• Basic Research •

The protective effect of zeaxanthin on human limbal and conjunctival epithelial cells against UV-induced cell death and oxidative stress

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Abstract

• AIM: To explore the protective effect of zeaxanthin on human limbal and conjunctival epithelial cells against UV-radiation and excessive oxidative stress.

• METHODS: Human limbal and conjunctival epithelial cells were isolated from cadaver and cultured *in vitro.* They were challenged with UVB radiation and H_2O_2 with and without zeaxanthin pretreatment. Cell viability, p38 and c-JUN NH(2)-terminal kinase (JNK) phosphorylation, IL-6, IL-8 and MCP-1 secretion and malondialdehyde (MDA) content were measured.

• RESULTS: Zeaxanthin had no measurable cytotoxicity on limbal or conjunctival epithelial cells when used at concentrations of 5 μg/mL and below. At 30 mJ/cm^2 UVB, the pretreatment of zeaxanthin increased the percentage of live cells from 50% to 69% (*P*=0.01) and from 66% to 75% (*P*=0.05) for limbal and conjunctival epithelial cells, respectively. The concentrations of IL-6, IL-8 and MCP-1 in the culture medium reduced to 66% (for IL-6 and MCP-1) and 56% (for IL-8) of the levels without zeaxanthin. This was accompanied by reduced p38 and JNK protein phosphorylation. Pretreatment of zeaxanthin also reduced intracellular MDA content caused by H_2O_2 stimulation from 0.86 μmol/L to 0.52 μmol/L (*P*=0.02) in limbal epithelial cells and from 0.96 μmol/L to 0.56 μmol/L in conjunctival epithelial cells (*P*=0.03). However, zeaxanthin did not have significant effect on H_2O_2-induced cell death in limbal or conjunctival epithelial cells.

• CONCLUSION: Zeaxanthin is an effective reagent in reducing the detrimental effect of UV-radiation and oxidative stress on ocular surface epithelial cells.

• KEYWORDS: zeaxanthin; UV-radiation; oxidative stress; malondialdehyde; limbal epithelial cells; conjunctival epithelial cells

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INTRODUCTION

Zeaxanthin [(3R,3'R)-β,β-carotene-3,3'-diol] is one of the two main carotenoid (tetraterpenoids) pigments that exists in high concentration in human retina, especially in the foveal cone cell axons and the Henle fiber layer[1-2]. The other one is lutein which can be metabolized to meso-zeaxanthin [(3R,3'S)-β,β-carotene-3,3'-diol]. Population-based epidemiological studies showed that dietary supplement of zeaxanthin could prevent the progression of age-related macular degeneration (AMD)[3-4]. Dietary supplement of zeaxanthin and lutein may also have protective effect against the development of cataract, diabetic retinopathy and were beneficial for the maintenance of cognitive function in the elderly[5-7]. In healthy humans, combined zeaxanthin and lutein supplement also enhanced visual performance, including reduced glare disability, better visual acuity and contrast sensitivity[8].

Zeaxanthin protects retinal cells from light-induced damage by both physical and biochemical mechanisms. With a maximal absorption of 450 nm, zeaxanthin can serve as a filter for blue light[9]. It can also quench singlet oxygen, scavenge superoxide and hydroxyl radicals, therefore protects retina from the deleterious effects of lipid peroxidation[9-10]. Studies on animal models with added zeaxanthin in the diet and *in vitro* cultured cells showed that it could protect retinal pigment epithelial (RPE) cells, retinal ganglion cells and lens epithelial cells against light-induced toxicity and oxidative stress[10-17].

Ultraviolet (UV) radiation poses a significant risk for the eye throughout life. The cells of the ocular surface, corneal and palpebral conjunctival epithelial cells, in particular, are constantly exposed to UV radiation[18]. Acute high-dose or chronic UV-exposure is associated with ocular surface pathologies including UV keratitis, climatic droplet keratopathy, dry eye disease, pterygium, basal cell carcinoma and squamous cell carcinoma[19]. While many ocular tissues such as the RPE, choroid, peripheral retina, ciliary body and iris contained zeaxanthin, cornea has no detectable amount of zeaxanthin[20]. The effect of zeaxanthin on ocular surface epithelial cells remains unknown. In this study, we analyzed the effect of zeaxanthin on primary cultured human limbal and conjunctival epithelial cells. Our results suggested that zeaxanthin had protective roles for ocular surface cells against UV insult and oxidative stress.

MATERIALS AND METHODS

Ethical Approval The study protocol was approved by the Institutional Review Board of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine and followed the tenets of the Declaration of Helsinki.

Materials Unless otherwise specified, all cell culture medium and supplements were purchased from ThermoFisher Scientific (Gibco). All plastic ware for cell culture was purchased from Greiner Bio-One (Frickenhausen, Germany). General reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and Western blot were purchased from Bio-Rad (Hercules, CA, USA). All primary and secondary antibodies used in this study were purchased from Cell Signaling Technology (Dancers, MA, USA). Zeaxanthin powder was purchased from Sigma-Aldrich (St. Louis, MO, USA). It was dissolved in dimethyl sulfoxide (DMSO), aliquoted in small volume and stored in -80℃.

Limbal and Conjunctival Epithelial Cell Isolation and Culture Primary human conjunctival and limbal epithelial cells were isolated from cadaver corneal tissue as described previously[21]. Briefly, after antibiotics/phosphate-buffered saline (PBS) washing, the small conjunctival tissue attached to the cornea was cut and the limbal rim was excised at the width of about 5 mm for further process.

To isolate conjunctival epithelial cells, the antibiotic-rinsed conjunctival tissue strip was cut into small pieces and placed on cell culture plate with one drop of full medium which contained equal volume of Dulbecco's modified Eagle's medium (DMEM) and F12, 10% fetal bovine serum (FBS), 0.5 μg/mL hydrocortisone, 10 nmol/L cholera toxin, 10 ng/mL human epidermal growth factor (hEGF), 5 μg/mL insulin and antibiotics. Epithelial cell outgrowth was observed 2-3d later and the culture was maintained for 4-5d before the tissues were discarded. The cells were then submerged in the same medium and cultured for further propagation. Passage 2 to 3 cells were used in this study.

Limbal epithelial cells was dissociated from the limbal rim by dispase and trypsin digestion as previously described[21]. Isolated limbal epithelial cells were cultured in supplemented hormonal epithelial medium (SHEM) medium which contained equal volume of DMEM and F12, 2 ng/mL recombinant human epidermal growth factor (EGF), 1 μg/mL bovine insulin, 0.1 μg/mL cholera toxin, 0.5 μg/mL hydrocortisone and 10% FBS in the presence of mitomycin-C inactivated 3T3 fibroblasts. Limbal cells were passaged when more than 70% of the culture dish area was covered by colonies and the majority of the colonies had more than 100 cells.

Cell Viability Assay Twenty thousand cells in 100 μL serum- and growth factor-free culture medium per well were inoculated into 96-well plate and allowed to grow overnight. Different concentrations of zeaxanthin or DMSO at the volume of 5 μL per well was added to desired wells and incubated for another 24h at 37℃ with 5% CO_2. The number of viable cells was analyzed using an MTT-based cell viability assay kit purchased from Sigma-Aldrich. For each experiment, the number of viable cells of the experimental groups were calculated as percentage of the controls according to the following formula: $(OD_{exp}-OD_{con})/(OD_{con}-OD_{blank})$. Here OD_{exp} was the absorbance of the experimental group and OD_{con} was the absorbance of the control group. OD_{blank} was the absorbance of the well which contained the same volume of culture medium but no cells.

Ultraviolet Light Exposure UVB light bulb was purchased from Philips (Philips UVB Narrowband TL 20W/01). The energy delivered to cells was measured using a UV meter (ST513, Sentry Optronics Corp. Taiwan, China). Twenty-five hundred thousand cells were plated on 6-well plates in serum- and growth factor-free medium with 5 μg/mL zeaxanthin (+Z) or DMSO (-Z). After 24h of incubation, cells were gently rinsed with PBS twice and 80 μL of PBS was added to each well. They were then exposed to 0 (-UV), 30 (+UV30) or 45 (+UV45) mJ/cm^2 UVB. The dose of the UV light was monitored by the UV meter which was placed immediately adjacent to the cell culture dish. After the UV exposure, PBS was carefully removed and full culture medium was added to the treated cells. The exposed cells were incubated for 24h before the number of live cells were analyzed as described above.

Hydrogen Peroxide Stimulation Stabilized 30% hydrogen peroxide (H_2O_2) was purchased from Sigma-Aldrich and was added to serum- and growth factor-free culture medium immediately prior use to reach the desired final concentration. Cells were cultured in 6-well plates and gently rinsed with PBS twice before adding the above medium. They were incubated for 1h in H_2O_2 in a standard cell culture chamber with 5% CO_2 at 37℃. After the incubation, the medium was aspired and cells

were gently rinsed twice in PBS. They were given normal medium and resumed culture until further analysis.

Cytokine Analysis Enzyme-linked immune sorbent assay (ELISA) kits for interleukin (IL)-6, monocyte chemoattractant protein-1 (MCP-1) and IL-8 were purchased from BD Biosciences (San Diego, CA, USA) and used following the manufacturer's instruction. Cell culture medium was diluted 5 times in assay buffer for the measurement of IL-6 and IL-8. At the end of the reaction, the plate was read at 450 nm on a microplate reader (BioTek Synergy H4, BioTek Instruments, USA).

Western Blot Analysis Cultured conjunctival and limbal epithelial cells were lysed in RIPA buffer containing 10 mmol/L Tris pH7.5, 150 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, 1 mmol/L ethylenediaminetetraacetic acid (EDTA) and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Total lysates (25 μg) were loaded on SDS-PAGE, transferred to nitrocellulose paper and blotted with antibodies as indicated. Each primary antibody was diluted and incubated as specified by the manufacturer. The membrane was further blotted with specific horseradish peroxidase conjugated secondary antibodies and visualized with SuperSignal chemiluminescent substrates purchased from Pierce Biotechnology (Rockford, IL, USA). For each blot, tubulin was detected simultaneously as loading control.

Malondialdehyde Analysis Malondialdehyde (MDA) content was measured using a commercial kit from Abcam (Abcam, Shanghai, China). Cells in 6-well plate were washed with PBS three times and lysed in 200 μL lysis solution (prepared immediately before use following the manufacturer's instruction). They were scraped off the plate and homogenized in a Dounce homogenizer. The homogenized cell mixture was spun at 13 000 g for 10min at 4℃ and the supernatant was used for analysis. Total cellular protein in the supernatant was also measured and the concentration of MDA was expressed as μmol/L MDA/gram total cellular protein.

Statistical Analysis Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) Version 19 (IBM Corporation, Armonk, NY). Student's *t*-test was used to determine the differences between groups. A probability level of $P<0.05$ was considered statistically significant.

RESULTS

Effect of Zeaxanthin on Limbal and Conjunctival Epithelial Cell Proliferation The effect of zeaxanthin on limbal and conjunctival epithelial cell viability was tested at the concentrations of 1, 5, 25 and 50 μg/mL (Figure 1). At 1 and 5 μg/mL, zeaxanthin had no inhibitory effect on cell viability. However, at 25 μg/mL zeaxanthin, the number of live limbal and conjunctival cells was 72% and 86% of controls, respectively. At 50 μg/mL zeaxanthin, the live limbal and conjunctival cells reduced to 50% and 32% of controls.

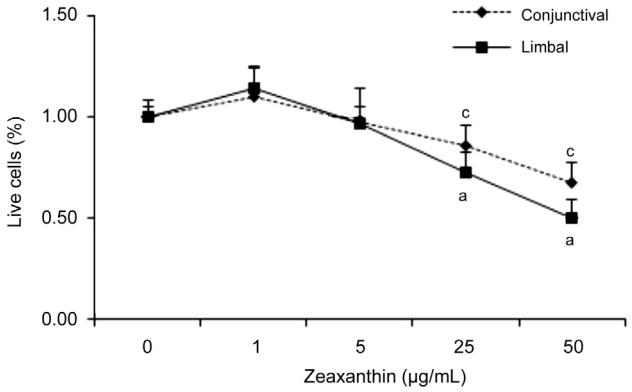

Figure 1 The effect of zeaxanthin on human limbal and conjunctival epithelial cell survival The cells were isolated and treated with 0 (DMSO), 1, 5, 25 and 50 μg/mL zeaxanthin. The experiment was repeated three times in quadruplicated wells each time using cells isolated from three different donor tissues. The data presented here were the averaged results of three experiments. Error bars represented the standard error means. [a,c]Significant differences between the stimulated- and DMSO-treated limbal and conjunctival epithelial cells, respectively ($P<0.05$ by unpaired Student's *t*-test).

Zeaxanthin Protected Limbal and Conjunctival Epithelial Cells from UVB-induced Cell Death Next we examined the effect of zeaxanthin on UVB-induced cell death. The cells were treated with either DMSO (-Z) or 5 μg/mL zeaxanthin (+Z) prior to different doses of UVB exposure. The number of live cells were analyzed at the end of the incubation and the results were presented in Figure 2.

For both cells, UVB radiation caused significant cell death. At the dose of 30 mJ/cm^2, more limbal epithelial cell death (50%) was observed than conjunctival cells (33%). However, both cells showed similar extent of cell death at 45 mJ/cm^2 UVB (about 55%). Pre-incubation with zeaxanthin reduced UVB-induced cell death in both cells. The percentage of live limbal and conjunctival epithelial cells after 30 mJ/cm^2 UV radiation increased from 50% to 69%, and from 66% to 75% with pre-incubation of zeaxanthin, respectively. Similar increase was also found when cells were exposed to 45 mJ/cm^2 UVB.

Zeaxanthin Reduced UVB-induced Secretion of Proinflammatory Cytokines in Limbal and Conjunctival Epithelial Cells UV radiation is known to cause inflammatory responses in cells, which are featured by increased proinflammatory cytokine secretion. To examine if zeaxanthin affected UV-induced cytokine secretion, we measured IL-6, MCP-1 and IL-8 concentrations in conjunctival epithelial cells exposed to 30 mJ/cm^2 UVB radiation with and without 5 μg/mL zeaxanthin pretreatment. The results were presented in Figure 3.

Low concentrations of IL-6, MCP-1 and IL-8 were measured in cells without exposure to UVB and there was no difference between cells with and without pretreatment of zeaxanthin.

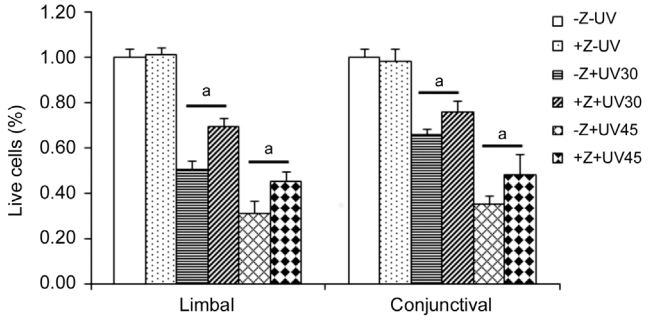

Figure 2 The effect of zeaxanthin on UVB-induced limbal and conjunctival epithelial cell death "-Z" indicated DMSO-treated group. "+Z" indicated 5 μg/mL zeaxanthin-treated group. "-UV" indicated no UV exposure. "+UV" indicated UVB-treated group, and the number indicated the dose. The experiment was repeated three times. In each experiment, the number of live cells in "-Z-UV" group was taken as 100% and used to calculate the percentage of live cells of other groups. Error bars represented the standard error means. [a]Significant difference between the paired conditions (P<0.05 by unpaired Student's t-test).

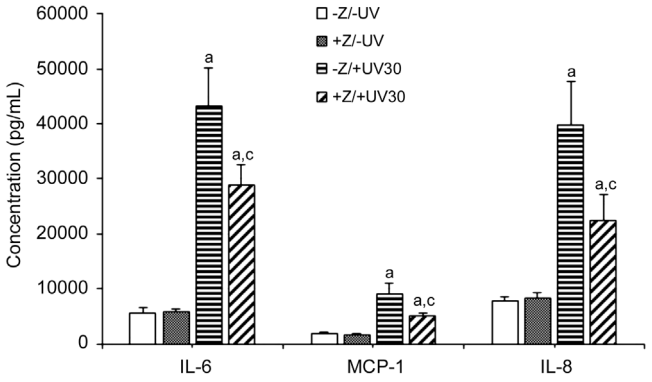

Figure 3 Effect of zeaxanthin on UVB-induced IL-6, MCP-1 and IL-8 secretion in conjunctival epithelial cells The experiment was repeated three times with triplicated wells each time. Error bars represented the standard error means. [a]Significant difference when compared to -Z/-UV controls; [c]Significant difference when compared to -Z/+UV30 (P<0.05 by unpaired Student's t-test).

After 30 mJ/cm^2 UVB radiation, the cells without zeaxanthin pretreatment showed 7.64-, 4.81-, and 5.04-fold increase in IL-6, MCP-1 and IL-8 concentrations, respectively. However, in the presence of zeaxanthin, the fold increase reduced to 5.12-, 3.19- and 2.84-fold for IL-6, MCP-1 and IL-8, respectively. When expressed in percentage, UVB-induced cytokine secretion in cells pre-treated with zeaxanthin reduced to 66% (for IL-6 and MCP-1) and 56% (for IL-8) of what was in cells without zeaxanthin. Similar responses were also observed in limbal epithelial cells (data not shown).

Protective Role of Zeaxanthin Against UVB-induced Cell Death Associated with Reduced Activation of MAP Kinase UV exposure is associated with the activation of protein tyrosine kinases such as p38 and c-Jun N-terminal kinase (JNK). To explore the mechanism of zeaxanthin induced protective effect against UVB-exposure, we examined

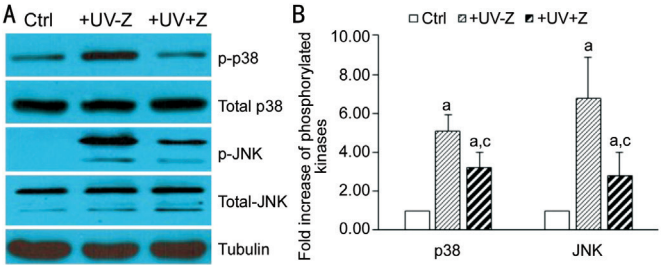

Figure 4 The effect of zeaxanthin on UVB-induced p38 and JNK phosphorylation in human limbal epithelial cells A: Representative Western blot results showing the phosphorylation of p38 (p-p38) and JNK kinase (p-JNK) 30min after UVB-radiation. Ctrl: Cells pretreated with DMSO without subsequent UVB exposure; +UV-Z: Cells pretreated with DMSO and subsequently exposed to 30 mJ/cm^2 UVB; +UV+Z: Cells pretreated with 5 μmol/L zeaxanthin and subsequently exposed to 30 mJ/cm^2 UVB. Tubulin was probed as loading control. B: The averaged densitometry data of p-p38 and p-JNK corrected by total p38 and JNK protein, respectively. The experiment was repeated 4 times using cells from 2 different donors. Data presented were average of all experiments and error bars represented the standard error means. [a]Significant difference when compared to controls; [c]Significant difference when compared to +UV/-Z group (P<0.05 by unpaired Student's t-test).

the phosphorylation of p38 and JNK in limbal epithelial cells with and without zeaxanthin pretreatment prior to 30 mJ/cm^2 UVB exposure. The results were presented in Figure 4. Phosphorylation of p38 and JNK was observed 30min after UVB. In cells pretreated with zeaxanthin, less phosphorylation of both kinases was observed. The results suggested that the pretreatment of zeaxanthin could reduce UV-induced kinase activation.

Zeaxanthin Decreased H_2O_2-induced Lipid Peroxidation but not Cells Death In order to see if the protective effect of zeaxanthin was limited to UV-induced cell damage, we used H_2O_2 to induce oxidative stress in limbal and conjunctival epithelial cells. At the concentration of 100 μmol/L, H_2O_2 caused 2.26- and 1.88-fold increase in cellular MDA concentrations in limbal and conjunctival cells, respectively (Figure 5). When these cells were pretreated with 5 μmol/L zeaxanthin, the MDA concentration reduced from 0.86 μmol/L to 0.52 μmol/L (P=0.02) in limbal epithelial cells and from 0.96 μmol/L to 0.56 μmol/L in conjunctival epithelial cells (P=0.03), which corresponded to 1.37- and 1.36-fold increase, respectively.

Next, we analyzed the effect of zeaxanthin on H_2O_2-induced cell death. Cells isolated from different donors exhibited big differences in the sensitivity to H_2O_2-induced cell death. On average, no statistically significant limbal epithelial cell death was observed when they were exposed to 100 μmol/L H_2O_2. An averaged 16% conjunctival epithelial cell death was observed at the same H_2O_2 concentration. When the concentration of H_2O_2 was increased to 200 μmol/L, 32% limbal and 43%

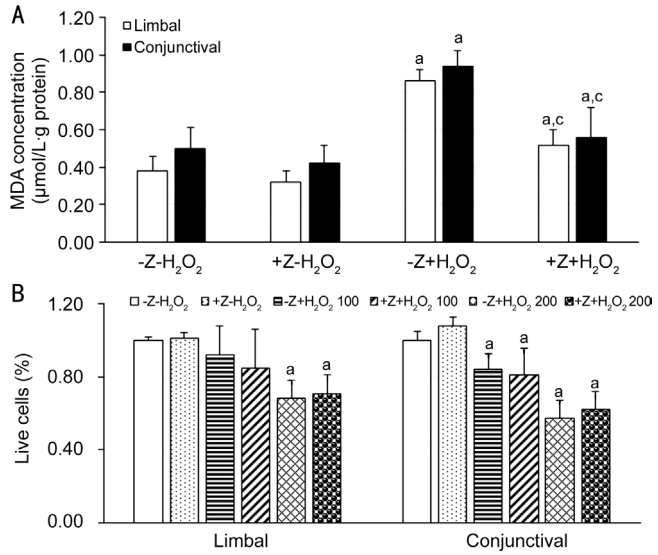

Figure 5 The effect of zeaxanthin on H_2O_2-induced MDA accumulation and cell death A: The concentrations of MDA in limbal and conjunctival epithelial cells in the presence or absence of zeaxanthin and H_2O_2; B: Percentage of live limbal and conjunctival epithelial cells under the treatment of zeaxanthin and H_2O_2. All experiments were repeated three times and the averaged data were presented here. Error bars represented the standard error means. [a]Significant difference when compared to controls (-Z-H_2O_2); [c]Significant difference when compared to -Z+H_2O_2 group (P<0.05 by unpaired Student's t-test).

conjunctival epithelial cells were found dead. However, pretreatment with zeaxanthin did not rescue cells from H_2O_2-induced cell death.

DISCUSSION

In this study, we showed that zeaxanthin alleviated human limbal and conjunctival epithelial cells from UVB-induced cell death and proinflammatory responses. These protective effects were associated with reduced activation of p38 and JNK kinase. We also showed that zeaxanthin was able to reduce hydrogen peroxide-induced lipid peroxidation in the above cells. Collectively, our study demonstrated a protective role of zeaxanthin against UV-induced damage on ocular surface cells.

We showed that a safe and effective concentration of zeaxanthin for corneal surface epithelial cells was 5 μg/mL. At higher concentrations of 25 and 50 μg/mL, a cytotoxic effect of zeaxanthin was observed after 24h of incubation. These results were in agreement with what was reported for RPE and lens epithelial cells[13,15,22-23]. However, zeaxanthin alone or in combination with lutein were also used as biological dye for the visualization of intraocular structures during vitreoretinal surgery and Descemet membrane endothelial keratoplasty[24-28]. In these procedures, it was used at concentrations as high as 20 mg/mL for a short time. Although no abnormality of intraocular structures or other side-effects were reported during the follow-ups of the patients, it would be prudent to reduce the concentration and limit the duration of the application.

The protective role of zeaxanthin on limbal and conjunctival epithelial cells were measured in three aspects: protection against cell death, secretion of proinflammatory cytokines, and lipid peroxidation. In lens epithelial cells, zeaxanthin was shown to reduce UVB-induced lipid peroxidation[11,22]. An anti-inflammatory effect of zeaxanthin was also reported in cultured RPE cells[23]. Our results extended the protective role of zeaxanthin to ocular surface epithelial cells. At molecular level, the mechanisms responsible for the protective roles of zeaxanthin included direct photon absorption, free radical scavenging and the reduction of lipid and protein peroxidation[4]. Here we found that zeaxanthin was effective in alleviating UVB-induced, but not H_2O_2-induced cell death, suggesting that direct photon absorption could be a dominant protective mechanism. Although no significant changes in cell viability was observed in the zeaxanthin-pretreated, H_2O_2-challenged cells, the lipid peroxidation was markedly reduced. The results confirmed the anti-oxidative effect of zeaxanthin on ocular surface epithelial cells.

Our results also showed that limbal epithelial cells were more sensitive to UV radiation than conjunctival epithelial cells. Limbal epithelial cells were different than the terminally differentiated corneal epithelial cells in that they had higher proliferation capacity. The basal layer of human limbal epithelium contained progenitor cells with growth and differentiation capacity to replenish the corneal epithelial cells[29]. A more sensitive apoptotic mechanism upon excessive oxidative stress may be advantageous to preserve the functional integrity of these cells. However, under high dose of 45 mJ/cm^2, the extent of cell death was similar between limbal and conjunctival cells (close to 70%), indicating the lethal effect of acute excessive UV radiation. On the other hand, cultured RPE cells were more resistant to UV radiation as 50% cell death was observed under 50 mJ/cm^2 UVB radiation[15]. This may be due to the presence of melanin in RPE cells.

As the forefront structure of the eye, cornea absorbs most of the UV light reaching the ocular surface, making it a vulnerable tissue to develop various acute and chronic UV radiation related conditions[30]. While the cells of the ocular surface have powerful build-in antioxidant mechanisms, additional UV-absorbing and antioxidative mechanisms are helpful, especially for people who had a high risk of excessive UV exposure, such as welders, mountaineers, surfers and skiers. The results of this study suggested that zeaxanthin could be a useful anti-UV and anti-oxidative stress reagent to offer protection for ocular surface. With more rigorous test of its safety, it may be conceivable to use it as a supplement in topical eye ointment.

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