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Oxidative hazard from blue-light on corneal epithelial cells: protective and anti-oxidant efficiency of lutein and astaxanthin.

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Abstract: Aim of this study was to evaluate *in vitro* the protective and antioxidant properties of the natural compounds lutein and astaxanthin on human primary corneal epithelial cells (HCE-F) exposed to high intensities of blue light. To this purpose, HCE-F cells were irradiated with a blue-light lamp (415-420 nm) at different energies (0 – 20 – 50 - 80 J/cm^2). Lutein and astaxanthin at doses found to be nontoxic (50 – 100 – 250 μM) were added to HCE-F right before blue-light irradiation at the selected dose of 50 J/cm^2. In all experiments, viability was evaluated by the MTT assay and by the production of reactive oxygen species (ROS) using the H2DCF-DA assay. Viability of HCE-F cells progressively decreased from 20 J/cm^2 to 80 J/cm^2, and ROS production dramatically increased at 50 and 80 J/cm^2. The presence of lutein or astaxanthin protected the cells from phototoxicity, with lutein slightly more efficient than astaxanthin also on the blunting of ROS. The association of lutein and astaxanthin was equally effective, and apparently did not give an advantage over the use of lutein alone likely because of the higher efficiency of lutein in blunting blue light radiation, while astaxanthin is more efficient towards the higher wavelengths.

Keywords: Blue-light; lutein; astaxanthin; ROS; corneal cells

1. Introduction

The portion of the electromagnetic spectrum visible to the human eye comprises wavelengths from 400 to 700 nm. Visible light, like all electromagnetic radiations, has energy, which is function of wavelength, so shorter wavelengths are most energetic. Blue–violet light (380 – 500 nm) is the highest-energy portion of the visible spectrum [1]. Sunlight represents the major source of blue-light (25 - 30%), but technological devices such as LED screens also have emission peaks in the blue-light range [2]. Blue light has a pivotal role in color vision, but overexposure to blue light may have harmful effects. Many authors have shown the toxicity of blue light on the posterior segment of the eye because of excessive ROS production, which tend to damage photoreceptor cells and retinal pigment epithelial cells (RPE) [3]. Few studies have investigated the role of blue light on the ocular surface. Blue light triggers ROS generation, which in turn may cause inflammation of the cornea and apoptosis of corneal cells, finally resulting in ocular surface dysfunctions, such as dry eye [4].

In this study, we have developed a model of blue-light hazard on the human corneal epithelial cell line HCE-F. We have tested the known anti-oxidant molecules lutein and astaxanthin in this model to evaluate their ability to protect the corneal surface from blue light hazard.

2. Materials and Methods

2.1 Anti-oxidant moleucles

Lutein was purchased from Molekula Group (cat. no. 29291878) and astaxanthin from Sigma Aldrich (cat. no. 38028884).

2.2 Cell culture

HCE-F cells were isolated from the human cornea of a donor patient after keratoplastic surgery [5]. Cells were cultivated in DMEM-F12 (ATCC, cat. no. 12634010) supplemented with 1% penicillin/streptomycin, 2% FBS (Sigma Aldrich, St. Louis, Missouri, USA, cat. no. F7524) and specific corneal epithelial growth factors (ATCC, cat. no. PCS-700-040), at 37°C in a humidified atmosphere containing 5% CO_2.

2.3 LED Light Source

The LED light lamp emitting at 415-420 nm and with a power of 25 Watt was purchased from Taoyuan Electron (Hk) Ltd (China). The irradiance of the lamp was measured using a power-meter (Thorlabs, Germany) when the lamp was placed 5 cm over the cell layer.

2.4 Blue light irradiation

HCE-F cells were seeded at a density of 2.5 x 10^4 cells per well into 96-well plates and left to adhere overnight at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours, the culture medium was replaced with serum-free medium (SFM) and cells were placed under the blue light LED lamp at a distance of 5 cm for different times so to attain 20, 50, 80 J/cm^2. After each exposition, ROS intracellular content and cell viability were measured as described below.

2.5 Measurement of intracellular ROS

HCE-F cells were seeded at a density of 2.5 x 10^4 cells per well into 96-well plates. The day after, HCE-F cells were incubated in SFM with 0 – 50 – 100 – 250 μM lutein, or 0 – 50 – 100 – 250 μM astaxanthin, or with a mix of 100 μM lutein and 100 μM astaxanthin, and then exposed to blue-light at 50 J/cm^2 (for 30 minutes.). The levels of intracellular ROS were analyzed using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Life Technologies, InvitrogenTM, USA; cat. no. D-399). HCE-F cells were washed once with SFM and incubated with 10 μM H2DCFDA (loading buffer) at 37°C for 30 minutes. The loading buffer was then removed, and the cells returned to SFM. The fluorescence intensity (λ_{ex} = 492 nm, λ_{em} = 517 nm) was measured with the Varioskan™ (Thermo Fisher Scientific, USA).

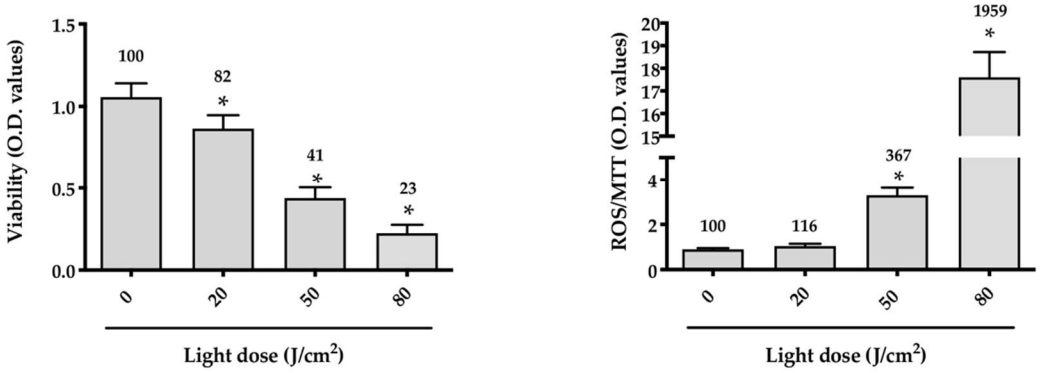
2.6 Cell viability

After the measurement of ROS, the cell viability with the CKK-8 assay (Sigma-Aldrich, cat. no. 96992) was performed in the same plate. Briefly, 10 µl of CKK-8 solution in 100 µl of serum-free medium were added to each well for 1.5 hours at 37°C in a humidified atmosphere containing 5% CO_2. At the end of the incubation time, the absorbance was measured at 450 nm by the plate reader Synergy 2 (BioTek, Thermo Fisher Scientific, USA). O.D. values were used to evaluate cell survival and to normalize ROS levels, according to the manufacturer's protocol.

3. Results

3.1 Blue-LED dose-effect

ROS levels and cell viability of HCE-F cells were evaluated after irradiation with the blue-LED lamp at 0, 20, 50, 80 J/cm². Survival of HCE-F progressively decreased from 20 J/cm² (Figure 1 a) and the amount of ROS increased directly proportional to the irradiation energies (Figure 1 b).

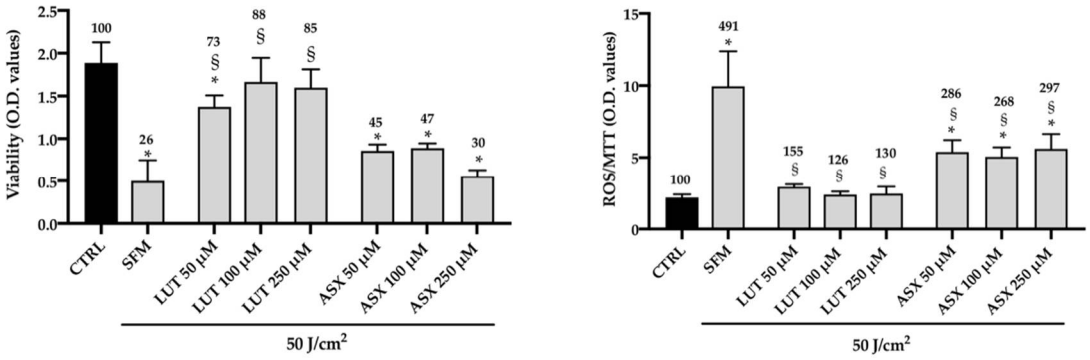


(**a**) (**b**)

Figure 1. (**a**) Cell viability after 0, 20, 50, 80 J/cm² blue light irradiance was evaluated by the CKK-8 assay and reported as mean ± SD of O.D values. (**b**) H2DCFDA assay estimated intracellular ROS content after 0, 20, 50, 80 J/cm² of blue light irradiance; O.D. values were normalized to the respective viability O.D. values. Percent values are indicated over each bar. *$p \leq 0.05$ *vs.* 0 J/cm². One-way ANOVA, followed by Tukey's test.

3.2 Dose-effect response of lutein and astaxanthin

The anti-oxidant power of different doses (50, 100, 250 µM) of lutein and astaxanthin in this photo-stress model was tested at the irradiation energy of 50 J/cm². In the absence of any protection, viability of HCE-F cells decreased by 74% (Figure 2 a) and ROS increased by 391% (Figure 2 b). In the presence of lutein a full recovery of viability could be observed at the highest doses of 100 and 250 µM (Figure 2 a), and all three doses returned the ROS level to control values (Figure 2 b). Astaxanthin was much less efficient than lutein in protecting cell viability (Figure 2 a), despite a significant reduction of ROS, which was however less important than what obtained with lutein (Figure 2 b).

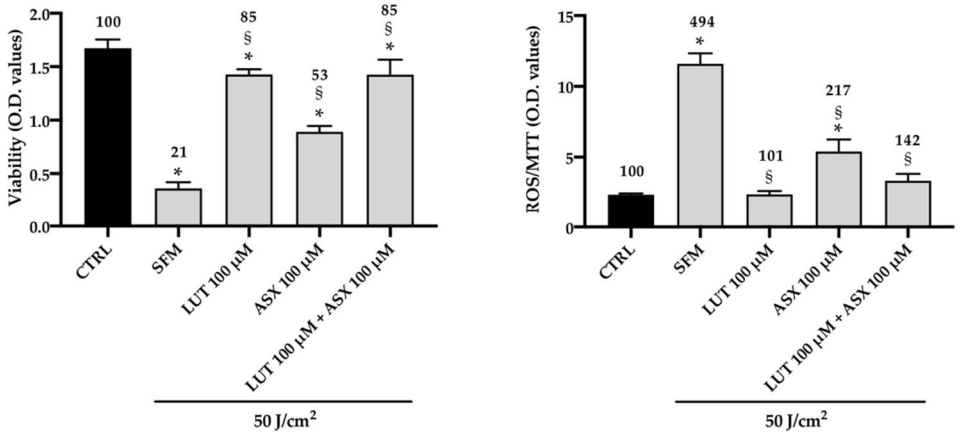


(**a**) (**b**)

Figure 2. (**a**) Cell viability after 50 J/cm² blue light irradiance in presence of lutein 50 – 250 µM, and astaxanthin 50 – 250 µM was evaluated by CKK-8 assay and reported as mean ± SD of O.D values. (**b**) H2DCFDA assay estimated intracellular ROS content in HCE-F cells irradiated at 50J/cm²; O.D. values were normalized to the respective viability O.D. values. Percent values are indicated over each bar. * $p \leq 0.05$ *vs.* CTRL; § $p \leq 0.05$ *vs.* SFM. One-way ANOVA, followed by Tukey's test.

3.2 Cumulative effect of lutein and astaxanthin

To identify a potential additive protective effect of lutein and astaxanthin, HCE-F cells were incubated with a mix of both molecules (100 µM of each anti-oxidant) and exposed at 50 J/cm² blue light dose. The mix resulted to be no better than 100 µM lutein alone in protecting cell viability (Figure 3 a), or decreasing ROS content (Figure 3 b).



(**a**) (**b**)

Figure 3. (**a**) Cell viability after 50 J/cm² blue light irradiance in presence of 100 µM lutein and 100 µM astaxanthin, alone and mixed, was evaluated by CKK-8 assay and reported as mean ± SD of O.D values. (**b**) Intracellular ROS content, performed by H2DCFDA assay; ; O.D. values were normalized to the respective viability O.D. values. Percent values are indicated over each bar. * $p \leq 0.05$ *vs.* CTRL; § $p \leq 0.05$ *vs.* SFM. One-way ANOVA, followed by Tukey's test.

4. Discussion

A moderate light exposure is usually not dangerous to the eye structures, which contain enough endogenous anti-oxidants to cope with this amount of irradiation. However, when the

radiation is more intense and prolonged over time, such as it may happen to people on the snow, or on the sea during a clear and sunny day, it can cause temporary or even permanent blindness [6]. Moreover, most of us are exposed daily to artificial lighting coming from white LED lamps, or LEDs used in the flat screens of TVs, computer monitors, tablets and smartphones. LED lamps generate white light by three methods: 1) by coupling a short wavelength diode with a phosphor emitting a longer wavelength; 2) by coupling an emitting diode in the UV range, coupled to one or more phosphors; 3) by using multiple emitting diodes at different wavelengths in the visible range [7]. In addition, these lamps decay over time, mainly through a bleaching of the phosphor, which is no longer able to absorb blue light: therefore these lamps emit progressively more blue light with the passing of time [8]. It is nowaday well known that blue light can damage ocular tissues, in the back and the front of the eye. Exposure of rats to common LEDs caused apoptosis and necrosis of the photoreceptors in the retina, a significant increase in ROS production and activation of inflammatory cytokines [9,10]. It has also been reported that the blood-retinal barrier architecture was altered by exposure to white LED light [11,12]. A systematic metanalysis showed the correlation between photodamage and AMD onset in humans [13]. Besides, also the eye surface suffers from photo-oxidative damage [14]. In mouse models, it has been shown that over-exposure to blue light could cause oxidative damage and apoptosis of corneal cells, further to eye dryness and increased inflammation of the ocular surface [15–18].

Because photo-oxidative cellular damage is closely related to a dramatic increase of ROS, in this work we have investigated the efficacy of two well-known natural antioxidants: lutein and astaxanthin. Lutein is an antioxidant taken with the diet which accumulates preferentially in the macula, but is found also in other tissues [19]. Lutein is a carotenoid, belonging to the xanthophyll group; its chemical structure counts 40 carbon atoms bound by alternating double and single bonds which make the molecule able to absorbe blue light [20]. It has been demonstrated both *in vitro* and *in vivo* that lutein can protect the retina, not only from photo-oxidative damage but also from inflammation by reducing cytokine expression levels [21,22]. Clinical studies have shown the protective power of lutein against retinal photodamage [23,24]. Astaxanthin is also a carotenoid, present in *Haematococcus pluvialis, Chlorella zofingiensis, Chlorococcum, and Phaffia rhodozyma,* having anti-tumor, anti-diabetic and anti-inflammatory properties [25]. Astaxanthin, having a structure similar to lutein, can absorb the short visible wavelengths too, even if with somewhat lower efficiency. Nonetheless, astaxanthin protects the retina from photodamage by attenuating the apoptosis of ganglionar cells and reducing oxidative stress [26,27]. *In vitro*, it has been reported that astaxanthin exerted its protective activity through activation of the Nrf2-ARE pathway, which increased the expression of phase II antioxidant enzymes such as NAD(P)H quinine oxidoreductase 1 (NQO1) and heme-oxygenase-1 (HO-1) [28–30].

The beneficial effects of plant extracts against the toxic effects of blue light (410–480 nm) on human corneal epithelial cells have also been addressed by some authors, showing the protective role of increased expression of HO-1, Prx-1, CAT and SOD-2 and the attenuation of ROS production [31].

Liu and colleagues developed a topical formulation of lutein with increased stability and residence time on the corneal surface [32]. It has been shown that both oral and topical administration of astaxanthin may effectively protect corneal cells from apoptosis and significantly reduce oxidative stress following UV-induced photokeratitis damage [33,34]. Our results show for the first time the

comparison between lutein and astaxanthin in the protection of human corneal epithelial cells from blue light damage, and indicate a higher efficacy of lutein over astaxanthin, which might be correlated to the different absorption spectrum of the two molecules, with astaxanthin showing an absorption peak at 500 nm, and lutein showing an absorption peak at 450 nm, thus closer to the blue light irradiation at 415 nm used in these experiments [35].

5. Conclusion

The results here illustrated show that both astaxanthin and lutein may protect the corneal epithelium from blue light photo-oxidative damage, though with different efficacy. The association of the two molecules could be expected to be more protective versus white light damage, because the two together would give a wider protection over the full length of the white light wavelengths.

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