Journal of Carcinogenesis & Mutagenesis

Chamorro et al., J Carcinog Mutagen 2013, S6
DOI: 10.4172/2157-2518.S6-008

Research Article Open Access

Photoprotective Effects of Blue Light Absorbing Filter against LED Light Exposure on Human Retinal Pigment Epithelial Cells *In Vitro*

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Abstract

Background: Over the recent years, several researches have speculated about the effects of Light Emitting Diodes (LEDs) radiation on retinal epithelium cells (RPE). Worldwide, most people live exposed to LEDs irradiation incorporated in screens of PCs, phones and TV sets. These lights give rise to the formation of reactive oxygen species and induce mutagenic mechanisms which lead to apoptosis and consequently to degenerative eye diseases, such as age-related macular degeneration (AMD). Thus, it is a priority interest to develop appropriate solutions for the growing industry field of LED light phototoxicity. The aim of this study was to investigate the protective effects of blue light absorbing filters in order to decrease induced apoptosis on human retinal pigment epithelial cells.

Methods: Human retinal pigment epithelial cells were exposed to 3 light-darkness (12 hours/12 hours) cycles of white (T^{a}5400°K), blue (468 nm), green (525 nm) and red (616 nm) LED light. Light irradiance was 5 mW/cm^2. Oxidative stress was evaluated by H2DCFDA staining, mitochondrial membrane potential by TMRM staining, DNA damage by H2AX histone activation, apoptosis by caspase-3 activation, and cell viability by DAPI.

Results: Our results have shown that the use of a blue light absorbing filter decreased cellular apoptosis by 56-89% and DNA damage by 57-81%. A decrease in ROS level production and an increase in cellular viability was also obtained.

Conclusion: This study suggests that blue light absorbing filters may protect against LED lighting photo toxicity and, consequently, provides a photo protector effect.

Keywords: Retinal light toxicity; Apoptosis; Retinal degeneration; Epithelial defects; Free radicals

Background

Due to the relationship between visible-light exposure and increased risk of retinal degenerative diseases observed in humans, mainly Age-Related Macular Degeneration (AMD), over the recent years, several researches have focused on retinal light-induced damage. Given the aging population, the socioeconomic impact of AMD is increasing and, consequently, therapeutic strategies to delay the progression of the pathology are a concern of interest [1].

The idea that light contributes to retinal cell damage arose several years ago. Since then, light damage has been classified into two classes according to the wavelengths of light that gives rise to the greatest level of damage. Noell was the first to determine that the action spectrum of phototoxicity was similar to the action spectrum of rhodopsin, thus it was described as a photochemical damage caused by long exposures to low irradiances which first manifestations are seen in the photoreceptors [2]. Later, Ham described a light damage caused by higher irradiances and shorter exposures than those described by Noell; Ham's damage was explained as a retinal phototoxicity that peaks around 425 nm, and which is initiated in the RPE [3,4].

The human retina is exposed to a high number of artificial lights of different spectra and intensities. Light-emitting diodes (LEDs) are novel light sources that reduce the negative effects of conventional bulbs, regarding to energy use, lifetime and temperature. In the coming years, LEDs will progressively replace incandescent or fluorescent lamps. It is estimated that as of September 1st 2016 there will be no incandescent lights in Europe [5]. However, recent studies have speculated that LED light radiation may cause ocular damage (apoptosis and other cell changes) as LEDs present specific spectral composition and energetic characteristics [5-8].

On the other hand, several studies have demonstrated that ocular photo-oxidative processes can be reduced by the use of lenses with appropriate transmission properties that filter shorter wavelength of the visible spectrum [9-12]. Thus, the purpose of our study was to evaluate the protective effects of a blue light absorbing filter on RPE cells exposed to LED light, focusing on oxidative stress, mitochondrial membrane potential, DNA damage, apoptosis and cell viability.

Methods

Cell culture of human RPE

A human RPE cell line HRPEpiC (ScienceCell Research Laboratories, USA) was used in the study. Cell cultures were grown

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Received September 15, 2013; **Accepted** December 12, 2013; **Published** December 18, 2013

Citation: Chamorro E, Carralero SF, Bonnin-Arias C, Pérez-Carrasco MJ, de Luna JM, et al. (2013) Photoprotective Effects of Blue Light Absorbing Filter against LED Light Exposure on Human Retinal Pigment Epithelial Cells *In Vitro*. J Carcinog Mutagen S6: 008. doi:10.4172/2157-2518.S6-008

in a low-serum epithelial cell culture medium (ScienceCell Research Laboratories, USA). Once confluent, the cells were removed from the culture dish with Trypsin/EDTA solution (Sigma-Aldrich, USA). Cells were plated in a 96 well, black clear Imaging Plate (Becton, Dickinson and Company, USA) with Poly-L-Lysine (Sigma-Aldrich, USA) Coating (density=5000 cells/well). The cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C, and the culture medium was changed every 24 h, following each light phase.

Light exposure

The culture medium was exposed to white light (Tª5400°K), blue light (468 nm), green light (525 nm) or red light (616 nm), in well chambers at an intensity of 5 mW/cm². The light was delivered from a LED-based system for 3 light-darkness (12 hours/12 hours) cycles. The exposition conditions were determined in previous studies; based on the results obtained, we selected 3 cycles of light/darkness as the irradiation provided is enough to produce damage in the RPE cells. On the other hand, we selected a circadian cycle with a darkness phase in order to allow a cell-recovery period in which cells can regenerate partially. Cells were taken for experimentation after the last dark phase (12 h) of the total exposure cycle; thus measures of phototoxicity were done after 12 hours of darkness, under two situations: (1) RPE cells exposed to light without a blue absorbing lens and (2) RPE cells exposed to light with a blue light filtering lens attached under the surface of the culture well and centered over the light beam. Control groups consisted of RPE cells kept in darkness. Figure 1 shows a schematic diagram of the LED light irradiation system, its spectral irradiance and the spectral transmission of the blue light filtering lenses. Table 1 shows the irradiance level (mW/cm²) of LED light on RPE cells with/without protection with a blue light absorbing filter.

Intracellular ROS production

Light-treated and untreated HRPEpiC cells were washed with phosphate-buffered saline, (PBS, Sigma-Aldrich, USA). Then, diluted (1:1000 5-(and-6)chloromethyl-2',7'-dichlorodihydrofluorescein diactate acetyl ester (H2DCFDA, Invitrogen, Germany) was added to the culture medium and incubated at 37°C for 30 minutes. In the presence of ROS, this probe was oxidazed to the highly fluorescent dichlorofluorescein compound (DCF). The fluorescence intensity of the intracellular tapped DCF indicates the amount of intracellular ROS. The average of cellular fluorescence intensity was measured using BD Pathway 855 Bioimager (Becton, Dickinson and Company, USA). Cells were imaged at 492-495 nm (excitation band pass filter) and 517–527 nm (emission cutoff filter) and images were analyzed using Attovision Software (Becton, Dickinson and Company, USA).

Mitochondrial membrane potential (MMA)

After treatment, cells were stained with Tetramethylrhodamine,

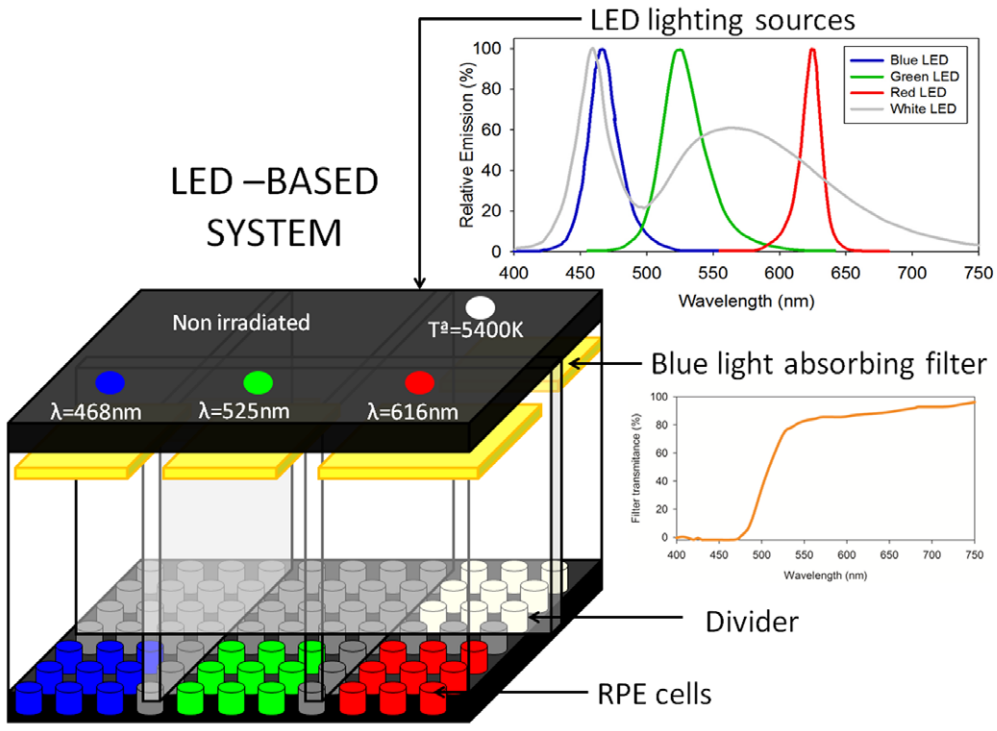

Figure 1: Schematic diagram of the main components of the LED-based system. Spectral irradiance of the different LED lighting sources: white (Tª5400°K), blue (468 nm), green (525 nm) and red (618 nm) and transmittance of the blue light absorbing filter.

	No filter	Filter
White	4.68	3.64
Blue (468 nm)	5.11	1.15
Green (525 nm)	4.54	3.94
Red (618 nm)	5.00	4.67

Table 1: Irradiance level (mW/cm²) of LED light on protected and non-protected RPE cells with a blue light absorbing filter.

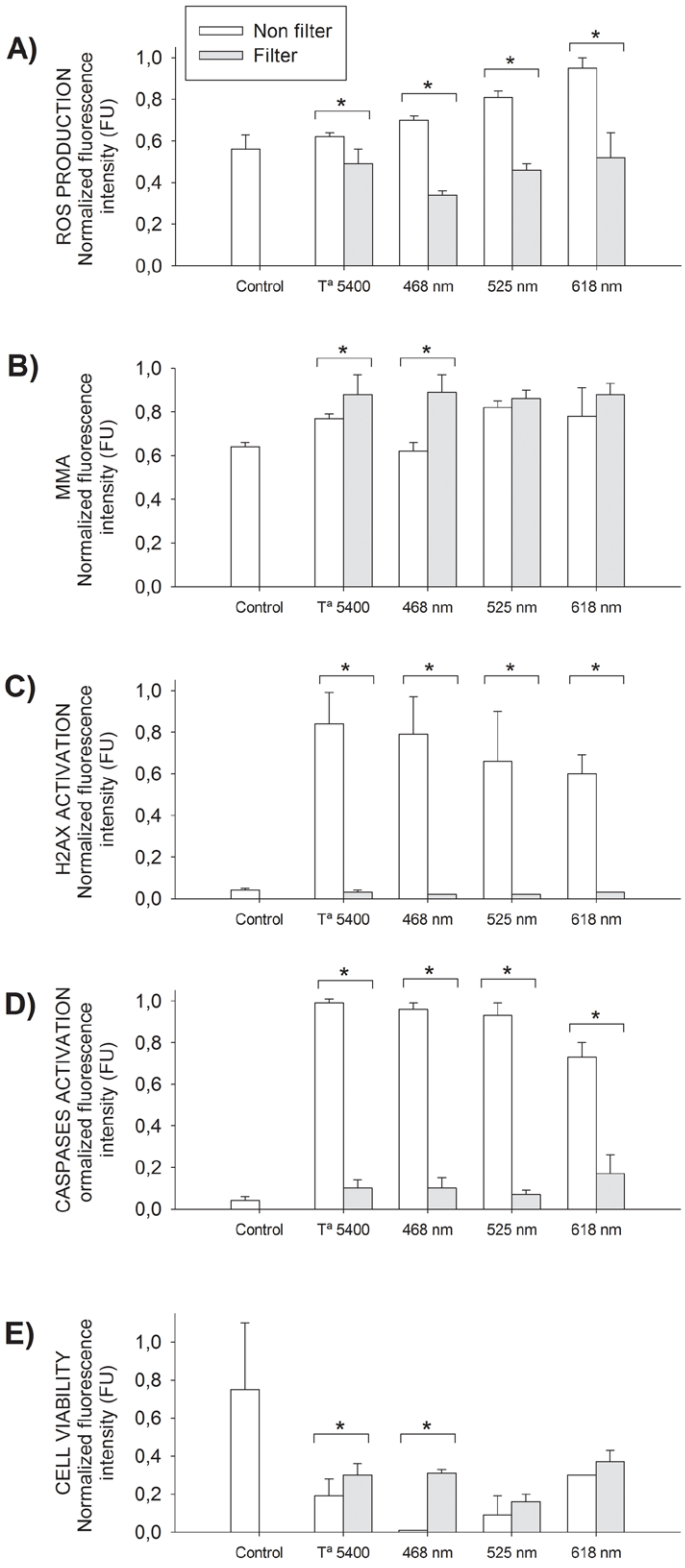

Figure 2: Photoprotective effects of blue light absorbing filter on human RPE cells in vitro. HRPEpiC cells were exposed to white (Tª5400°K), blue (468 nm), green (525 nm) and red (616 nm) LED lighting (irradiated cells) in the presence or not of a blue light absorbing filter for 3 light-darkness cycles (12 hours/12 hours). Control groups consisted of RPE cells kept in darkness. The graph displays mean normalized fluorescence intensity radios of protected cells versus non protected cells. Bars represent mean ± SD from n=3-5 experiments. (A) ROS production determined by the H2DCFDA staining. (B) Mitochondrial membrane potential determined by the TMRM staining. (C) DNA damage determined by the activation of H2AX histone. (D) Apoptosis determined by the activation of caspase-3. (E) Cellular viability of HRPEpic cells determined by labeling all nuclei with DAPI. *p<0.05, t-student test.

methyl ester, TMRM probe (Invitrogen, Germany), a red-orange fluorescent dye that is readily sequestered by active mitochondria. Briefly, cells were incubated with 50 μM TMRM solution for 30 minutes at 37°C. Fluorescence intensity was imaged using BD Pathway 855 Bioimager (Becton, Dickinson and Company, USA) and an excitation band pass filter at 549 nm and an emission cutoff filter at 572 nm.

Caspase 3 activation assay

Caspase 3 activation was assessed by Immunocytochemistry using a caspase 3 antibody (Cell Signaling Technology, USA) that detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175. This antibody does not recognize full length caspase-3 or other cleaved caspases. After light exposure, the cells were washed with PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich, USA) for 1 hour. After the fixation step cells, were washed three times with PBS and permeabilized with 0.3% Triton X100-PBS (Sigma-Aldrich, USA) for 10 minutes, followed by three washing steps. The samples were then blocked with 3%Bovine Serum Albumin (BSA, Sigma-Aldrich, USA) in PBS for 30 minutes and finally anti-Caspase3 antibody was added at 1/350 in PBS-0.5% BSA solution and then cells were incubated for 60 minutes at room temperature. After three washing steps, the secondary antibody Alexa 633 was added at 1/400 in PBS-0.5% BSA solution and then samples were incubated for 60 minutes at room temperature. After three washing steps, the fluorescence of the samples was measured in the Pathway 855 automated fluorescence microscope using an excitation band pass filter at 632 nm and an emission cutoff filter at 647 nm. Caspase 3 is primarily located in the cytoplasm and the algorithm for the image analysis was set to sample an area around the nucleus previously stained with DAPI.

Phosphorilated H2AX detection

After treatment, cells were fixed for 60 minutes with pre-warmed formaldheyde, 4% in PBS, washed with PBS and permeabilized with 0.3% Triton X100-PBS for 10 minutes. Cells were washed twice with PBS and blocked using 3% BSA (in PBS) for 30 minutes. For detection of Anti-gamma H2A.X (phospho S139), an antibody (Abcam, UK) at 3 ug/ml in 0.5% BSA was used. After 1 h at room temperature, the primary antibody was removed. Three washed prepared the cells for the addition of the Alexa Fluor 633 conjugated secondary antibody.

Cell viability

The DNA dye nuclear stain 4'6-diamidine-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich, USA), was added at this point and removed with the secondary antibody after a 1 hour incubation period. Cells were washed and then plates were imaged on the BD 855 Pathway Bioimager. Analysis of the image data was then performed, using Attovision software (Becton, Dickinson and Company, USA).

Statistical analysis

Each experiment was repeated three times. The values were given as mean ± SD. Data was analyzed using an unpaired two-tailed t-test by Statgraphics version Centurion XVI.I (USA). A p value less than 0.05 was considered statistically significant.

Results

Measurement of intracellular ROS production

After 3 light-darkness (12 hours/12 hours) cycles exposure to LED light, ROS production levels increased in the absence of a blue light filter in comparison with non-irradiated cells. However, we found a lower level of reactive oxygen species production in RPE cells protected by a blue light filter. With the blue light filter, differences in ROS production levels were significantly lower than those without the filter for the four light sources: white (T^{a}5400°K), blue (468 nm), green (525 nm) and red (616 nm) LED light (Figure 2A).

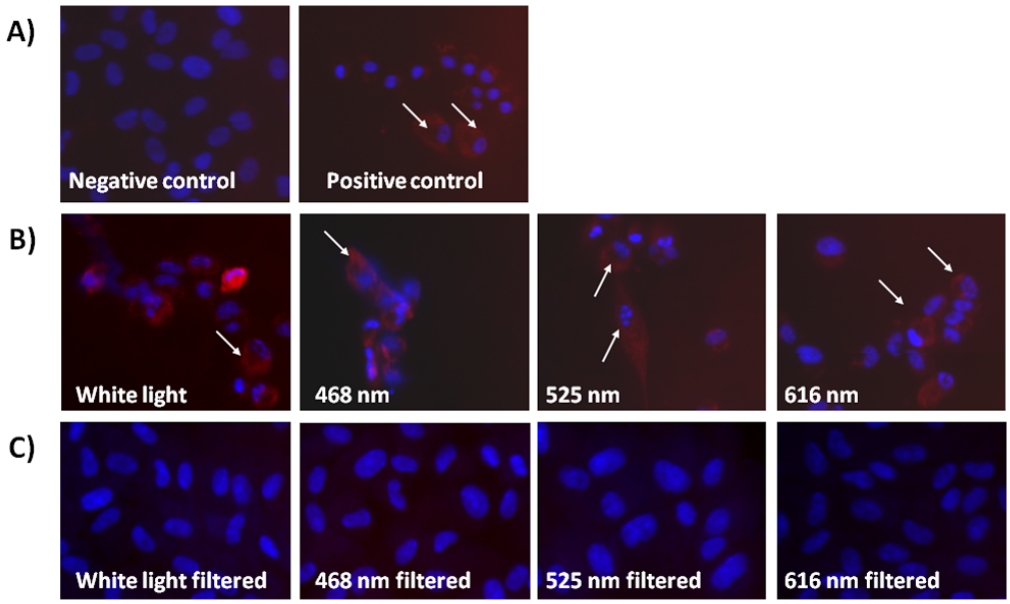

Figure 3: Apoptosis in HRPEpiC cells exposed to white (T^{a}5400°K), blue (468 nm), green (525 nm), and red (618 nm) LED lighting (irradiated cells) for 3 light-darkness cycles (12 hours/12 hours). Apoptosis determined by the activation of caspase-3 is observed as a pink coloration around DAPI-stained cells. The white arrows indicate apoptotic cells. (A) Representative images of RPE cells maintained in darkness (negative control) and RPE cells treated with H_2O_2 to provocate toxicity (positive control); (B) Effects of LED lighting on human RPE cells in vitro without filter; (C) Photoprotective effects of blue light absorbing filter on human RPE cells.

Measurement of mitochondrial membrane potential

After 3 light-darkness cycles of irradiation, no significant effect on mitochondrial membrane potential was detectable compared to control cells (non-irradiated) for any of the different LED lights. Nevertheless, cells exposed to blue (468 nm) and white (T^a5400°K) light have shown a significant increase in mitochondrial membrane potential when cells were protected with a blue light absorbing filter (Figure 2B).

Effects of light on DNA damage of RPE

Significant H2AX activation was observed on not protected RPE cells exposed to LED light, suggesting DNA damage. The fluorescence microscopic data for all irradiated RPE cells show an increased degradation of nucleic acids in comparison with the control cells. However, RPE cells protected with a blue light absorbing filter, normal values of H2AX were found for the four light sources. The inhibitory effects of the blue light absorbing filter on H2AX activation was 81% for white (T^a5400°K) LED light, 77% for blue (468 nm), 64% for green (525 nm), 57% for red (616 nm) monochromatic LED light (Figure 2C).

Detection of apoptosis

Apoptosis determined by the activation of caspases-3 is observed as a pink coloration around DAPI-stained cells. In Figure 3, representative images of RPE cells protected and not protected with a blue light absorbing filter are shown. Fluorescence microscopy shows that the percentage of apoptotic cells was reduced on light exposed RPE cells protected with a blue light absorbing filter in comparison with not protected RPE cells. Cell death reduction due to the filter effects was 89%, 86%, 86% and 56% for white (T^a5400°K), blue (468 nm), green (525 nm) and red (616 nm) LED light irradiated RPE, respectively (Figure 2D).

Cell viability

Under DAPI staining, it was observed that illuminated RPE cells became nonviable. The difference in the cell number of non-protected RPE cells irradiated by LED light and RPE cells protected with a blue light absorbing filter was statistically significant for blue (468 nm) and white (T^a5400°K) LED light ($p<0.01$). Maximum damage reduction was observed in cells exposed to blue LED light. In the experiments 99% and 81% of not protected irradiated cells became nonviable after exposure to blue (468 nm) or white (T^a5400°K) light. The filter inhibits light mediated cell death by 30% for blue (468 nm) light and by 11% for white (T^a5400°K) light. Blue light absorbing filter caused a slight decrease in the number of nonviable RPE cells irradiated with green (525 nm) and red (618 nm) light; however the difference was not statistically significant (Figures 2E).

Discussion

Recent studies have speculated that LED light radiation may cause ocular damage, mainly oxidative stress, which leads to apoptosis [5-8]. However, the potential protective effects of blue light absorbing filter against phototoxicity caused by these new light sources have not been explored. The present study shows that absorbing filters in the blue region of the spectrum may protect RPE cells from the damaging effects of LED light. The results of this study clearly show that a blue light absorbing filter decreases the apoptotic cellular death by 50-89% and inhibits DNA damage by 57-81%, decreases ROS production and increases mitochondrial membrane potential.

The results obtained in our study provide preliminary data that may be clinically relevant in retinal pathologies progress. Some epidemiological studies have demonstrated an association between visible-light exposure and an increased risk in age-related macular degeneration (AMD) progression [9,13-16]. Age-related macular degeneration (AMD) is the leading cause of legal blindness in the population over 60 years in developed countries [17-21]. It is known that the RPE cells play an important role in the development and progression of this pathology. In AMD, the photochemical reaction and oxidative stress induced by light may be the main responsible in the degeneration of RPE cells [16,20,22-25]. The exposure to blue light induces the production of ROS in the mitochondria of RPE cells and leads to an increase in DNA changes, which subsequently result in apoptosis [20,26-29]. The RPE cell induced dysfunction is considered as a prelude to the photoreceptor cell degeneration that characterizes the visual impairment associated with AMD [30,31]. The reduction in phototoxic damage provided by this blue light absorbing filter may be implemented in eyeglasses and contact lenses to protect the retina of the phototoxic effects.

Our results demonstrate an experimental evidence for the damage of white (T^a5400°K) light to RPE cells and the protective effect of a blue light absorbing filter, after 3 light-darkness (12 hours/12 hours) cycles. In cells exposed to white (T^a5400°K) LED light, the filter provides a reduction in light transmission of 22%, that resulted in an 89% decline in apoptotic cells, an 81% reduction in DNA damage and an 11% increase in cell viability. It is relevant to keep in mind that every day the human visual system is exposed to different types of lights, including sunlight and artificial lights. Taking into account blinking time and 8 hours per day of sleep time, the human eye is exposed to 5000 hours of irradiation per year. On the other hand, according to previous measures carry out by the Neuro-Computing and Neuro-Robotics Research Group, general white light contains a 24% of short wavelengths, between 400 and 500 nm. In a society exposed to high amount of phototoxic radiations it seem obvious that eye protection against such types of light is necessary.

Despite transmittance differences of the blue light absorbing filter used in different experiments, our results are consistent with previous reports that a blue light absorbing filter incorporated in an intraocular lens attenuates light-induced damage. Sparrow et al. suggested that a partial blue light absorbing filter produces approximately 80%, 82% and 78% decrease in the death of A2E-laden RPE exposed to blue (430 nm), green (550 nm) and white (T^a5400°K) light [30]. However, Hui et al. demonstrated that partial blue light absorbing filter protects against RPE damage, increasing cell viability to 79.5% in the non-irradiated control, growing free glutathione (GSH) levels and pigment epithelium-derived factor (PEDF) and, decreasing ROS and vascular endothelial grown factor (VEGF) levels in A2E-laden RPE cells [31]. Also, Yanagi et al. found that VEGF production induced by white light exposure was inhibited with a partial blue light absorbing filter in A2E-laden RPE cells and described as an inhibitory effect of light-induced cell damage with the partial blue light absorbing filter. As a result cell survival enhanced up to 42% in the non-irradiated control [17].

The research carried out by Zhou et al. is relevant to our case. Zhou et al. tested the in vitro response of RPE cells exposed to blue light and protected by polycarbonate filters. These filters contain different levels of a blue light absorbing pigment. Cells were irradiated with 430 ± 20 nm light at an output power of 8 mW/cm^2 or 1 mW/cm^2 for 24 hours. The data showed a significantly reduction in cell viability (40%). Moreover, the range of protection was dependent on the transmittance of the filter. Blue light absorbing filter induced an 18-47% reduction in cell death and a 23-50% increase in percent of viable cells, according to the absorption of the filter [9].

It is important to note that previous studies have supplemented the medium with fluorophore A2-E, so RPE cells have A2E accumulation. It has been shown that the accumulation of A2E by cultured RPE confers sensitivity to light-induced damage. Specifically, the short wavelength visible spectrum was found to induce apoptosis of A2E-laden RPE cells [32].

Following Rezai et al. and Kernt et al. [20,33], our study experiments were performed with human RPE cells which did not contain lipofuscin fluorophore A2E accumulation. Our results indicate that, even in the absence of lipofuscin LED light may be toxic and that blue light absorbing lens protects RPE cells from blue light-induced apoptosis. The recent study of Rezai et al. found that partial blue light absorbing filter reduced approximately 49% of RPE cell apoptosis [20]. On the other hand, Kernt et al. showed that the reduction in RPE cell death was accompanied by a significant decrease of the antiapoptotic protein Bcl-2 and increase in Bax and VEGF-A. Thus, supporting the theory that a partial blue light absorbing filter can help to prevent apoptosis in the human RPE exposed to light [33].

We suggest that light with varying intensity and exposure times may have different effects on RPE cells. Apoptosis induced by light is preceded by several events; it is reasonable to consider that illumination initially caused sublethal cellular stress, including a decline of mitochondrial activity, and continued illumination for a longer period induced lethal stress causing cell apoptosis [17]. H2AX activation and Caspases activation were the variables that showed a greater protective effect induced by the blue light absorbing filter. However, no differences were found in mitochondrial membrane potential. This may be due to the characteristics of the light exposure. We used a high light intensity of 5 mW/cm^2 produced by a LED-based system for 3 light-darkness cycles (12 hours/12 hours) with white light (T^a5400°K), blue light (468 nm), green light (525 nm) or red light (616 nm) in well chambers that induced lethal stress. This value can be observedin everyday life situations. So, we have selected this value in order to match with similar studies on this subject [30,31]. This light intensity implies 34.150 lux for an incandescent light source or 33.446 lux for a D65 (skylight) light source. It is alike to the horizontal irradiance for a person looking up to a clear sky day when the sun is around 37.5°C [34] or a person at 20 cm of a 100 w incandescent lamp [35]. Regarding our results, it is necessary to clarify the fact that the blue-light filter decreased the toxic effects of all types of lights used. As it is shown in Figure 1, the filter mainly absorbs the short wavelength. However, it also partially absorbs a smaller percentage of the rest of wavelengths, including long wavelengths. Also, due to the interposition of an element in the light path, there is a decrease in light intensity. This explains why a decrease of damage was found in cells exposed to the long wavelength when protected by a blue light absorbing filter.

Regarding ROS production results, a high level of ROS production could be expected in RPE cells exposed to short wavelength (blue or white light) in comparison to cells exposed longer wavelength (green or red light). However our results show a higher level of ROS production in cells exposed to red and green light than in cells exposed to white and blue light. This is explained by the level of apoptosis. In cells exposed to blue and white light, we observed a high level of apoptosis, thus a low level of surviving cells which can produce ROS. However in cells exposed to red and green light the apoptosis is lower, and so there are more cells that can produce ROS, showing a higher level in this value. However, bearing in mind that the existence of photosensitizes such as riboflavin in the culture medium can affect light-dependent ROS generation [36-38], our results on ROS production must be taken cautiously.

Considering that the results seem to play a very important role in the applied biology in this specific field of the human health, we also have done studies to demonstrate the effects of light in human retina in vivo, in order to demonstrate also in vivo the results obtained using only cellular cultures. These results are part of a PhD and the results are in consideration for another manuscript.

In conclusion, this study has found that the use of a blue light absorbing filter reduces phototoxic damage in human RPE cells exposed to LED light, providing an ocular photoprotector effect. After 3 light-darkness cycles (12 hours/12 hours) exposure to LED light, RPE cells protected by a blue light absorbing filter showed a decrease in apoptotic death accompanied by a decrease in DNA damage and a reduction in ROS levels. Future investigation are necessary in order to develop appropriate solutions for the growing industry field of LED-light phototoxicity.

Acknowledgement

This work has been supported in part by Fundación Mapfre (Spain). This work has been supported in part by Fundación Mapfre (Spain).

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This article was originally published in a special issue, **Apoptosis** handled by Editor(s). Dr. Nancy Turner, Texas A&M University, USA