, and neuroblastoma cells *in vitro* [48, 49]. Zeaxanthin had moderate effects in reversing multidrug resistance in mouse lymphoma and human breast cancer cells [48, 50]. Zeaxanthin inhibited the invasion of rat ascites hepatoma cells *in vitro* [51]. Baudelet et al. reported that the extracts of the Glaucophyte *Cyanophora paradoxa* could inhibit the growth of cutaneous melanoma, mammary carcinoma, and lung adenocarcinoma cells *in vitro*. Further analysis indicated that zeaxanthin was one of the three main pigments or derivatives responsible for the cytotoxicity of *Cyanophora paradoxa* fractions in cancer cells [52]. For the experimental animal study, Firdous et al. reported that oral administration of meso-zeaxanthin, another xanthophyll carotenoid, could significantly increase tumor latency period in 3-methylcholanthrene-induced sarcoma in mice. Survival of tumor-bearing mice was significantly increased by meso-zeaxanthin treatment [53]. All of these results are consistent with the results from the present study.

In conclusion, we have demonstrated in the present *in vivo* study that intraocular administration of zeaxanthin significantly inhibits the growth and invasion of human uveal melanoma in nude mice. The results of the present study may be useful for the development of a novel therapeutic approach to the management of uveal melanoma, especially for the combination of zeaxanthin with other aggressive uveal melanoma treatments.

Conflict of Interests

Dr. Dennis L. Gierhart is the chairman of ZeaVision, LLC. Dr. Rosen and Dr. Hu have intellectual property related to malignant tumors and zeaxanthin. None of the other authors have financial interests relevant to the contents of this paper.

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Review Article

Management of Ocular Diseases Using Lutein and Zeaxanthin: What Have We Learned from Experimental Animal Studies?

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Zeaxanthin and lutein are two carotenoid pigments that concentrated in the retina, especially in the macula. The effects of lutein and zeaxanthin on the prevention and treatment of various eye diseases, including age-related macular degeneration, diabetic retinopathy and cataract, ischemic/hypoxia induced retinopathy, light damage of the retina, retinitis pigmentosa, retinal detachment, and uveitis, have been studied in different experimental animal models. In these animal models, lutein and zeaxanthin have been reported to have beneficial effects in protecting ocular tissues and cells (especially the retinal neurons) against damage caused by different etiological factors. The mechanisms responsible for these effects of lutein and zeaxanthin include prevention of phototoxic damage by absorption of blue light, reduction of oxidative stress through antioxidant activity and free radical scavenging, and their anti-inflammatory and antiangiogenic properties. The results of these experimental animal studies may provide new preventive and therapeutic procedures for clinical management of various vision-threatening diseases.

1. Introduction

Zeaxanthin and lutein are two carotenoid pigments that belong to the xanthophylls subclass. They cannot be synthesized in mammals and must be obtained from the diet for distribution to various tissues, especially the retina [1–3]. Zeaxanthin and lutein are most dense at the center of the fovea in the yellowish pigmented area called the macula lutea and are referred to as macular pigment. The macular pigment is tissue protective, acting via antioxidant, anti-inflammatory, and light-screening properties [1–6]. Low systemic and retinal levels of lutein and zeaxanthin are adversely associated with the risk of age-related macular disease (AMD) and diabetic retinopathy [7–10]. Various observational and interventional studies have suggested that the supplementation of lutein and zeaxanthin might reduce the risk of AMD [1, 3, 11–18].

Numerous reports have been published studying the effects of lutein and zeaxanthin on various ocular diseases (AMD, diabetic retinopathy and cataract, ischemic/hypoxia induced retinopathy, light damage of the retina, retinitis pigmentosa, retinal detachment, and uveitis) in experimental animal models [19–51] (Table 1). In this review, we describe the effects of lutein and zeaxanthin and their underlying molecular mechanisms in experimental animal models for various ocular diseases and explore the role of these xanthophylls in the clinical management of vision-threatening diseases.

2. Diabetic Retinopathy

Diabetic retinopathy is one of the most common causes of blindness in developed countries. Despite the tighter control of blood glucose and the advances in treatment of diabetic eye diseases, rates of diabetic retinopathy in the United States have increased by 89% over the last decade. Therefore, search for the novel procedures for the prevention and treatment of diabetic retinopathy is urgently required [1].

Table 1: Effects of lutein and zeaxanthin on experimental animal models for ocular diseases.

	Authors	Year	Animal	Methods for inducing disease	Pathologic changes	Xanthophylls used	Effectiveness
Diabetic retinopathy	Muriach et al. [20]	2006	Mice	Diabetes Alloxan-induced	NFκB, ROS & MDA (+) ERG damage Antioxidant (−)	L (oral)	Prevent all changes
	Sasaki et al. [21]	2010	Mice	Diabetes STZ-induced	ROS & extracellular signal-regulated kinase (+) Brain-derived neurotrophic factor (−) ERG damage RGC apoptosis	L (oral)	Prevent all changes
	Kowluru et al. [22]	2008	Rats	Diabetes STZ-induced	ROS, VEGF, ICAM & reactive nitrogen species (+) Antioxidant (−)	Z (oral)	Prevent all changes
	Kowluru et al. [23]	2014	Rats	Diabetes STZ-induced	ERG damage NFκB, VEGF & IL-1β (+) Cell apoptosis	Z, L, and others (oral)	Prevent all changes
	Tang et al. [24]	2011	Mice	db/db mice	RPE & RGC damage Mitochondria dysfunction	Wolfberry (L Z) (oral)	Prevent all changes
	Yu et al. [25]	2013	Mice	db/db mice	VEGF (+) antioxidant (−) Mitochondria dysfunction	Wolfberry (L Z) (oral)	Prevent all changes
Diabetic cataract	Arnal et al. [26]	2009	Rats	Diabetes STZ-induced	Lens opacity MDA (+) GSH (−)	L (oral)	Prevent all changes except GSH
Age-related macular degeneration	Fernández-Robredo et al. [29]	2009	Mice	ApoE−/− mice	RPE & Bruch's membrane changes VEGF (+)	Z & other antioxidants (oral)	Prevent all changes at high doses
	Fernández-Robredo et al. [28]	2013	Mice	ApoE−/− mice	RPE & Bruch's membrane changes MMP-2 & VEGF (+)	L & others or L alone (oral)	Only effective in L with others
	Ramkumar et al. [30]	2013	Mice	DKO mice	RPE & PR damage COX-2, IL-1, iNOS & VEGF (+)	L, Z, and others (oral) (AREDS2)	Prevent all changes
	Yu et al. [31]	2012	Mice	β5−/− mice	RPE & PR damage Lipofuscin (+) Actin solubility (−)	L, Z, and others (oral)	Prevent all changes
	Izumi-Nagai et al. [32]	2007	Mice	LASER-induced CNV	CNV NFκB, VEGF, ICAM-1 & MCP-1 (+)	L (oral)	Prevent all changes at high doses
Ischemic/hypoxia retinopathy	Li et al. [38]	2009	Mice	Carotid artery block	RGC & ERG damage Muller cells activation ROS (+)	L (IP)	Prevent all changes
	Li et al. [37]	2012	Mice	Carotid artery block	ERG damage ROS (+) Muller cells activation	L (IP)	Prevent all changes
	Choi et al. [40]	2006	Rats	High IOP	Cell death nNOS & COX-2 (+)	L (IP or IVt)	Prevent all changes
	Dilsiz et al. [39]	2006	Rats	High IOP	MDA & caspase-3 (+) GSH (−)	L (IC)	Prevent all changes

Table 1: Continued.

	Authors	Year	Animal	Methods for inducing disease	Pathologic changes	Xanthophylls used	Effectiveness
Light damage retinopathy	Thomson et al. [42]	2002	Quails	Intensive white light	PR apoptosis	Z & L (oral)	Prevent all changes
	Sasaki et al. [43]	2012	Mice	Intensive white light	PR apoptosis & DNA damage, ERG damage	L (oral)	Prevent all changes
Retinitis pigmentosa	Miranda et al. [45]	2010	Mice	rd1 mice	PR apoptosis GSH & GPx (−) MDA (+)	L, Z & other antioxidants or L & Z alone (oral)	Effective only in L, Z & other antioxidants
Retinal detachment	Woo et al. [44]	2013	Rats	Hyaluronate subretinal injection	PR apoptosis Caspase-8 & caspase-3 (+) Glial cell activation	L (IP)	Prevent all changes
Uveitis	Jin et al. [48]	2006	Rats	LPS (SCI)	Proinflammatory factors, NFκB & iNOS (+)	L (IV)	Prevent all changes
	He et al. [49]	2011	Mice	LPS footpad injection	NO & MDA (+) SOD & GPX (−)	L (oral)	Prevent all changes
	Sasaki et al. [50]	2009	Mice	LPS (IP)	PR & ERG damage ROS, IL-6, STAT-3 (+)	L (IC)	Prevent all changes
Misc.	Zou et al. [51]	2014	Rats	Normal	—	Z (oral)	Akt, Nrf2 & GSH (+) Peroxidation (−)

L: lutein; Z: zeaxanthin; IC: subcutaneous injection; IP: intraperitoneal injection; IV: intravenous injection; IVt: intravitreous injection. (−): decreased; (+): increased.
For other abbreviations, see the text.

Zeaxanthin and lutein have been reported to have therapeutic effects in experimental animal models of diabetic retinopathy through their antioxidant and anti-inflammation effects. Injection of streptozotocin (STZ), a compound that is toxic to the insulin-producing beta cells of the pancreas, can be used to produce an experimental model of diabetes mellitus in mice, which will go on to develop diabetic retinopathy. Alloxan injection can also induce diabetes in experimental animals [19–23]. db/db mice spontaneously develop diabetes [24, 25].

2.1. STZ- or Alloxan-Induced Diabetic Retinopathy. Muriach et al. reported the effects of lutein on diabetic retinal changes in alloxan-induced diabetic mice [20]. These mice showed an increase of malondialdehyde (MDA, a marker for lipid peroxidation) and nuclear factor κB (NFκB) levels, along with a decrease in glutathione (GSH) levels and glutathione peroxidase (GPx) activities in the retina. Electroretinography (ERG) b-wave amplitude decreased in diabetic mice. Lutein [70% purity, 0.2 mg/kg body weight (wt), administered by stomach tube] was used as daily treatment started on day 4 after alloxan injection and lasted until the end of the experiment. While lutein treatment did not alter the hyperglycemic status of alloxan diabetic mice, supplementation of lutein restored levels/activities of NFκB, MDA, GSH, and GPx in the retina. ERG b-wave amplitude was also restored to normal after lutein treatment, suggesting that although the high blood glucose levels were not normalized, biochemical and functional changes in the diabetic retina were improved by supplementation of lutein [20].

Sasaki et al. studied the effects of lutein on diabetic retinopathy which developed in STZ-induced diabetic mice [21]. Extracellular signal-regulated kinase signal pathway was activated in diabetic retina. Brain-derived neurotrophic factor was depleted. ERG showed a decrease of oscillatory potentials, which reflected degeneration of neurons in inner retina. At the later stage, the thicknesses of the inner plexiform layer and inner nuclear layer (INL) were decreased; the numbers of retinal ganglion cells (RGC) and inner retinal cells were reduced, together with the appearance of apoptotic cells. Reactive oxygen species (ROS) levels in the retina were also increased, which may play a role in the development of degenerative changes in the retina [21]. Mice were constantly fed either a lutein-supplemented diet [0.1% of lutein (wt/wt) added to the mouse chow] or a control diet (the same chow without addition of lutein) from the onset of diabetes until the end of the experiment. Lutein again did not affect the body weight and blood glucose levels. However, ROS levels in the retina were reduced and all diabetic pathologic changes in the retina could be avoided by the supplementation of lutein, suggesting that lutein protects the retina against diabetic damage mainly through its antioxidant effect [21]. Lutein may have potential therapeutic value in protecting visual function in patients with diabetes [19, 21].

The protective effects of zeaxanthin against diabetic retinal changes have been studied in STZ-induced diabetes in rats by Kowluru et al. [22]. Rats received powdered diet with or without supplementation of 0.02% or 0.1% zeaxanthin (equal to 8.4 and 44 mg/d) soon after induction of diabetes. The zeaxanthin levels in the retinas of normal and diabetic rats were in the range of 130 to 180 pg/mg protein and were elevated to approximately 300 and 1,500 pg/mg protein in diabetic rats receiving 0.02% and 0.1% zeaxanthin, respectively. Zeaxanthin also did not lower the high blood glucose levels in diabetic rats. In diabetic rats, the retinal levels of lipid peroxide, oxidatively modified DNA, nitrotyrosine (a parameter of oxidative stress), inducible NO synthase (iNOS), vascular endothelial cell growth factor (VEGF), and intercellular adhesion molecule- (ICAM-) 1 were all significantly increased; the expression of electron transport complex III was decreased [22]. Supplementation of zeaxanthin significantly decreased elevation of lipid peroxide, oxidatively modified DNA, nitrotyrosine, iNOS, VEGF, and ICAM-1; and the levels of electron transport complex III were increased to normal [22]. The nature defense system against oxidative stress, Mn superoxide dismutase (SOD), and GSH were also decreased significantly in the diabetics retinas [22]. Zeaxanthin supplementation increased retinal MnSOD levels to normal but the GSH levels were not completely recovered [22]. The effects of zeaxanthin were comparable in the two groups supplemented with different dosages of zeaxanthin. This study suggested that zeaxanthin has the potential to inhibit the development of diabetic retinopathy via ameliorating oxidative stress and inhibition of VEGF expression and inflammation, which raises the possibility that it could be used as an adjunct therapy to help prevent vision loss in patients with diabetes.

Effects of zeaxanthin, lutein, and other nutrients on the development of diabetic retinopathy in STZ-induced diabetic rats were recently reported by Kowluru et al. [23]. Following induction of diabetes in male rats by injection of STZ, ERG showed decreasing of the amplitudes of both a- and b-waves. Apoptotic cells increased in the retinal vasculature and degenerative capillaries could be found in the retina. Diabetes in rats caused a significant activation of retinal NFκB with increase of VEGF and interleukin- (IL-) 1β levels in the retina [23]. Supplementation with zeaxanthin (2 mg/d), lutein (1 mg/d), lipoic acid, omega-3 fatty acids, and other nutrients ameliorated diabetes-induced capillary cell apoptosis, prevented ERG changes and activated NFκB, and ameliorated increased levels of VEGF and IL-1β [23]. However, the severity of hyperglycemia in diabetic rats was not decreased by the supplementation, suggesting that the beneficial effects of these antioxidants on diabetes-induced retinal pathology are not due to control of hyperglycemia [23].

2.2. Diabetic Retinopathy in Spontaneous Diabetic Mice (db/db Mice). In an experimental diabetes type 2 model, diabetes occurs spontaneously in the db/db mice [24, 25]. At the early stage of diabetes, retinal blood microvessels are still intact, but hyperglycemia-induced cellular oxidative stress occurs, which causes mitochondrial dysfunction, endoplasmic reticulum stress, thinning of INL and photoreceptor layers,

apoptosis of RGC cells, and loss of retinal pigment epithelium (RPE) layer integrity [24]. Supplementation of wolfberry, a Chinese traditional medication containing high levels of zeaxanthin (1.76 mg/gm fruit) and lutein (0.05 mg/gm fruit), prevented retinal damage in diabetic mice. Zeaxanthin and lutein were able to mimic wolfberry's protective effect in cultured RPE cells, suggesting that they were the active agents in wolfberry's protection retinal cells against a high glucose challenge [24].

The effects of wolfberry on diabetic eye changes were investigated in a diabetic mouse model (db/db mice) by Yu et al. [25]. Wolfberry did not lower the fasting blood glucose level in db/db mice. However, in diabetic mice which showed lower levels of lutein and zeaxanthin in the retina compared with normal controls, wolfberry treatment significantly elevated zeaxanthin and lutein levels in both the retina and liver [25]. Lowered expressions of retinal scavenger receptor class B type I, glutathione S-transferase Pi, and β,β-carotene 9′,10′-oxygenase proteins were also observed in db/db mice; wolfberry elevated these protein levels back to normal, suggesting that diabetes might cause inhibition of uptake, binding and transport, and degradation of lutein and zeaxanthin within the retinal cells [25].

Hypoxia and angiogenic factors such as hypoxia-inducible factor-1α and VEGF and mitochondrial stress biomarker were significantly increased in the retinas of diabetic mice [25]. Wolfberry reversed these changes and increased mitochondrial biogenesis. Wolfberry also reversed mitochondrial dispersion in the RPE, increased mitochondrial copy number, and elevated citrate synthase activity [25]. All of these findings suggest that, in diabetic mice, hyperglycemia and subsequent hypoxia were causative factors leading to changes in lutein and zeaxanthin metabolic homeostasis via inhibition of metabolic gene expression producing mitochondrial dysfunction and subsequent diabetic retinal pathology [25].

3. Diabetic Cataract

Diabetic cataract is a common eye complication of diabetes. The effects of lutein on the prevention of diabetic cataract have been studied in a rat model [26]. Persistent hyperglycemia, high glycated hemoglobin, and loss of body weights were observed in rats treated with STZ [26]. Supplement of lutein (0.5 mg/kg orally) did not significantly affect blood glucose, glycated hemoglobin, or body weights in diabetic rats. Diabetic rats developed lens opacity in 81% (13/16) of eyes and mature cataract occurred in 7/16 eyes. In diabetic rats treated with lutein, lens opacity only developed in 38% (6/16) eyes and no mature cataracts were observed [26]. The lens in diabetic rats showed an increase of MDA levels and a decrease of GSH levels. Lutein treatment reduced the MDA levels but did not raise GSH, indicating that lutein may prevent the development of diabetic cataract through the inhibition of lipid peroxidation [26]. This study suggested that lutein might be used as an adjuvant treatment combined with the proper glycemic control to prevent the occurrence of diabetic cataract [26].

4. Experimental Animal Models Mimicking AMD

AMD is a major cause for irreversible blindness among the elderly in the Western world. The effects of lutein/zeaxanthin on AMD have been studied in several experimental animal models that mimic pathological changes in AMD [28–32]. The effects of lutein and zeaxanthin deficiency on the RPE and macula have been studied in nonhuman primates [33–35].

4.1. Apolipoprotein E-Deficient Mice. AMD is a multifactorial disease. Abnormal lipid levels and oxidative stress may contribute to the development of AMD [28, 29]. Apolipoprotein E-deficient mice (apoE−/−) are a well-established experimental animal model of hypercholesterolemia and display morphological and ultrastructural alterations in RPE similar to those in human AMD.

The effects of zeaxanthin on ocular changes in (apoE−/−) mice have been studied [29]. ApoE(−/−) mice showed elevation of plasma total cholesterol and triglycerides level. In the apoE−/− mice eyes, vacuoles in the RPE, basal laminar deposits, and an increase in Bruch's membrane thickness could be observed associated with an elevation of VEGF levels in the retina [29]. Supplementation with zeaxanthin (4 g/kg of diet) and other antioxidants (vitamin C, vitamin E, and zinc) significantly increased the retinal and liver zeaxanthin levels, decreased the VEGF levels in the retina-choroid, and improved the status of Bruch's membrane but did not change plasma cholesterol levels [29].

The effects of supplementation of lutein alone (0.093 mg/kg/d by gastroesophageal cannula) or lutein with multivitamins and GSH (vitamin A, vitamin C, vitamin E, various vitamin B compounds, β-carotene, GSH, and various minerals, such as zinc and selenium) on the ocular changes in apoE−/− mice were tested by the same groups of authors [28]. ApoE−/− mice showed higher plasma and retinal lipid peroxidation, with increased VEGF expression and matrix metalloproteinase- (MMP-) 2 activity in the retina-choroid, associated with ultrastructural alterations similar to AMD, such as basal laminar deposits and vacuoles, and an increase in Bruch's membrane thickness, but without drusen or neovascularization [28]. Supplements of lutein alone only partially prevented the retinal morphological changes [28]. Lutein alone caused decrease of expression of VEGF and MMP-2 in the retina-choroid; however, the difference between this group and the controls was not statistically significant [28]. Supplementation of lutein with large dose of multivitamin substantially ameliorated all retinal morphological alterations and significantly reduced VEGF levels and MMP-2 activity [28]. These results suggest that ocular changes in apoE−/− mouse could be prevented by efficient antioxidant treatments including lutein, multivitamins, and GSH [28].

4.2. DKO Mouse Model. The DKO mouse model is generated by knocking out a chemokine (monocyte chemoattractant protein-1 (MCP-1)) and a chemokine receptor (Cx3cr1) on

a Crb1rd8 background. DKO mice develop RPE pathologic changes (lipofuscin accumulation, hypertrophy, and hypotrophy) and photoreceptor and synaptic degeneration that mimic the pathologic changes in AMD [30].

Ramkumar et al. used the DKO model to investigate the effects of lutein (1.76 μM), zeaxanthin (35.1 μM), long-chain n3 polyunsaturated fatty acid, docosahexaenoic acid, and eicosapentaenoic acid, which is similar to the diet used in the Age-Related Eye Diseases Study 2 (AREDS2) clinical trial [30]. DKO mice developed progressive focal photoreceptor (PR) loss and RPE mottling, whereas the controls (Crb1rd8 mice) did not develop any retinal lesions [30]. DKO mice developed accumulation of liposomes and lipofuscin in the RPE, focal RPE hypertrophy and hypopigmentation, loss of the outer and inner segments layers, and abundant PR loss [30]. AREDS2-treated DKO mice showed a significantly more healthy RPE and PR [30]. DKO mice had high A2E levels and mRNA levels of inflammatory genes [*tumor necrosis factor-α, cyclooxygenase-2* (*COX-2), IL-1β, and iNOS*] and angiogenic genes (*VEGF*), which were significantly greater than those of AREDS2-treated DKO mice, indicating that AREDS2 could downregulate the expression of inflammatory and proangiogenic genes linked to advance AMD [30]. This study demonstrated a benefit of the AREDS2 diet on retinal AMD-like lesions in DKO model [30].

4.3. Mice Lacking αvβ5 Integrin. Mice lacking αvβ5 integrin (β5−/− mice) have a primary defect in phagocytic activity due to lack of the outer segment recognition receptor αvβ5 integrin; this causes accelerated age-accumulation of lipofuscin, increases in 4-hydroxynonenal-adducts, and decreased solubility of actin in the RPE, accompanied by a decrease of a-wave amplitude in ERG that indicates dysfunction of photoreceptors [31]. Accumulation of lipofuscin, damage to the RPE and PR in aged β5−/− mice, mimics the pathologic changes of dry-type AMD in human [31]. Supplementation of grapes or marigold extract containing lutein/zeaxanthin (52 mg lutein/2 mg zeaxanthin per kg body wt/d) given to β5−/− mice prevented 4-hydroxynonenal-adduct formation and decreased actin solubility and lipofuscin accumulation and age-related photoreceptor dysfunction. This suggests that consumption of an antioxidant-rich diet might prevent the destabilization of the actin resulting from a physiological, sublethal oxidative burden on RPE cells, which appears to be associated with age-related blindness [31].

4.4. LASER-Induced Choroidal Neovascularization. Choroidal neovascularization (CNV) is an important pathological change in the neovascular AMD. CNV can be induced in mice by LASER photocoagulation. Izumi-Nagai et al. reported that pretreatment with oral supplements of lutein (10–100 mg/kg body wt per day) significantly suppressed the index of CNV volume induced by LASER photocoagulation [32]. Macrophage infiltration into CNV is decreased in lutein-treated mice. LASER-induced elevation of ICAM-1, VEGF, and MCP-1 levels in RPE-choroid was also significantly inhibited by lutein treatment [32]. LASER-induced CNV was accomplished with NFκB activation, which was also prevented by supplementation of lutein [32]. This study suggested that lutein suppressed LASER photocoagulation-induced CNV through inhibition of NFκB activation which upregulates inflammatory molecules and angiogenic factors, suggesting that lutein supplementation may have therapeutic value in suppressing the development of CNV [32].

4.5. Lutein and Zeaxanthin Deficiency Studies in Monkeys. Effects of lutein and zeaxanthin on the RPE have also been studied in nonhuman primates [33–35]. Comparison of monkeys on a standard diet or xanthophyll free diet demonstrated that deficiency of lutein and zeaxanthin leads to an absence of macular pigment, increased hyperfluorescence in the foveal region [33], and decreased RPE density at the foveal center [34], suggesting that the RPE cells are sensitive to the absence of macular pigment. In monkeys fed a normal diet with normal macular pigmentation, the fovea was less sensitive to blue-light-induced damage than the parafovea [35]. In xanthophyll-free monkeys, the fovea sensitivity to blue-light-induced damage was increased to the same level as that of parafovea. Lutein and zeaxanthin supplementation decreased foveal sensitivity to normal, indicating that lutein and zeaxanthin provide significant protection against short-wavelength photochemical damage in the fovea [35]. This study suggested that supplementation of lutein and zeaxanthin might contribute to the reduction of risk for AMD, especially for persons with reduced macular xanthophyll levels due to retinal disease, poor diet, or genetic predisposition [35].

5. Retinal Ischemic/Hypoxic Injury

Effects of lutein on retinal ischemic/hypoxic injury have been studied in two different experiment animal models: blockade of internal carotid artery-induced ischemia model in mice [36–38] and high intraocular pressure- (IOP-) induced ischemia model in rats [39, 40].

5.1. Retinal Ischemic/Hypoxic Injury Induced by Blockade of Internal Carotid Artery. Retinal ischemia is a common feature of retinal vasculopathies, such as retinopathy of prematurity and diabetic retinopathy. In ischemic retinopathy, decrease in the normal retinal blood supply results in oxidative stress and retinal neovascularization [36].

Retinal ischemia/reperfusion (I/R) was induced by a blockade of internal carotid artery in mice [37, 38]. Lutein (0.2 mg/kg) or vehicle (DMSO) was given by intraperitoneal injection 1 h before and after reperfusion. In the ischemic group, Muller cell gliosis was induced by retinal I/R injury and combined with a marked cell loss and appearance of apoptotic cells in the ganglion cell layer (GCL) and the INL, suggesting the involvement of RGC and amacrine cells. Bipolar and horizontal cells seemed relatively unaffected by I/R [37, 38]. In flash ERG, the b-wave/a-wave ratio and oscillatory potentials were significantly reduced [38]. Oxidative stress and oxidative DNA damage were increased in GCL and INL layers [38]. Lutein treatment prevented the deterioration of ERG, decreased the oxidative stress, decreased the cell loss and the apoptotic cells in the retina, inhibited upregulation

of glial fibrillary acidic protein, which avoided Muller cell gliosis, and preserved retinal function [37, 38]. These studies suggested that lutein protected the retina, especially the RGC, from I/R damage by its antioxidative, antiapoptotic, and anti-inflammatory properties [37, 38].

5.2. High Intraocular Pressure Induced-Ischemia Model. It is well known that I/R generates retinal damage, particularly if ischemia persists for ≥60 min [39]. Most of the retinal damage is caused during reperfusion rather than during ischemia [40, 41]. ROS and reactive nitrogen species play important roles in the pathogenesis of I/R induced retinal damage [40].

The effect of lutein on high IOP-induced retinal ischemic/reperfusion damage has been tested in a rat model [40] by increasing IOP above systolic blood pressure for one hour in Sprague-Dawley rats. Lutein was injected intravitreally (10 μL of 0.5 mg/mL) 30 minutes before ischemia or intraperitoneally (0.5 mg/kg) one hour before and one hour after ischemia. The experimental rats were sacrificed 24 hours after reperfusion. Acute ischemic injury caused loss of 46% and 32% cells in the GCL and INL, respectively [40]. Lutein injected intraperitoneally (0.5 mk/kg of body weight) significantly increased survival of retinal neurons to 85% and 88% in the GCL and INL, respectively. Neuronal nitric oxide synthase (nNOS) was increased in early ischemic retina and this can induce excessive nitric oxide (NO), which results in neuronal cell death and activation of glial cells [40]. nNOS and COX-2 (related to prostaglandin metabolism) were increased in ischemic retinas and these increases were inhibited by lutein [40]. This study confirmed that lutein functions as an anti-ischemic drug by inhibiting nNOS and COX-2 expressions and suggested that lutein supplementation may protect against ischemia-mediated cell death in the retina [40].

In another study in high IOP-induced ischemic retina model in rats, the effects of subcutaneous injections of lutein (100 mg/kg/d) have been compared to three other antioxidants (vitamin E and two extracts from plants: Trigonella and Teucrium) [39]. I/R caused severe retinal damage as indicated by increased levels of MDA and caspase-3 activity (biomarker of apoptosis) and decreased GSH contents. Lutein significantly prevented all deteriorative changes caused by I/P and showed the strongest effect of the antioxidants tested, whereas other antioxidants only had partially protective effects [39]. This study suggested that lutein showed superior efficacy compared to all other tested antioxidants. Therefore, the future therapeutic use of lutein against I/P damage of the retina deserves further experiments and clinical trials [39].

6. Light-Induced Retinal Damage

The effects of zeaxanthin on light-induced photoreceptor cell death have been studied in quail fed a carotenoid-deficient diet [42]. Light irradiated retina showed numerous apoptotic PR with apoptotic rods outnumbering cones, accompanied by activated microglial invasion of the damaged PR layer. Oral supplementation of zeaxanthin produced a rapid enrichment in the zeaxanthin fraction of total serum xanthophylls and a slow increase in the zeaxanthin fraction of retinal xanthophylls. Zeaxanthin supplementation decreased apoptosis and activation of microglia. The numbers of apoptotic PR and activated microglia correlated negatively and significantly with the concentration of retinal zeaxanthin, suggesting that zeaxanthin could protect PR against light-induced apoptosis [42].

The effects of lutein in light-induced retinal degeneration have been tested in a mouse model of light-induced retinal degeneration [43]. In this model, after the light irradiation, PR cells gradually degenerate and become apoptotic, resulting in thinning of the PR layer. ERG shows a reduction of a-wave and b-wave amplitudes, indicating visual impairment. Apoptosis is caused by upregulation of double-stranded breaks in DNA [43]. Supplementation of lutein in powder chow (170 mg/kg body wt/d) attenuated apoptosis of PR by suppression of double-stranded breaks in DNA. Light-induced oxidative stress of the retina and functional and histological damage in the retina were suppressed by the treatment of lutein. This study indicated that a lutein-supplemented diet could attenuate light-induced visual impairment by protecting the DNA of the PR cells [43].

7. Rescue of PR in Retinal Detachment

The neuroprotective effect of lutein on the PR in a rat model of retinal detachment has been studied [44]. Retinal detachment was induced by subretinal injections of sodium hyaluronate in rats. Daily injections of corn oil (control group) or lutein (0.5 mg/kg) in corn oil (treatment group) were administered intraperitoneally. In the control group, the average number of viable cells in the outer nuclear layer (ONL) was significantly decreased. Apoptotic cells could be detected in the cells in the ONL. Lutein treatment, commenced after four hours, significantly increased the number of viable cells and decreased the number of apoptotic cells. Cell loss in the ONL was reduced from 24% in the control group to 11% in lutein treatment group, demonstrating that lutein could prevent 54% of cell death caused by retinal detachment. Western blotting results showed that there was a decrease of cleaved caspase-8 and caspase-3, but not caspase-9, in lutein treated group, indicating that lutein probably acted on the extrinsic apoptosis pathway rather than the intrinsic apoptosis pathway [44]. Lutein treatment group also showed significantly reduced glial fibrillary acidic protein immunoreactivity and preserved rhodopsin expression. Similar results were detected when lutein was given 36 hours after retinal detachment induction [44]. This study suggests that lutein is a neuroprotective agent that could rescue the apoptosis of PR in rats with retinal detachment even when given 36 hours after the occurrence of retinal detachment [44]. The use of lutein in retinal detachment patients may serve as an adjunct to surgery for the improvement of visual outcomes [44].

8. Retinitis Pigmentosa

Retinitis pigmentosa is a group of inherited disorders characterized by progressive PR degeneration leading to loss of

vision. The rd1/rd1 mouse has an insertion of viral DNA in the β-subunit of the *cGMP phosphodiesterase* gene. This mutation leads to toxic accumulation of cGMP and high Ca2+ levels in the rd1 photoreceptors and causes the death of rods followed by cone death [45–47]. A mutation in the same gene has been found in human forms of autosomal recessive retinitis pigmentosa, making the rd1 mouse an ideal spontaneous model of retinitis pigmentosa [45]. It has been reported that cone cell death after rod cell death in the rd1 mouse model was due to oxidative damage caused by reduced oxygen utilization by rods and that antioxidant therapy might prevent the cone cell death in this model [45–47].

The effects of oral administrated antioxidant mixture which contained zeaxanthin and lutein, α-lipoic acid, and GSH plus an extract obtained from *Lycium barbarum* that contains various antioxidants, such as zeaxanthin and β-carotene, have been tested in rd1 mouse [45]. Apoptotic cells could be detected in the ONL of rd1 mice retina by terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Treatment with lutein or zeaxanthin alone decreased apoptotic cells in this model by 11% and 20%, respectively, which was not statistically significant [45]. But treatment with the full antioxidant mixture resulted in a statistically significant decrease (39%) of apoptotic cells [45, 47]. Cells with oxidative DNA damage could be detected in the ONL of rd1 mice retina by avidin staining. Treatment with lutein or zeaxanthin alone decreased avidin-positive cells by 14%, which was also not statistically significant. Only the full antioxidants mixture reduced the avidin-positive cells by a statistically significant 21% [45, 47]. Supplement of antioxidants mixture also increased GPx activity and GSH levels and decreased cystine levels in rd1 retinas, whereas no change was observed in glutathione disulfide reductase activity [45, 47]. These studies suggest that oxidative stress plays a role in the photoreceptor death in retinitis pigmentosa and an antioxidant mixture containing zeaxanthin and lutein is able to reduce photoreceptor death in rd1 retina [45, 47].

9. Endotoxin-Induced Uveitis

Intraperitoneal or subcutaneous injection of lipopolysaccharide (LPS) into mice or rats can generate endotoxin-induced uveitis (EIU), which is an important experimental animal uveitis model [19, 48–51, 53, 54].

Intravenous injection of lutein suppresses the development of EIU in rats in a dose-dependent manner [48]. The anti-inflammatory effect of lutein (100 mg/kg) is comparable to dexamethasone (1 mg/kg). During the development of EIU, various inflammatory factors significantly increased in the aqueous humor, including tumor necrosis factor-α, IL-6, MCP-1, macrophage inflammatory protein-2, prostaglandin E2, and NO. Lutein significantly decreased the levels of these inflammatory factors in the aqueous humor [48]. The mechanism of the anti-inflammatory effect of lutein may be related to the suppression of proinflammation signal pathways. Lutein inhibited the activation of NFκB in the iris-ciliary body and the expression of iNOS and COX-2 in cultured mouse macrophage cells, suggesting that lutein can have a potent anti-inflammatory effect on EIU [48].

Another study of the effects of lutein in EIU showed that levels of NO and MDA were increased in ocular tissues in EIU model [49]. The levels of oxygen radical absorbance capacity, GSH, vitamin C, total superoxide dismutase SOD, and GPx activities and mRNA levels of *copper-zinc SOD*, *manganese SOD*, and *GPx* were reduced in ocular tissues in EIU [49]. Supplementation of lutein in drinking water (0.125–0.25 mg/kg/d) for five days before LPS injection significantly avoided the above-mentioned changes related to oxidative stress. These results suggested that the protective effects of lutein against the LPS-induced inflammation might be related to the alleviation of oxidative stress [49].

In EIU, inflammation processes involve both uvea and retina. Inflammation in the retina causes dysfunction of retina and loss of vision. The outer segments of PR are shortened, accompanied by a reduction in rhodopsin and a decrease of photoreceptor cell function. ERG shows a decrease in the a-wave amplitude [19, 50]. Sasaki et al. reported that lutein had a neuroprotective effect during retinal inflammation in EIU [50]. Supplementation of lutein by subcutaneous injection (100 mg/kg body wt) prevented the shortening of the outer segments and the reduction of rhodopsin. ERG a-wave amplitude was preserved by the treatment of lutein. Pathological changes of Muller cells and activation of signal transducer and activator of transcription 3 were also decreased by lutein. This study indicated that lutein protected the neuroretina against inflammatory damage by reducing oxidative stress, suppressing inflammatory signal pathway, and preservation of rhodopsin protein [19, 50].

10. Miscellaneous

The effects of zeaxanthin on the retinal antioxidative capacity, the levels of lipid and protein peroxidation, and their signal pathway were tested in normal rats [51]. Rats fed with high dosages of zeaxanthin showed a significant increase of Akt phosphorylation in the retina that might activate the NF-E2-related factor 2 (Nrf2) pathway. GSH levels and the expression of Nrf2 target genes in the retina were significantly increased by zeaxanthin supplementation [51]. The biomarker of lipid and protein peroxidation (4-hydroxynonenal and the carbonyl protein) were significantly decreased in the retina of zeaxanthin-supplemented rats [51]. Rats fed with a lower dosage of zeaxanthin showed a tendency toward similar changes as the high dosage zeaxanthin group but statistical significance was reached in only some of the parameters [51]. The authors mentioned that this is the first time that zeaxanthin was presented as a phase II enzymes inducer, instead of an antioxidant [51].

11. Summary

The effects of lutein and zeaxanthin on the prevention of various eye diseases, including AMD, diabetic retinopathy and cataract, ischemic/hypoxia induced retinopathy, light damage of the retina, retinitis pigmentosa, retinal detachment, and

uveitis [19–51], have been studied in a variety of experimental animal models. In these animal models, lutein and zeaxanthin have been reported to have beneficial effects in protecting ocular tissues and cells, especially the retinal neurons, against damage caused by different etiological factors [19–51]. The mechanisms responsible for these effects of lutein and zeaxanthin include prevention of phototoxic damage by absorption of blue light [53], reduction of oxidative stress through antioxidant activity and free radical scavenging [54], and their anti-inflammatory and antiangiogenic properties [4].

The therapeutic effects of lutein and zeaxanthin have been evaluated clinically in several eye diseases such as AMD, diabetic retinopathy, retinopathy of prematurity, and cataract [1, 3, 10, 23] and also in a small group of retinitis pigmentosa patients [3]. Lutein and zeaxanthin have not been tested clinically in other eye diseases, such as retinal detachment (for neuroprotective effects on the photoreceptors) and uveitis (anti-inflammation and improvement of survival of retinal neurons). Future studies to explore the effects of supplementation of lutein and zeaxanthin in these severe eye diseases may prove to be clinically valuable.

Experimental animal studies have provided important information on the effects of lutein and zeaxanthin in various eye diseases. However, animal models also have their limitations and many problems should be addressed prior to clinical application.

The first limitation involves species differences between human and experimental animals. Most of these studies on the effects of lutein and zeaxanthin were tested in the murine models. Rats and mice have the advantage that they are relatively inexpensive, easily performed, and reproduced. However, rodents have no macula and their retinal photoreceptor cells are predominantly rods, rather than the cone cells that predominate in the human macula. These differences should be considered in the translation of results obtained from animal studies to human eye diseases, especially in AMD [10].

Second, the dosages used in animal studies are usually much higher than those used clinically. The tested dosages of lutein (10 mg/d) and zeaxanthin (2 mg/d) in AREDS2 are much lower than the dosages used in animal studies. Many animal studies have indicated that the effects of both lutein and zeaxanthin are dose-dependent [29, 32, 51]. The effective dosages are usually at the range of 0.5–100 mg/kg body wt/d (oral administration), which translates to 30–6000 mg/d in a 60 kg weight adult [26, 31, 32, 42, 49, 51], which is usually 60- to 300-fold greater than previously tested clinical dosages. For example, Zou et al. reported that rats fed with high dosages of zeaxanthin (24 mg/kg/d, equal to 1440 mg/d in a 60 kg weight adult) showed a significant activation of the Nrf2 pathway in rats [51]. A lower dosage (8 mg/kg/d, equal to 480 mg/d in a 60 kg weight adult) showed similar tendency but the difference between treated rats and the controls was not statistically significant [51]. Therefore, it may be necessary to increase clinical trial dosages of lutein and zeaxanthin in order to reproduce the results obtained in animal studies. This will require study of the safety and toxicity of lutein and zeaxanthin at large dosages to determine maximum dosages that could be used clinically safely.

Third, there are currently three known macular pigments, lutein, zeaxanthin, and meso-zeaxanthin [52]. In the past, most animal studies tested only the effects of lutein and zeaxanthin. Very little is known on the function of meso-zeaxanthin [55–57]. Therefore, future studies will need to include the effects of meso-zeaxanthin in various animal models to elucidate the full picture of the role of macular pigment in ocular disease.

Conflict of Interests

None of the authors have financial interests relevant to the contents of this paper.

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Research Article

Effects of Lutein and Zeaxanthin on LPS-Induced Secretion of IL-8 by Uveal Melanocytes and Relevant Signal Pathways

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The effects of lutein and zeaxanthin on lipopolysaccharide- (LPS-) induced secretion of IL-8 by uveal melanocytes (UM) were tested in cultured human UM. MTT assay revealed that LPS (0.01–1 μg/mL) and lutein and zeaxanthin (1–10 μM) did not influence the cell viability of cultured UM. LPS caused a dose-dependent increase of secretion of IL-8 by cultured UM. Lutein and zeaxanthin did not affect the constitutive secretion of IL-8. However, lutein and zeaxanthin decreased LPS-induced secretion of IL-8 in cultured UM in a dose-dependent manner. LPS significantly increased NF-κB levels in cell nuclear extracts and p-JNK levels in the cell lysates from UM, but not p-p38 MAPK and p-ERG. Lutein or zeaxanthin significantly reduced LPS-induced increase of NF-κB and p-JNK levels, but not p38 MAPK and ERG levels. The present study demonstrated that lutein and zeaxanthin inhibited LPS-induced secretion of IL-8 in cultured UM via JNK and NF-κB signal pathways. The anti-inflammatory effects of lutein and zeaxanthin might be explored as a therapeutic approach in the management of uveitis and other inflammatory diseases of the eye.

1. Introduction

Zeaxanthin and lutein are two natural bioactives that belong to the xanthophyll class. They are present in the eye in high concentrations. The retina and lens in general and the macular region in the center of the retina in particular are highly enriched in these two xanthophylls [1, 2]. There is increasing evidence that lutein and zeaxanthin may play an important role in protecting against several eye diseases, such as age-related macular degeneration (AMD) [3–8]. Therefore, lutein and zeaxanthin are widely used as nutrient supplements for the prevention and treatment of AMD and other eye diseases. The protective effects of lutein and zeaxanthin may be related to their short wave light-screening effect and antioxidant properties [1–9]. Recent studies indicated that they also influence cell function through various signal pathways or transcription factors and have anti-inflammatory effect [9, 10]. It has been reported that lutein can suppress the development of uveitis caused by injection of lipopolysaccharide (LPS) in rats and mice [10, 11].

LPS is an endotoxin that can induce a potent inflammatory response [12]. Injection of LPS into mice or rats can generate endotoxin-induced uveitis (EIU), which is an important experimental uveitis model in animals [10–16]. LPS injection causes significant increase of several cytokines and chemokines in the eye [10]. Interleukin-8 (IL-8)/CXCL8, a proinflammatory chemokine, plays an important role in the pathogenesis of LPS-induced uveitis. Injection of anti-IL-8 antibodies can block LPS-induced uveitis [15].

Animal studies indicated that lutein reduces the secretion of proinflammatory cytokines and chemokines and this may be the mechanism of inhibition of LPS-induced uveitis by lutein [10]. However, the cell type and the signal pathways involved in this process remain to be studied.

Uveal melanocytes (UM) are the predominant cell type in the uvea. In the past, very little was known about the function of UM and the role of UM in the pathogenesis of various eye diseases. In the past decades, after the development of methods for the culture of UM and the establishment of in vitro models for studying the function of UM, it has been reported that UM produce various growth factors, cytokines, and chemokines, in addition to their functions related to melanin [17–19]. Hu et al. reported that UM produce IL-8 constitutively and the secretion of IL-8 could be increased significantly by the stimulation of LPS [19]. This suggested that UM might play a role in the pathogenesis of ocular inflammatory diseases and may be involved in the inhibitory effects of lutein on LPS-induced uveitis. However, the effects of lutein and zeaxanthin on the secretion of chemokines by UM have not been reported previously. The purposes of this study were to investigate the effects of lutein and zeaxanthin on LPS-induced secretion of IL-8 in human UM in vitro and to study the signal pathways involved in this process.

2. Material and Methods

2.1. Reagents. Cell culture medium, fetal bovine serum, and trypsin were obtained from GIBCO (Grand Island, NY, USA). LPS, dimethyl sulfoxide (DMSO), collagenase, lutein, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBF), and protease inhibitor cocktail were obtained from Sigma (St. Louis, MO, USA). Zeaxanthin was obtained from ZeaVision LLC (Chesterfield, MO, USA). Quantikine IL-8 ELISA kit was obtained from R&D System (Minneapolis, MN, USA). p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1/2 (ERK1/2), and c-Jun N-terminal kinase 1/2 (JNK1/2) ELISA kits and cell extraction buffer, PMSF, hypotonic buffer, and nuclear factor-kappa B (NF-κB) ELISA kits were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Cell Culture. Human UM were isolated from donor eyes by trypsin-collagenase sequential method and cultured in FIC medium as previously reported [17–19]. Cells from a primary culture of melanocytes isolated from choroid at passage levels 3–5 were used in this study.

2.3. Cell Viability Assay. MTT assay was used to determine the effects of LPS, lutein, and zeaxanthin on cell viability of cultured UM as previously described [19]. For each experiment, 5×10^3 cells were seeded into each well in 96-well plates. After incubation for 24 h, LPS, lutein, or zeaxanthin at different levels was added. Twenty-four hours later, 50 μL of 1 mg/mL MTT was added and culture plates were incubated at 37°C for 4 h. The medium was removed and 100 μL of DMSO was added. The optical density as the parameter of cell viability was measured at 540 nm with a microplate reader (Multiskan EX, Thermo, Ventana, Finland).

2.4. LPS and Secretion of IL-8 by UM. Human UM (1×10^5 cells/well) were seeded into the 24-well plates and cultured. When cells reached 80–90% confluence, they were rinsed with PBS and incubated in serum-free culture medium with or without LPS. After 24 h, the conditioned media were collected and centrifuged. The supernatants were stored at −80°C.

2.5. Lutein and Zeaxanthinon Constitutive Secretion of IL-8 by UM. Human UM (1×10^5 cells/well) were seeded into the 24-well plates and cultured. When cells reached 80–90% confluence, they were rinsed with PBS and incubated in serum-free culture medium with or without lutein or zeaxanthin. After 24 h, the conditioned media were collected and centrifuged. The supernatants were stored at −80°C.

2.6. Lutein and Zeaxanthin on LPS-Induced Secretion of IL-8 by UM. Human UM (1×10^5 cells/well) were seeded into the 24-well plates and cultured, and culture medium was replaced as described above. Lutein or zeaxanthin at different final levels was added. Two hours later, LPS at the final levels of 0.1 μg/mL was added to the cultures. After 24 h, the conditioned media were collected and centrifuged. The supernatants were stored at −80°C.

2.7. Measurement of IL-8 Protein Levels. Enzyme-linked immunosorbent assay (ELISA) was used for the measurement of IL-8 protein levels in the supernatant of cultured cells. A commercially available Quantikine IL-8 ELISA kit was used to determine IL-8 protein levels according to the protocol provided by the manufacturer. The optical density of the ELISA samples was measured at 450 and 540 nm using a microplate reader. The sensitivity of the assay for IL-8 was 3.5 pg/mL.

2.8. LPS, Lutein, and Zeaxanthin on MAPK and NF-κB. For the study of phosphorylated- (p-) p38 MAPK, p-ERK1/2, and p-JNK1/2 levels, human cultured UM (1×10^6 cells/well) were seeded into 6-well plates and cultured for 24 h and culture medium was replaced as described above. Lutein or zeaxanthin at different final levels was added. Two hours later, LPS at the final levels of 0.1 μg/mL was added into cultures and cultured for 60 min. The cultured media were removed and the cultures were washed with PBS. UM were harvested by scraping with a rubber policeman. Cell were washed with PBS and centrifuged. Cell extraction buffer with protease inhibitor cocktail and PMSF were added to the pellets and cultured for 30 min and centrifuged. All of these procedures were done under 4°C. The supernatants were collected and stored at −80°C. For the study of NF-κB, cells were cultured, treated with or without lutein, zeaxanthin, and LPS, and collected as described above. Collected cells were treated with hypotonic buffer and centrifuged. The pellets that contain nuclear fraction were collected, treated with cell extraction

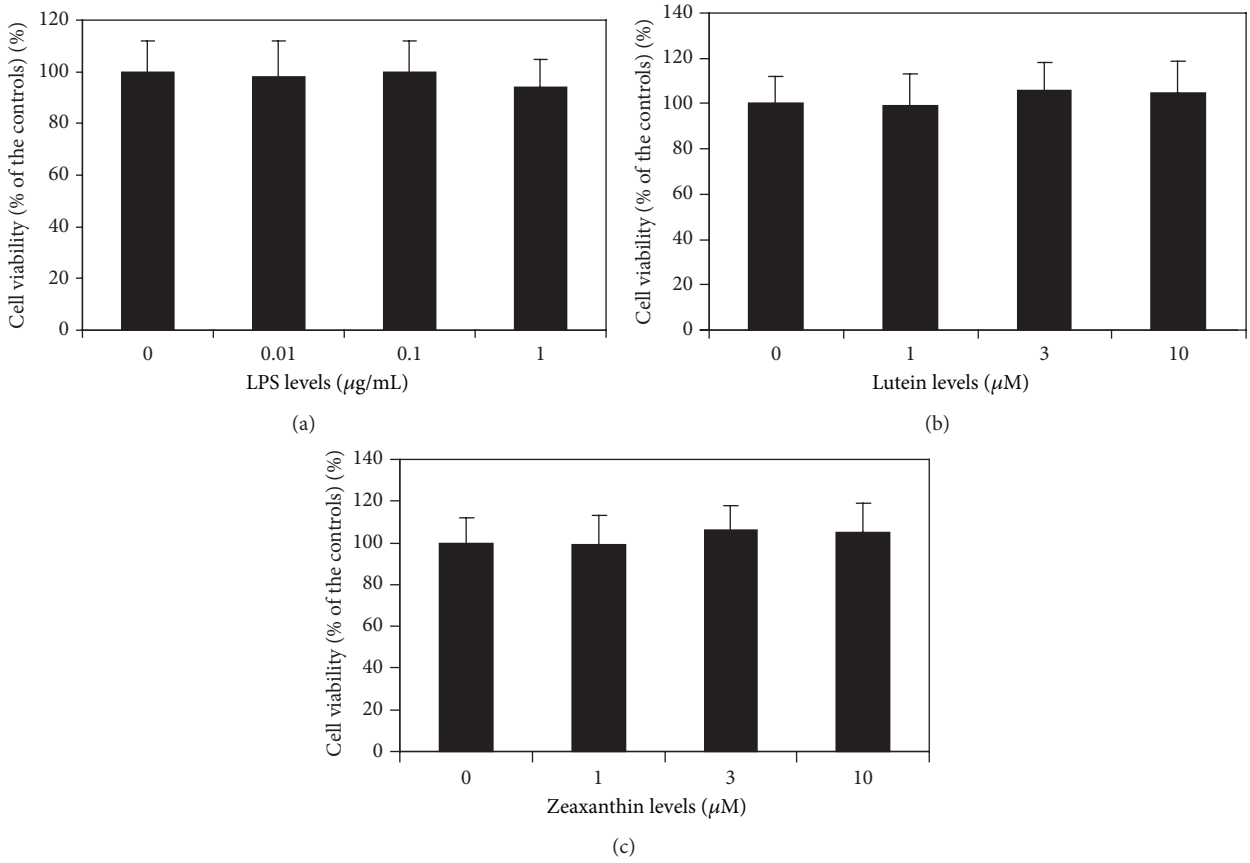

Figure 1: Effects of LPS, lutein, and zeaxanthin on viability of uveal melanocytes (UM). Cells were seeded into 96-well plates and treated with LPS, lutein, and zeaxanthin at different levels. Cell viability was determined by MTT assay (see Material and Methods). LPS (a), lutein (b), and zeaxanthin (c) at all tested levels did not affect the viability of UM (expressed as percentage of the controls) ($p > 0.05$).

buffer, vortexed, and centrifuged. The supernatants were stored at −80°C.

2.9. MAPK and NF-κB Assay. ELISA was used for the measurement of MAPK and NF-κB. Commercially available p38 MAPK, ERK1/2, and JNK1/2 ELISA kits were used to determine p-p38 MAPK, p-ERK1/2, and p-JNK1/2 levels in cell extracts, respectively. The test was performed according to the protocol provided by the manufacturer. The sensitivity of these kits was 0.8 U/mL. NF-κB ELISA kits were used to determine NF-κB levels in cell nuclear extracts according to the protocol provided by the manufacturer. The sensitivity of this kit was <50 pg/mL.

2.10. Statistical Analysis. Each experiment was replicated 3 times and the data were presented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) test was performed to assess the significance. Values of $p < 0.05$ were considered statistically significant. All data analysis was performed using specific software (SPSS 19.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cell Viability Assay. MTT assay showed that LPS at the final levels of 0.01, 0.1, and 1 μg/mL did not influence the cell viability of cultured human UM ($p > 0.05$, compared with cells not treated with LPS) (Figure 1(a)). Lutein and zeaxanthin at the final levels of 1, 3, and 10 μM also had no effects on the cell viability of cultured UM ($p > 0.05$, compared with cells not treated with LPS) (Figures 1(b) and 1(c)). Therefore, level ranges of 0.01–1 μg/mL of LPS and 1–10 μM of lutein and zeaxanthin were chosen for subsequent experiments.

3.2. LPS and Secretion of IL-8 by UM. ELISA analysis of cell supernatants of UM cultured with serum-free culture medium detected a low level of IL-8 protein (9.20 ± 0.90 pg/mL), indicating a low level of constitutive secretion of cultured UM. LPS at 0.01–1.0 μg/mL caused a dose-dependent significant increase of IL-8 levels ($p < 0.05$ at all levels of LPS as compared with cells not treated with LPS, Figure 2).

3.3. Lutein and Zeaxanthin on Constitutive Secretion of IL-8 by UM. IL-8 protein levels in cell supernatants from cells

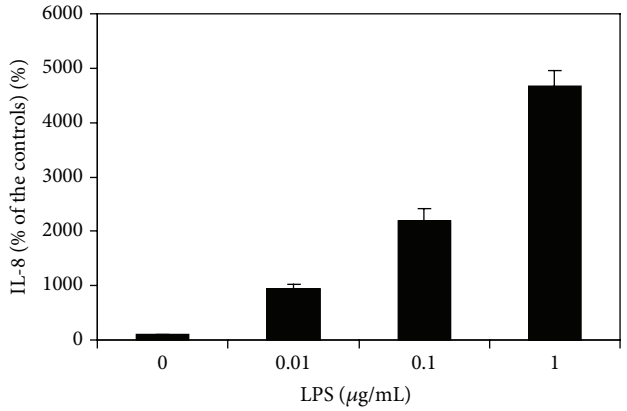

Figure 2: LPS stimulates secretion of IL-8 by uveal melanocytes. Cells were plated into 24-well plates and treated with LPS at different levels. IL-8 protein levels in the conditioned medium were determined by ELISA kit and expressed as percentage of the controls. LPS significantly increased the secretion of IL-8 dose-dependently ($p < 0.05$).

treated with lutein or zeaxanthin (1, 3, and 10 μM) did not significantly differ from those from cells not treated with lutein and zeaxanthin ($p > 0.05$, Figure 3), suggesting that lutein and zeaxanthin do not affect the constitutive secretion of IL-8 from UM.

3.4. Lutein and Zeaxanthin on LPS-Induced Secretion of IL-8 by UM. Lutein (1, 3, and 10 μM) dose-dependently decreased LPS-induced secretion of IL-8 in cultured UM ($p < 0.05$ as compared with cells treated by 0.1 μg/mL LPS only, Figure 4(a)). However, IL-8 levels in cells treated with lutein and LPS were still greater than those from cells not treated with LPS ($p < 0.05$), suggesting that lutein has a partial inhibitory effect on LPS-induced secretion of IL-8. Zeaxanthin (1, 3, and 10 μM) dose-dependently decreased LPS-induced secretion of IL-8 in cultured UM ($p > 0.05$, zeaxanthin 1 μM versus the controls and $p < 0.05$, zeaxanthin 3 and 10 μM versus the controls, Figure 4(b)). IL-8 levels in cells treated with zeaxanthin and LPS were still greater than those from cells not treated with LPS ($p < 0.05$), suggesting that zeaxanthin also has a partial inhibitory effect on LPS-induced secretion of IL-8.

3.5. LPS, Lutein, and Zeaxanthin on MAPK and NF-κB Pathways. LPS at 0.1 μg/mL level significantly increased p-JNK levels ($p < 0.05$ as compared with cells not treated with LPS; Figure 5(a)) but not p-p38 MAPK and p-ERG1/2 levels in cultured UM ($p > 0.05$, Figures 5(b) and 5(c)). Addition of lutein or zeaxanthin significantly reduced LPS-induced increase of p-JNK levels (both $p < 0.05$, Figure 5(a)) but did not affect p-p38 MAPK and p-ERG1/2 levels (both $p > 0.05$, Figures 5(b) and 5(c)) in cultured UM. NF-κB levels in cell nuclear extracts from cells treated with LPS were significantly greater than those from cells not treated with LPS ($p < 0.05$, Figure 5(d)). Lutein or zeaxanthin significantly reduced LPS-induced increase of NF-κB levels in cell nuclear extracts (both $p < 0.05$, Figure 5(d)). These results suggested that JNK1/2 and NF-κB (but not p38 MAPK and ERK1/2) play an important role in LPS-induced increased secretion of IL-8 and in the inhibitory effects of lutein and zeaxanthin on LPS-induced increased secretion of IL-8.

4. Discussion

In the present study, we demonstrated that lutein and zeaxanthin inhibited LPS-induced secretion of IL-8 in cultured human UM and this effect was mediated by JNK1/2 and NF-κB signaling pathways.

Uveitis is a common eye disease and a major cause of visual impairment throughout the world [10, 11, 20]. Intraocular or systemic injection of LPS can induce uveitis in experimental animals (EIU). EIU is a well-known model of experimental uveitis used for the study of human uveitis [10–16, 19].

LPS is an endotoxin and is the major component of the outer membrane of Gram-negative bacteria. LPS can induce a strong response from the immune system [12]. LPE-induced uveitis is thought to be the result of a cytokine-chemokine cascade [10, 14]. IL-8 is a critical chemokine in the development and regulation of EIU. IL-8 levels increased significantly in LPS-induced uveitis [15, 16]. Intraocular injection of anti-IL-8 antibody inhibits leukocyte accumulation and decreases the clinical and histological grades of inflammation in LPE-induce uveitis [15]. In vitro studies suggested that LPS induces expression of IL-8 in various cell types [21–23].

Chemokines act as chemoattractants and activators of specific leukocytes at the site of inflammation. Chemokines could be classified into four subfamilies based on the number and location of the cysteine residues at the N-terminus of the molecule and are named CC (with two adjacent cysteines near the N-terminus of the molecule), CXC (the two cysteines being separated by an amino acid), CX3C (having three amino acids between the two cysteines), and C (having a specific amino acid sequence of glutamic acid-leucine-arginine immediately before the first cysteine), in agreement with the systematic nomenclature. In the two main subfamilies (CC and CXC), CXC chemokines are important in the attraction of neutrophils and CC chemokines have powerful chemoattractants and activators for monocytes and lymphocytes [19, 21, 22]. IL-8 is a prototype of CXC chemokine family and is a potent stimulus for neutrophils recruitment and activation. It also triggers the migration and adhesion of T cells, monocytes, and basophils to vascular endothelium and leads to extravasation of these cells into the tissues [19, 21]. Biological activities of IL-8 are mediated by two cell surface G-protein-coupled receptors, CXCR1 and CXCR2 [21, 22]. IL-8 acts as a proinflammatory chemokine and plays an important role in the pathogenesis of the inflammatory process [21, 22]. In the eye, intraocular injection of IL-8 induces uveitis in experimental animals [24]. IL-8 levels are significantly increased in the aqueous humor or vitreous from patients with various types of uveitis [19, 20, 25, 26].

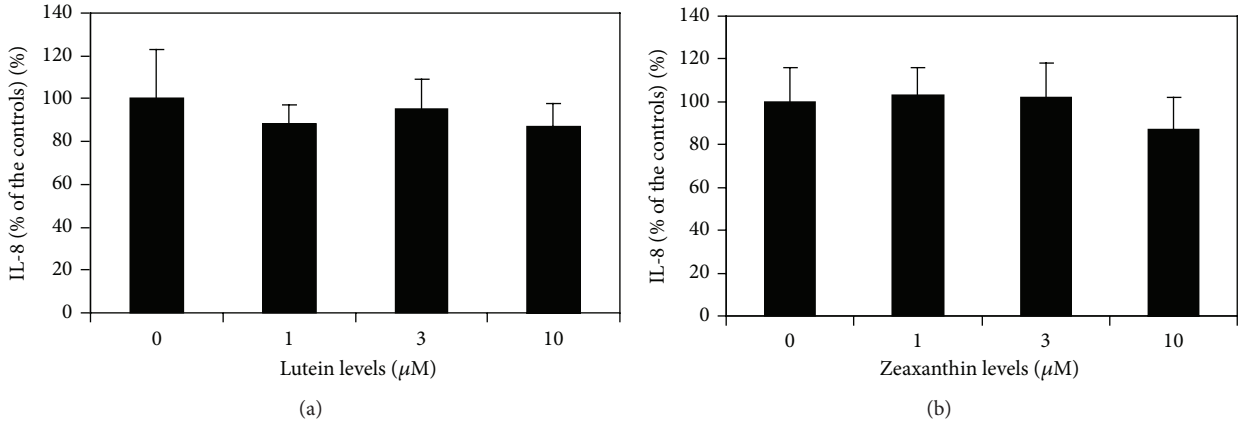

Figure 3: Lutein and zeaxanthinon constitutive secretion of IL-8 by uveal melanocytes. Cells were plated into 24-well plates and treated with lutein or zeaxanthin at different levels. IL-8 protein levels (expressed as percentage of the controls) in cell supernatants from cells treated with lutein (a) or zeaxanthin (b) at different levels did not significantly differ from those from cells not treated with lutein and zeaxanthin ($p > 0.05$).

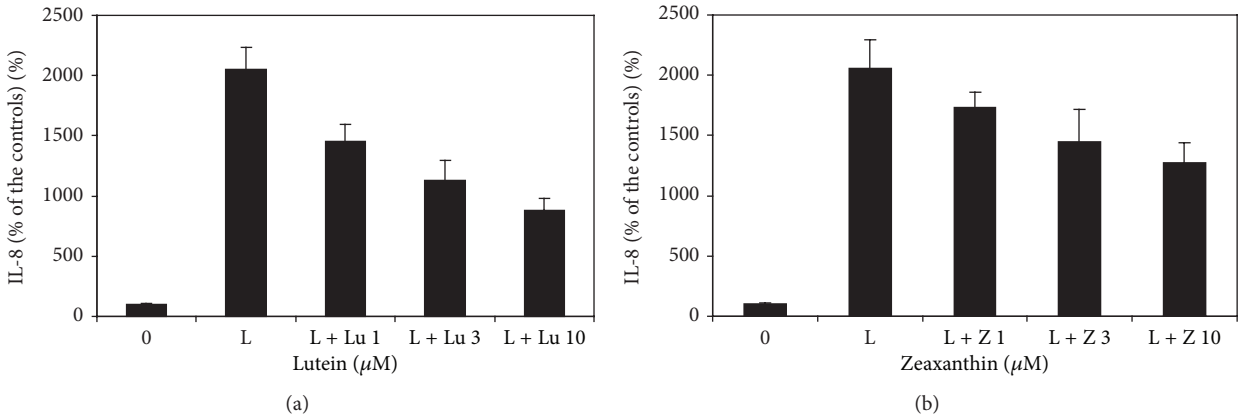

Figure 4: Lutein and zeaxanthin on LPS-induced secretion of IL-8 by uveal melanocytes (UM). Cells were plated into 24-well plates and treated with lutein or zeaxanthin, followed by addition of LPS (0.1 μg/mL) or not. Lutein (a) and zeaxanthin (b) dose-dependently decreased LPS-induced secretion of IL-8 in cultured UM ($p < 0.05$ as compared with cells treated by 0.1 μg/mL LPS only). IL-8 levels in cells treated with lutein or zeaxanthin and LPS were still greater than those from cells not treated with LPS ($p < 0.05$), suggesting that both xanthophylls have a partial inhibitory effect on LPS-induced secretion of IL-8.

It has been reported that intravenous injection or oral administration of lutein suppresses the development of LPE-induced uveitis in rats and mice, respectively [10, 11]. The anti-inflammatory effect of 100 mg/kg lutein was as strong as that of 1 mg/kg dexamethasone [10]. During the development of EIU, various inflammatory factors significantly increased in the aqueous humor [10]. Lutein significantly decreased the levels of these inflammatory factors in the aqueous humor. The mechanism of the anti-inflammatory effect of lutein may be related to the suppression of proinflammation signal pathways [10]. In addition to the anti-inflammatory effects, lutein has neuroprotective effects on retinal neurons during experimental uveitis and retinitis caused by LPS [27].

In the present study, LPS significantly induced the secretion of IL-8 by UM in a dose-dependent manner, which is consistent with previous reports [19]. Lutein and zeaxanthin dose-dependently inhibit LPS-induced increased secretion of IL-8 in cultured UM; this is consistent with the animal studies, which showed that lutein suppresses the occurrence of LPE-induced uveitis [10]. To the best of our knowledge, this is the first report showing that lutein and zeaxanthin inhibit LPS-induced expression of IL-8 in UM.

The mechanism of lutein and zeaxanthin inhibition of LPS-induced expression of chemokines in UM has not been previously reported. The present study demonstrated that lutein and zeaxanthin inhibited the secretion of IL-8 induced by LPS through the activation of JNK1/2 and NF-κB signal pathway, but not p38 and ERK pathway; this is consistent with the animal study, which showed that lutein inhibited the activation of NF-κB in the iris-ciliary body in LPS-induced uveitis and in cultured macrophages [9, 10].

In conclusion, this study demonstrated that lutein and zeaxanthin inhibited LPS-induced secretion of IL-8 in cultured human UM and this effect was mediated by JNK1/2

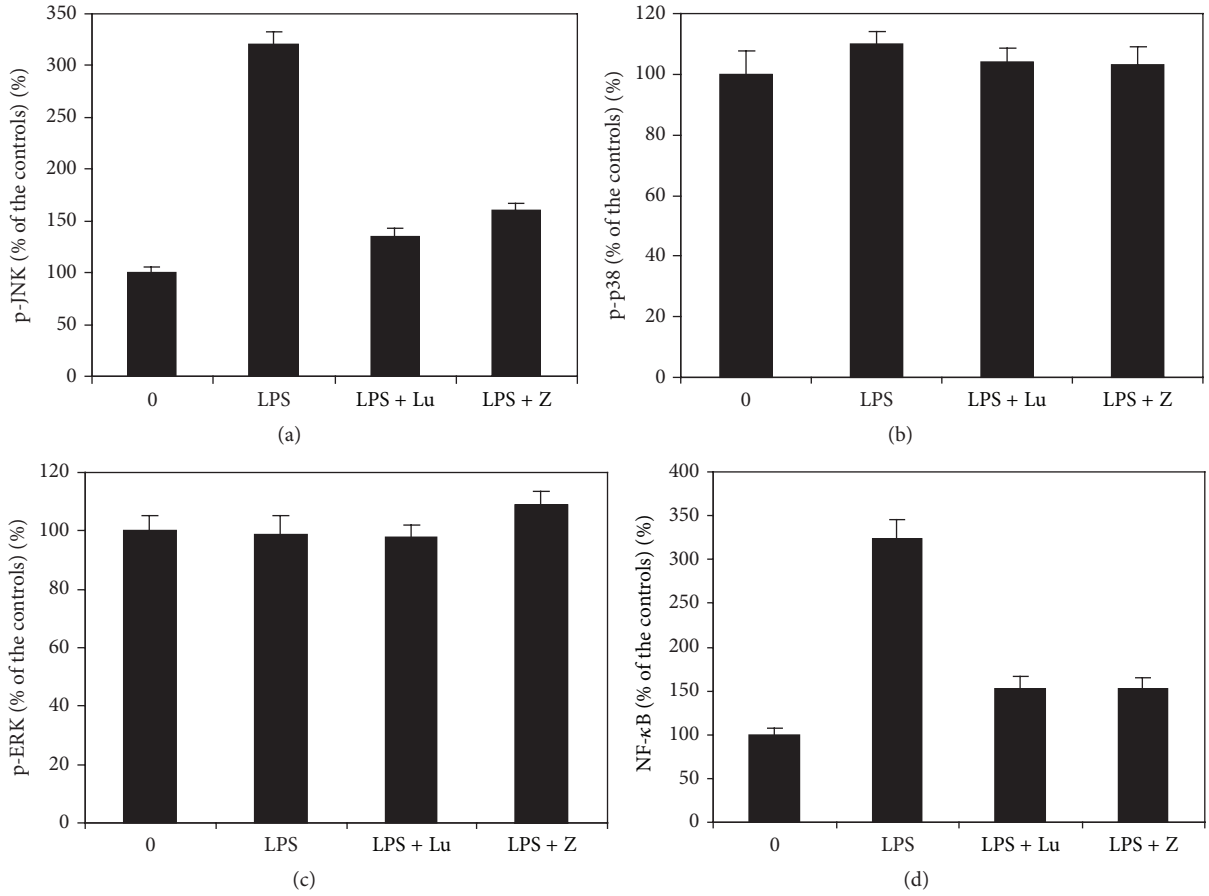

FIGURE 5: LPS, lutein, and zeaxanthin and various signal pathways. UM were seeded into the culture dishes, treated with LPS with or without previous treatment of lutein or zeaxanthin. p-MAPKs in the cells and NF-κB levels in the cell nuclear extracts were determined by ELISA kit and expressed as percentage of the controls. LPS significantly increased p-JNK (a) and NF-κB levels (d) ($p < 0.05$ as compared with cells not treated with LPS) but not p-p38 MAPK (c) and p-ERG1/2 levels (d) in cultured UM.

and NF-κB signaling pathways. Inhibition of secretion of IL-8 by lutein and zeaxanthin might be explored as a therapeutic approach in the management of uveitis and other inflammatory diseases of the eye.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Lutein Leads to a Decrease of Factor D Secretion by Cultured Mature Human Adipocytes

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Purpose. Complement plays an important role in the pathogenesis of age related macular degeneration (AMD) and trials are currently being conducted to investigate the effect of complement inhibition on AMD progression. We previously found that the plasma level of factor D (FD), which is the rate limiting enzyme of the complement alternative pathway, was significantly decreased following lutein supplementation. FD is synthesized by adipose tissue, which is also the main storage site of lutein. In view of these findings we tested the hypothesis whether lutein could affect FD synthesis by adipocytes. *Methods*. A cell line of mature human adipocytes was incubated with 50 μg/mL lutein for 24 and 48 h, whereafter FD mRNA and protein expression were measured. *Results*. Lutein significantly inhibited adipocyte FD mRNA expression and FD protein release into adipocyte culture supernatants. *Conclusions*. Our earlier observations showing that a daily lutein supplement in individuals with early signs of AMD lowered the level of circulating FD might be caused by blocking adipocyte FD production.

1. Introduction

Age related macular degeneration (AMD) is the leading cause of irreversible visual impairment among older adults in industrialized countries and is now recognized as the third cause of global blindness [1–3]. It is a multifactorial disease with age as the most important risk factor [4]. Modifiable factors like smoking and nutrition also play a role in the development of AMD [5–9]. Of the nutrient factors, the carotenoids lutein and zeaxanthin have been shown to be beneficial in the maintenance of proper vision [10–14]. Patients with AMD often exhibit lower dietary intake of lutein compared to control subjects [15–17]. Further, intervention studies have proven that intake of antioxidant supplements containing lutein can affect the progression to the advanced stages of AMD [10–13].

Lutein and zeaxanthin are the main constituents of the macular pigment [18, 19]. They absorb light between 390 and 540 nm [20–22], thereby shielding the retina from harmful blue light that causes photochemical light damage [23]. In addition, macular pigment is capable of scavenging free radicals [24] which also results in a protective antioxidant effect in the retina [18]. Recently it has been shown that lutein also has anti-inflammatory properties [25], having beneficial effects in various models of experimental ocular inflammation, such as endotoxin induced uveitis [26], retinal ischemia [27, 28], and diabetic retinopathy [29–31]. This may also have implications in AMD, due to an inflammatory mechanism involving the alternative complement pathway that has recently been implicated to play a major role in the pathogenesis of AMD [32].

Various complement proteins have been found to be associated with drusen [33]. These accumulations of extracellular material are located between Bruch's membrane and the retinal pigment epithelium of the eye and their presence is a common early sign of AMD. Genetic studies furthermore

revealed that variants in the genes of complement factors, especially complement factor H (CFH), increased the susceptibility to AMD [34–36]. In addition, various complement activation products were increased in the circulation of AMD patients, providing evidence for a systemic inflammatory component to the disease pathogenesis [37–39].

Whether lutein administration could affect the inflammatory component of AMD is not clear yet. The first clues came from studies showing that administering lutein had a beneficial effect in an experimental model of AMD [40, 41]. Recently, we have reported that daily supplementation with lutein over a time period of twelve months led to a significant decrease in the plasma levels of the complement factors: factor D (FD), C3d, C5a, and sC5b-9 [42, 43].

The activation of the alternative complement pathway involves a number of cleavage reactions and amplification steps whereby complement components interact with each other in a strictly regulated manner. FD is a rate limiting enzyme in the activation sequence of the alternative pathway and as such a key player in complement homeostasis [44, 45]. FD is also known as adipsin, since its main source is adipose tissues, where it is secreted by mature adipocytes [46]. Adipose tissue is also a main storage site for carotenoids such as lutein and zeaxanthin [47–49]. Whether lutein influences FD secretion by adipose cells is unknown and was the subject of the study described here.

2. Methods

2.1. Cell Culture. Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes were obtained from Professor Wabitsch (University of Ulm, Ulm, Germany) [50]. These cells originate from an adipose tissue specimen of an SGBS patient and have been used for a number of studies on adipose differentiation, adipocyte glucose uptake, lipolysis, apoptosis, regulation of expression of adipokines, and protein translocation [51]. SGBS preadipocytes at generation 20 were seeded in 25 cm^2 culture flasks in DMEM/F12 (Catalog number 31330, Invitrogen) containing 1% penicillin/streptomycin (P/S, Invitrogen, Catalog number 15140-122), 3.3 mM biotin (Sigma-Aldrich, Catalog number B-4639), 1.7 mM D-pantothenic acid (Sigma-Aldrich, Catalog number P-5155), and 10% fetal calf serum (FCS) (Gibco Invitrogen, Breda, The Netherlands) and cultured for 6 days to reach 90% confluence. Cells were washed 3 times with phosphate buffered saline (PBS) and then changed to a serum- and albumin-free differentiation medium consisting of DMEM/F12 supplemented with 2 μmol/L rosiglitazone (Cayman, Catalog number 71740), 25 nmol/L dexamethasone (Sigma-Aldrich, Catalog number D-1756), 0.5 mM methyl iso-buthylxantine (Sigma-Aldrich, Catalog number I-5879), 0.1 μM cortisol (Sigma-Aldrich, Catalog number H-0888), 0.01 mg/mL human transferrin (Sigma-Aldrich, Catalog number T-2252), 0.2 nM triiodothyronine (T3, Sigma-Aldrich, Catalog number T-6397), and 20 nM human insulin (Sigma-Aldrich, Catalog number I-1507) at day 0. Medium was refreshed after two days and at day 4 the medium was changed and cells were further cultured in DMEM/F12 supplemented with 0.1 μM cortisol (Sigma-Aldrich, Catalog number H-0888), 0.01 mg/mL human transferrin (Sigma-Aldrich, Catalog number T-2252), 0.2 nM triiodothyronine (T3, Sigma-Aldrich, Catalog number T-6397), and 20 nM human insulin (Sigma-Aldrich, Catalog number I-1507). Cells were incubated in this medium for several days and the culture medium was refreshed every two days. Small lipid droplets became visible after approximately 7 days. After 10 days, approximately 10% are differentiated and showed massive triglyceride accumulation. On culture day 20, the cells were filled with high amounts of intracellular lipids and the differentiation grade was approximately 50–60%. During the whole process, all cells were cultured under humidified atmosphere containing 5% CO_2 at 37°C.

2.2. Experimental Protocol. To study the effect of lutein (Sigma, Switzerland, Catalog number X6250, purity > 99%), the mature adipocytes (for culture detail, see above) were washed three times with phosphate buffered saline (PBS) and then further incubated in serum-free DMEM/F12 medium for one day before treatment. The medium was supplemented with only transferrin, D-pantothenic acid, and biotin. Lutein was dissolved in dimethyl sulfoxide (DMSO, Sigma, Switzerland, Catalog number D8418, BioReagent, for molecular biology, purity ≥ 99.9%) and then serially diluted to the working concentrations with DMEM/F12 culture medium. As vehicle control we used the same medium with 0.5% DMSO solvent. The lutein concentration of 50 μg/mL (v/v) was chosen after performing pilot experiments comparing different concentrations (0.5 μg/mL–50 μg/mL, data not shown).

2.3. Enzyme-Linked Immunosorbent Assay. Supernatants from mature adipocytes cultured with or without lutein were collected after 24 and 48 hours and stored at −80°C for FD measurements. The secretion level of FD was measured at a 1/100 dilution by using a commercially available development kit (DuoSet) for human complement factor D (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

2.4. Quantitative Real-Time PCR (qRT-PCR). The expression of FD mRNA was assessed by quantitative real-time PCR (qRT-PCR). Total RNA was isolated from the mature adipocytes incubated with or without lutein for 48 hours by using the RNeasy Mini Kit (Qiagen Westburg, Leusden, The Netherlands) according to the manufacturer's protocol. The quantity and purity of the mRNA were measured by using the NanoDrop system (Thermo Scientific, USA). 500 ng of each RNA sample was reverse transcribed for the first-strand cDNA by using the *iScript* cDNA synthesis kit protocol (Bio-Rad Laboratories B.V., The Netherlands) and then diluted 40 times with distilled water. The following primers were purchased from Operon (Sigma-Aldrich, The Netherlands) and used for real-time PCR (FD forward 5′-GTCCTGGTGGCGGAGC-3′, reverse 5′-AGAACCTGCACCTTCCCGTC-3′: β-actin forward 5′-GACTACCTCATGAAGATCCT-3′, reverse 5′-GCGGATGTCCACGTCACACT-3′). The mixture reaction contained 12.5 μL SYBR© Green Supermix (Bio-Rad), 5 μL diluted cDNA, and 0.3 μM primers in

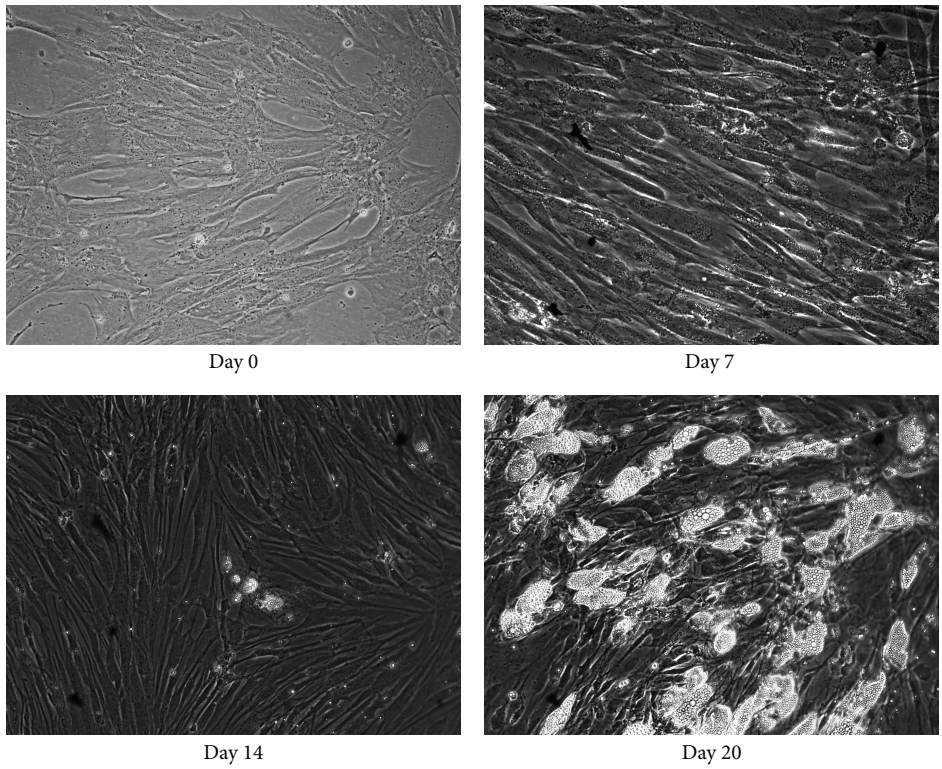

Figure 1: The differentiation process of SGBS preadipocytes to mature adipocytes at days 0, 7, 14, and 20.

a total volume of 25 μL. The two-step qRT-PCR was performed under the following cycling conditions which consisted of an initial denaturation at 95°C for 3 min, followed by 40 alternating cycles of 95°C for 10 sec and 55°C for 45 sec, respectively. All PCR reactions included a cDNA dilution curve to assess PCR efficiency and all reactions were followed by a melt curve (55–95°C). Data were analyzed by using MyiQ Software system (Bio-Rad) and the amount of target cDNA in each sample was determined by a fractional PCR threshold cycle number (Ct-value) and compared to the corresponding Ct-value for the housekeeping gene β-actin. The relative gene expression level for each gene was calculated by using the 2-Delta Delta C(T) method [52].

2.5. Statistical Analysis. Statistical analysis was performed using SPSS 20.0.0; one-way analysis of variance (ANOVA) with Post hoc Bonferroni test was used for data analysis. All values are expressed as mean ± standard error of the mean (SEM). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. The Differentiation Process of SGBS Adipocytes. SGBS preadipocytes were differentiated into mature adipocytes during 20 days. Lipid accumulation became visible after 7 days and increased further during the differentiation period (Figure 1). After 20 days, approximately 50–60% of the cells were fully differentiated as demonstrated by the massive triglyceride accumulation.

3.2. The Effect of Lutein on FD Secretion by Mature Adipocytes. Lutein was added to mature adipocyte cultures and the mean FD concentration in the culture medium was measured at 24 h and 48 h. As control, adipocyte cultures that contain the similar medium with DMSO only (vehicle control) or cells with medium only (blank group) were used (Figure 2). The mean FD concentration increased with time in the blank group from 155.3 ± 3.1 ng/mL at 24 h to 311.8 ± 10.2 ng/mL at 48 h. In the DMSO vehicle control group the mean FD concentration increased from 174.1 ± 4.3 ng/mL to 357.6 ± 14.5 ng/mL between the 24 h and 48 h time points, respectively. Addition of lutein to the cultures resulted in a decreased concentration of FD as compared to the two control groups. Particularly, a significantly decreased FD level was observed when comparing the lutein group with the vehicle control group: 174.1 ± 4.3 ng/mL (DMSO control) versus 133.3 ± 11.9 ng/mL (lutein) ($p < 0.0001$) at 24 h and 357.6 ± 14.5 ng/mL (DMSO control) versus 271.1 ± 38.7 ng/mL (lutein) ($p = 0.002$) at 48 h. When comparing the blank control with the DMSO vehicle control, it is clear that DMSO resulted in a slight increase in the FD production by the adipocytes: 155.3 ± 3.1 ng/mL (blank control) versus 174.1 ± 4.3 ng/mL (DMSO control) at 24 h ($p = 0.017$) and 311.8 ± 10.2 ng/mL (blank control) versus 357.6 ± 14.5 ng/mL (DMSO control) at 48 h ($p = 0.081$).

3.3. Lutein Downregulated the mRNA Expression of FD in Adipocytes. The aforementioned results showed that lutein inhibited FD protein secretion. To examine whether lutein also affects FD mRNA expression in adipocytes we harvested

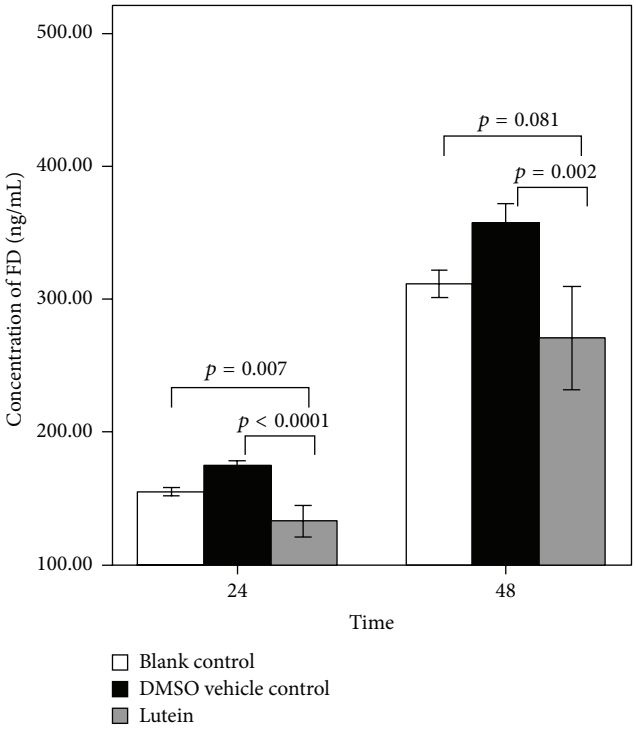

Figure 2: Factor D (FD) concentration in culture medium from 20-day differentiated SGBS adipocytes incubated for 24 h and 48 h in culture medium (blank control), 0.5% DMSO (DMSO vehicle control), or lutein (50 μg/mL) in the presence of 0.5% DMSO (lutein).

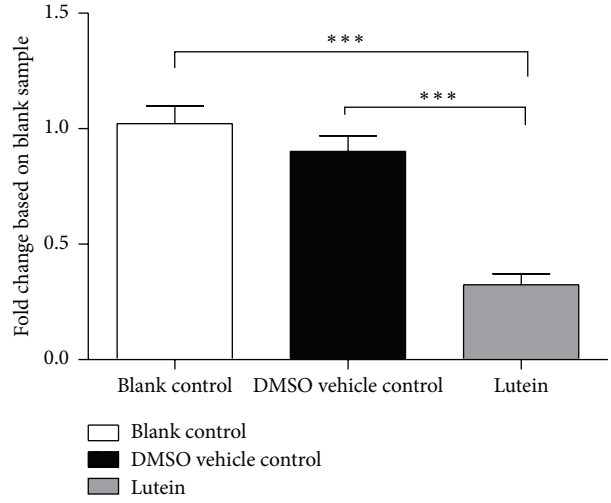

Figure 3: Factor D mRNA expression in 20-day differentiated SGBS adipocytes incubated for 48 h with culture medium (blank control), 0.5% DMSO (DMSO vehicle control), or lutein (50 μg/mL) in the presence of 0.5% DMSO (lutein). Bars represent fold change of FD mRNA expression compared to blank control ($^{***}p < 0.0001$).

20-day differentiated SGBS adipocytes that were incubated for an additional 48 h with lutein. We performed a qPCR analysis whereby FD mRNA expression was measured relative to β-actin mRNA (household gene). The results showed that incubation of mature SGBS adipocytes with lutein for 48 hours significantly downregulated FD mRNA expression when compared to both the blank control and the vehicle control (DMSO) ($p < 0.0001$ and $p < 0.0001$, resp.) (Figure 3). No difference was observed in the mRNA expression between the DMSO group and the blank group ($p = 0.37$).

4. Discussion

In this study we show that lutein suppresses factor D (FD) expression in mature SGBS adipocytes, both at the level of protein secretion and at the mRNA level. To the best of our knowledge, this is the first study to examine the influence of lutein on the expression of FD in human adipocytes. Adding lutein to SGBS adipocyte cultures resulted in a 23% reduction at 24 hours and a 24% reduction at 48 hours of the release of FD as compared to vehicle controls. Adipose tissue is the main source of FD [46] and earlier data using SGBS adipocytes already showed that these cells were able to secrete FD [53]. At the same time, adipose tissue also serves as the main storage site for carotenoids such as lutein and these facts prompted us to study a possible interaction between these two factors [54]. The *in vitro* observations from this study support our earlier *in vivo* findings showing that taking a lutein supplement reduces circulating FD levels [42].

How lutein is taken up by adipocytes is not clear yet. Lutein biosynthesis only occurs in plants, algae, bacteria, and certain fungi. Humans cannot make lutein and uptake is dependent on the dietary intake of certain fruits, leafy vegetables, or eggs [55]. In the gut, lutein is taken up by enterocytes, packed into chylomicrons, and then transported via lymphatics, thoracic duct, and bloodstream to the liver hepatocytes [56]. In the hepatocytes it is bound to lipoproteins and then transported via the blood to various sites in the body including the retina and adipose tissue [57]. Body fat is an important storage site for lutein and it may compete with other tissues, thereby making it less available for the macula [58]. Possible differences in adipose tissue composition and distribution may explain differences observed in lutein metabolism between men and women [59]. Both lutein and factor D have separately been implicated in the pathogenesis of AMD and our observation linking these two factors is a novel observation [60].

The presence of FD in adipose tissue has been attributed to its role in the local cleavage of complement component C3, thereby forming C3a [61]. The carboxy-terminal arginine of C3a is subsequently cleaved by carboxypeptidase N to generate C3a-desarg, also known as acylation stimulating protein (ASP) [62]. ASP interacts with the ASP receptor (C5L2) on adipocytes, thereby triggering triglyceride synthesis [62].

How FD expression is regulated in adipose tissue did not receive much attention yet. FD mRNA expression is increased during the differentiation of preadipocytes to adipocytes [63]. In addition, FD secretion was measured in the medium of SGBS adipocytes but not in the medium of SGBS preadipocytes [53]. On the other hand, *in vitro* stimulation with retinoic acid (RA) resulted in a 4-5-fold

suppression of FD mRNA expression by mouse adipocytes [64].

Under normal conditions an adipocyte will already have a lutein store inside the cells [59]. The amount of lutein in adipose tissue ranges between 0.09 Mol (men) and 0.36 Mol (women) [59], which is a few thousandfold higher than the dose (50 μg/mL is equivalent to 87 μMol) used in our study to load the cells. The SGBS cells we used were cultured under lutein-free conditions and will thus not yet have acquired lutein inside the cells. In our experiments we cultured the SGBS cells for up to 48 hours with lutein with a dose that is much higher than that found in human plasma (0.22 μMol) [25]. Using this short time period we found that lower doses than the 50 μg/mL did not have an effect. Further experiments should be done using both longer and shorter time intervals and with varying doses of lutein to investigate how different adipose lutein levels might affect FD expression in our *in vitro* model. As mentioned above, an adipocyte in the human body already contains a certain concentration of lutein and we believe that the level of lutein in an adipocyte will control the steady-state production of FD. Future experiments with adipose tissue taken from humans whereby lutein content is correlated with FD levels will provide evidence to show whether this hypothesis is correct.

The blood level of ASP (C3a-desarg) has been shown to be increased in patients with AMD and this has added support to the hypothesis that AMD is a disease caused by a hyperactive alternative complement pathway [65]. FD levels have also been found to be increased in the blood of AMD patients [36, 37]. As mentioned earlier, FD is the rate limiting enzyme of the alternative pathway and small changes in its concentration can potentially have profound effects on the generation of biologically active products such as C3a, C3a-desarg, and C5a.

Obesity has been shown to be a risk factor for AMD but the exact role of adipose tissue in the pathogenesis of AMD is not yet clear [7, 66]. Plasma levels of FD are associated with body mass index (BMI) and were shown to be higher in obese versus nonobese subjects [67]. The evidence shown above suggests a possible role for adipocyte biology in AMD pathogenesis [60].

A role for FD in retinal disease became apparent from an experimental mouse model showing that photoreceptors were protected from light induced damage in FD knockout animals [68]. Control of FD has now been brought to the clinic with the availability of a humanized IgG Fab murine anti-factor D antibody (FCFD4514S) that has been shown to block the formation of the alternative pathway C3 convertase [69]. The FD antibody was named Lampalizumab, and phase 1 and phase 2 trials, whereby it was given intravitreally in patients with geographic atrophy, have shown promising results [70] and are now followed by two phase-3 trials (these trials are registered with https://clinicaltrials.gov/).

In conclusion, we have shown that lutein suppresses FD expression in and secretion by human SGBS adipocytes. This observation may explain the decrease in circulating FD with daily lutein supplementation that we observed in an earlier study [42]. It might offer a novel therapeutic approach to prevent the progression of AMD and other inflammatory diseases that are modulated by FD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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