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Effects Of Light Emitting Diodes Radiations On Human Retinal Pigment Epithelial Cells In Vitro

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ABSTRACT

To investigate the effect of LED lighting on RPE cells, HRPEpiC cells were exposed to 3 light-darkness cycles (12hours/12hours) with blue light (468nm), green light (525nm), red light (616nm). Cellular viability of HRPEpic cells was evaluated by labeling all nuclei with DAPI, ROS production was determined by the H2DCFDA staining and fluorescence microscopy, Mitochondrial membrane potential was quantified by the TMRM staining and fluorescence microscopy, DNA damage was determined by the activation of H2AX histone and apoptosis was evaluated by the activation of caspases-3, -7. The clearly show that LED lighting radiation decrease 75-99% cellular viability and increase 66-89% cellular apoptosis, as well as, increase the production of ROS and cause DNA damage. This study indicates that 3 light-darkness cycles (12hours/12hours) exposure to LED lighting affect to in vitro RPE cells.

INTRODUCTION

Visible light spectrum can be absorbed by biologic chromophores in RPE cells, causing cellular dysfunction and even death of cells (6). The blue region of the spectrum (400-500 nm) although it is out of the UV-A range (1) has relatively high energy and can penetrate through tissues to cells and their organelles. Cell culture studied revealed that blue light directly induces the production of reactive oxygen species (ROS) in RPE mitochondria and leads to apoptosis, potentially triggered by ROS damage mitochondrial DNA (10).

Human observers are exposed to a limited numbers of natural and artificial lights. Light pollution is increasing exponentially in industrialized countries, with more sophisticated light sources, with specific spectra and high intensities. LEDs, or Light-Emitting Diodes, were developed as an energy-efficient option to traditional light bulbs. In the coming years, the light output of LEDs will continue to increase, enabling to progressively suppress the least efficient light sources and replace them by Light-Emitting Diodes (LED). By the first of September 2016, no more incandescent lights will be available in Europe for domestic lighting, and inorganic or organic LEDs could become the next generation light sources (3).

The potential risks of these new light sources need to be explored. Due to specific spectral and energetic characteristics of white LEDs as compared to other domestic light sources, some concerns have been raised regarding their safety for human health and particularly potential harmful risks for the eye (3).

The purpose of the study was to study the effects of LED lighting on RPE cells. Outcome measures included cell viability, oxidative stress, mitochondrial membrane potential, DNA damage and apoptosis.

MATERIALS AND METHODS

Cell culture of human RPE: The human retinal pigment epithelial cell line HRPEpiC (ScienceCell Research Laboratories, USA), was grown in a low-serum epithelial cell culture medium (ScienceCell Research Laboratories, USA). After primary cultures became confluent, the cells were detached from the culture dish with the use of the Trypsin/EDTA solution (Sigma-Aldrich, USA). Cells were plated on 96well, black clear Imaging Plate (Becton, Dickinson and Company, USA) with Poly-L-Lysine (Sigma-Aldrich, USA) Coating (density = 5000cells/well). The cells were incubated in a humidified atmosphere of 5% CO2 and 95% air at 37ºC, and the culture medium was changed every 24 h.

Light exposure: Illumination was produced by a LED-based system. Cells plated on imaging plate were exposed to 3 light-darkness cycles (12hours/12hours) with blue light (468nm), green light (525nm), red light (616nm) or white light in well chambers (light intensity was 5mW/cm2). Although this value is not very frecuent in daylive situations it can be found in several cases, beside we have select this value in order to compare with other studies about this subject (9, 11) This value imply 34.150 lux for an incandescent lightsource or 33.446 lx for a D65 (skylight) lightsource. It is similar to the horizontal irradiance for example for a lying person looking up for clear sky day when the sun is around 37,5º(12) or a person at 20 cm of a 100w incandescent lamp(2)

Control group consisted of RPE cells kept in the dark. Figure 1 shows a schematic diagram of the LED lighting irradiation system and spectral irradiance of LED lighting.

<Figure 1>

Cell viability: Cell nuclei were labeled by incubating the cells with the nuclear stain 4′6-

diamidine-2-phenylindole dihydrochloride, DAPI, (Sigma-Aldrich, USA) for 1 hour. The viable cells were counted under a BD Pathway 855 fluorescence microscope (Becton, Dickinson and Company, USA) and analysis of the image data was performed using Attovision software (Becton, Dickinson and Company, USA).

Measurement of intracellular ROS production: Oxydative stress was measure by using the dye (5-(and-6)chloromethyl-2',7'-dichlorodihydrofluorescein diactate acetyl ester H2DCFDA (Invitrogen, Germany) at a final concentration of 1:1000 for 30 min at 37 C in the dark. Excess dye was removed by washing in PBS. Fluorescence intensity was measured in a BD Pathway 855 Bioimager (Becton, Dickinson and Company, USA) using an excitation band pass filter at 492-495nm and an emission cutoff filter at 517-527nm.

Measurement of mitochondrial membrane potential (MMA): Mitochondrial damage was assessed by using the dye Tetramethylrhodamine, methyl ester, TMRM (Invitrogen, Germany) at a final concentration of 1:1000 for 30 min at 37 C in the dark. Excess dye was removed by washing in PBS. Fluorescence intensity was measured in a BD Pathway 855 Bioimager (Becton, Dickinson and Company, USA) using an excitation band pass filter at 549 nm and an emission cutoff filter at 572 nm.

Immunocytochemical Detection of Histone H2AX and Activated Caspase-3 and -7: DNA damage and apoptosis was evaluated by immunocytochemistry, evaluating the activation of histone H2AX and caspases-3 and -7. At designated time period, cells were washed with phosphate-buffered saline, (PBS, Sigma-Aldrich, USA) and fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) for 1 hour. Cells were suspended in 0.3% Triton X-100-PBS (Sigma-Aldrich, USA) in a 3% Bovine Serum Albumin, BSA (Sigma-Aldrich, USA) 1% (w/v) in PBS for 30 min to suppress. The cells were then incubated in 2.5% PBS+BSA containing either a

combination of 1:400 diluted antiphospho-histone H2AX (Abcam, UK) and 1:400 anticaspase-3 rabbit antibody (Cell Signaling Technology, USA). The cells were then incubated for 1 hour and washed twice with PBS, and resuspended in 1:400 diluted goat antimouse Alexa Fluor 633 conjugated (Invitrogen, Germany) and 1:400 diluted goat antirabbit Alexa Fluor 488 (Invitrogen, Germany) for 30 min at room temperature in the dark. After three washing steps, the fluorescence of the samples was measured in the Pathway 855 automated fluorescence microscope (Becton, Dickinson and Company, USA) using an excitation band pass filter at 632 nm and an emission cutoff filter at 647 nm for caspase -3,-7 detection. For histone H2AX detection, an excitation band pass filter at 488 nm and an emission cutoff filter at 594 nm were used.

Statistical analysis: Each experiment was repeated three times. The values were given as mean±SD. Data were 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

Cell viability

Nonirradiated RPE cells grew well but irradiation inhibited the growth of RPE cells. The difference in cell number of RPE cells irradiated by blue, green or white LED lighting and not irradiated was statistically very significant ($p<0.01$). Maximum damage was observed in cells exposed to blue LED lighting. In the experiments, 99%, 88% and 75% of the cells irradiated became nonviable after blue, green or white light. Red light caused a slight decrease of number of RPE cells. However, the difference in cell number of RPE cells irradiated by red light and not irradiated was statistically nonsignificant (Figures 3A and 4A).

Measurement of intracellular ROS production

Low level production of reactive oxygen species was observed in RPE cells maintained in the dark. However, a significant increase in the level of ROS was observed after 3 light-darkness cycles (12hours/12hours) with blue light, green light or red light. Non increase of cellular cytoplasm fluorescence was detected in cells exposed to white LED lighting in comparison with non irradiated cells (Figures 2A, 3B and 4B).

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 for any of the different LED lighting (Figures 2B, 3C and 4C).

Effects of light on DNA damage of RPE

Significant DNA damage was observed for light exposed RPE cells. The fluorescence microscopic data for all irradiated RPE cells show the increased degradation of nucleic acids in comparison with the control cells. Maximum damage was showed to cells exposed to blue LED lighting (Figures 2C, 3D and 4D).

Detection of apoptosis

Percentage of apoptotic cells was increased on light exposed RPE cells in comparison with RPE cells maintained in the dark. The death of non irradiated RPE cells reached a frequency of 3.7%. However, cell death was 86%, 84%, 66% and 89% for blue, green, red and white irradiated RPE, respectively (Figures 2D, 3E and 4E).

<Figure 2>

<Figure 3>

<Figure 4>

DISCUSSION

Epidemiological studies suggest an association between visible-light exposure and increased risk of advanced age-related macular degeneration (AMD). Visible light can affect the retina and RPE by photochemical, thermal and mechanical mechanism.(13). Experimental evidence has demonstrated that the retina and RPE are much more sensitive to blue light damage than red or green light (7, 8, 5). The most researches have been focused on evaluate the response of the retina to light from conventional lighting sources as halogen or fluorescent.

It has been speculated that LED lighting radiation may cause ocular damage (3), however, the potential risks of these new light sources has not been explored. In this study, we have demonstrated that LED lighting is able to damage RPE cells. The results of this study clearly show that LED lighting radiation decrease 75-99% cellular viability and increase 66-89% cellular apoptosis, as well as, increase the production of ROS and cause DNA damage.

The present results are consistent with previous reports that suggest that visible light of conventional light sources could be (por ser algo más dilomatico?) able to cause cell damage. Sparrow et al (11) analyzed human RPE cells irradiated with blue light (430nm, 8mW/cm2), green light (550nm, 8mW/cm2) and white light (246 mW/cm2).The light was delivered from a tungsten halogen source for 20 minutes and it was observed that illuminated RPE cells remained viable. In another study, Godley et al (6) exposed confluent cultures of human primary retinal epithelial cells to visible light (390-550nm at 2.8mW/cm2) of a metal halide

lamp for 0-9 hours and analyzed cell viability and ROS production. Cells maintained in the absence of blue light exposure showed no decrease in viability, no mitochondrial or nuclear DNA damage and low level production of ROS; however blue light-irradiated cells showed an increasing loss of viability (approximately 10%), time-dependent increase in the levels of ROS and maximal mitochondrial DNA damage 3 hours after exposure with evidence of some repair mechanism.

On the other hand, Chu et al (4) studied changes on viability of RPE as a result of blue and red halogen light irradiation. Early passages of human RPE cells were exposed to blue light (460nm, 0.4mW/cm2) and red light (640nm, 1mW/cm2) for 48 hours. Cell viability was not significantly affected by blue-light irradiation or red-light irradiation at low doses. After that, Youn et al (2009)(14) investigated light-induced retinal damage in human RPE cells exposed to specific narrow wavebands of blue light obtained using interference filters and an arc lamp system(400nm at an irradiance of 1.555 mW/cm2, 420nm at an irradiance of 1.466 mW/cm2 and 435.8nm at an irradiance of 1.351 mW/cm2) for 3-12 hours. Cells exposed to 400 nm light showed decrease in cell viability, degradation of mitochondria and nucleic acids damage; however, no alterations was observed for 420 and 435.8 nm light-exposed RPE cells.

It is relevant the researches carry out by Roehlecke et al (10) in which are evaluated the in vitro response of RPE cells exposed to blue LED lighting. Cells were irradiated with 405nm light at an output power of 0.3 mW/cm2 or 1 mW/cm2 for 3, 24 or 72 hours. The data shown a significantly stimulated ROS production and a decrease of mitochondrial membrane potential after 24 hours of exposure to blue light, but no apoptosis or viability changes was evidenced. They used low doses of light for up to 72 hours without a repair time, in order to establish an in vitro model system in which light irradiation induced mild stress without causing cell death.

It has been suggested that cells may adapt to the light-induced stress and therefore survive (10) so in the present study we have exposed cells to 3 light-darkness cycles (12hours/12hours) instead of continuous light.

In conclusion, 3 light-darkness cycles (12hours/12hours) exposure to LED lighting affect to growth RPE cells, produce cellular stress increasing ROS levels accompanying of an increasing of DNA damage and apoptotic cells. Future investigation will determine the intensities and wavelengths of LED lighting which are lethal and nonlethal for ocular tissues, as well as the effect of optical filters in RPE cells protection. This information will be necessary in order to develop appropriate normative for this growing industry field.

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FIGURE CAPTIONS

Figure 1. Schematic diagram of the LED lighting irradiation system and spectral irradiance of the different LED lighting sources: blue, green, red and white light

Figure 2. Representative images of effects of LED lighting on human retinal pigment epithelial cells in vitro. HRPEpiC cells were exposed to blue, green, red and white LED lighting (irradiated cells) or maintained in the dark (control) for 3 light-darkness cycles (12hours/12hours). A. Cellular viability of HRPEpic cells determined by labeling all nuclei with DAPI. B. ROS production determined by the H2DCFDA staining and fluorescence microscopy; an increase of fluorescence in cells indicates oxidative stress. C. Mitochondrial membrane potential determined by the TMRM staining and fluorescence microscopy. Reduction or absence of fluorescence indicates decrease of MMP. D. DNA damage determined by the activation of H2AX histone. E. Apoptosis determined by the activation of caspases-3,-7. The white arrows indicate apoptotic cells.

Figure 3. Effects of monochromatic LED lighting on human retinal pigment epithelial cells in vitro. HRPEpiC cells were exposed to blue, green and red LED lighting (irradiated cells) or maintained in the dark (control) for 3 light-darkness cycles (12hours/12hours). The graph displays mean fluorescence intensity radios of irradiated cells versus unirradiated controls. Bars represent mean ± SD from n=3-5 experiments. The asterisk (*) indicates significant differences as compared to controls ($p<0.05$, t-student test). A) Cellular viability of HRPEpic cells determined by labeling all nuclei with DAPI. B) ROS production determined by the H2DCFDA staining and fluorescence microscopy. C) Mitochondrial membrane potential determined by the TMRM staining and fluorescence microscopy. D) DNA damage determined

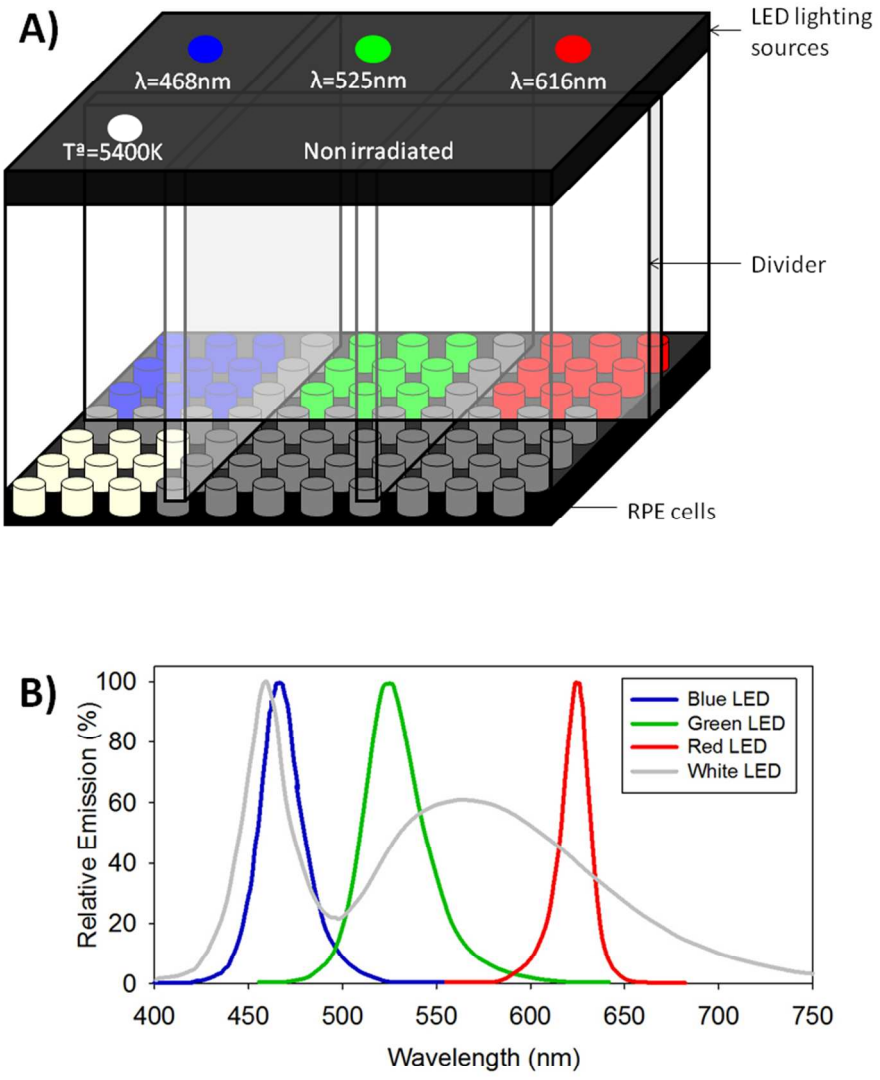
by the activation of H2AX histone. E) Apoptosis determined by the activation of caspases-3, -7 is observed as a pink coloration around DAPI stained cells.

Figure 4. Effects of white LED lighting on human retinal pigment epithelial cells in vitro. HRPEpiC cells were exposed to white LED lighting (irradiated cells) or maintained in the dark (control) for 3 light-darkness cycles (12hours/12hours). The graph displays mean fluorescence intensity radios of irradiated cells versus unirradiated controls. Bars represent mean ± SD from n=3-5 experiments. The asterisk (*) indicates significant differences as compared to controls ($p<0.05$, t-student test). A) Cellular viability of HRPEpic cells determined by labeling all nuclei with DAPI. B) ROS production determined by the H2DCFDA staining and fluorescence microscopy. C) Mitochondrial membrane potential determined by the TMRM staining and fluorescence microscopy. D) DNA damage determined by the activation of H2AX histone. E) Apoptosis determined by the activation of caspases-3, -7 is observed as a pink coloration around DAPI stained cells.

Table 1. Cell viability, ROS production, mitochondrial membrane potential, DNA damage and apoptosis of cultured RPE irradiated with blue, green, red and white LED lighting. Values indicate fluorescence intensity, mean ± SD.

	Control	Blue light	Green light	Red light	White light
Viability (FU)	855±403	10±2*	99±114*	339±1	217±108*
ROS (FU)	593±78	737±19*	855±30*	1004±49*	656±26*
MMA (FU)	634±19	620±39	823±30	780±128	770±18
DNA damage (FU)	131±41	2537±589*	2258±738*	1920±286*	2697±493*
Apoptosis (%)	3.7±0.02	86.1±0.03*	83.9±0.05*	65.5±0.07*	88.8±0.02*

*$p<0.05$ compared to the control



Schematic diagram of the LED lighting irradiation system and spectral irradiance of the different LED lighting sources: blue, green, red and white light
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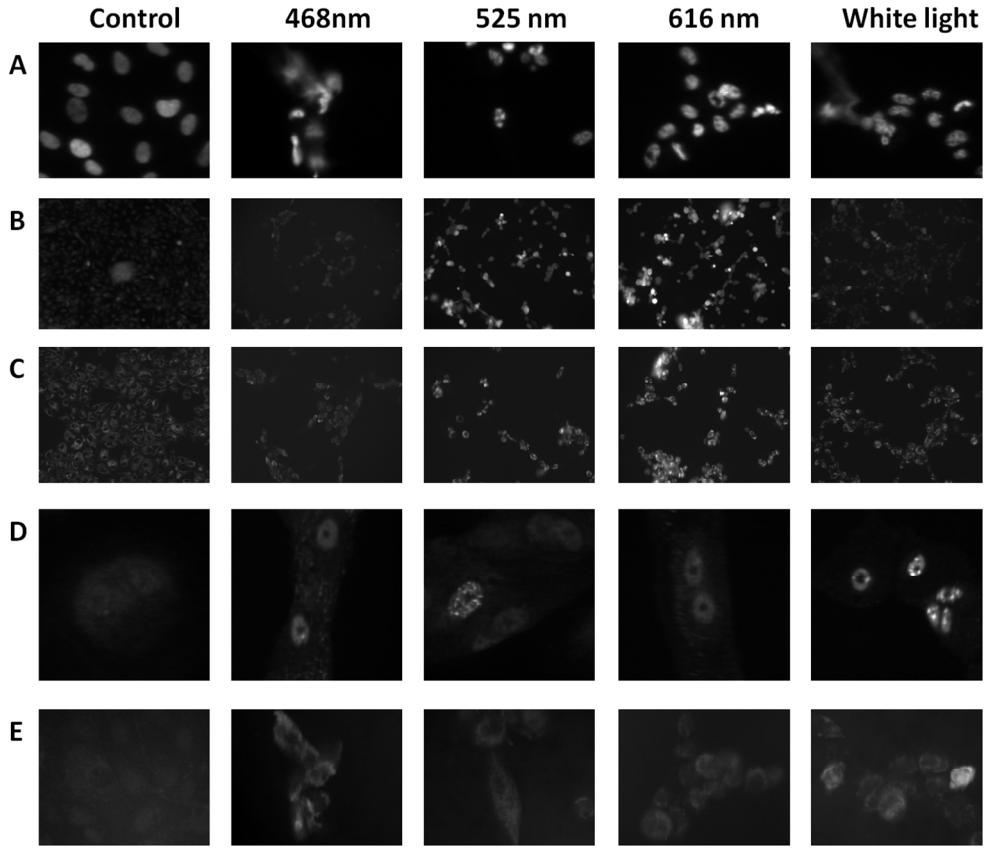

Representative images of effects of LED lighting on human retinal pigment epithelial cells in vitro. HRPEpiC cells were exposed to blue, green, red and white LED lighting (irradiated cells) or maintained in the dark (control) for 3 light-darkness cycles (12hours/12hours). A. Cellular viability of HRPEpic cells determined by labeling all nuclei with DAPI. B. ROS production determined by the H2DCFDA staining and fluorescence microscopy; an increase of fluorescence in cells indicates oxidative stress. C. Mitochondrial membrane potential determined by the TMRM staining and fluorescence microscopy. Reduction or absence of fluorescence indicates decrease of MMP. D. DNA damage determined by the activation of H2AX histone. E. Apoptosis determined by the activation of caspases-3,-7. The white arrows indicate apoptotic cells.
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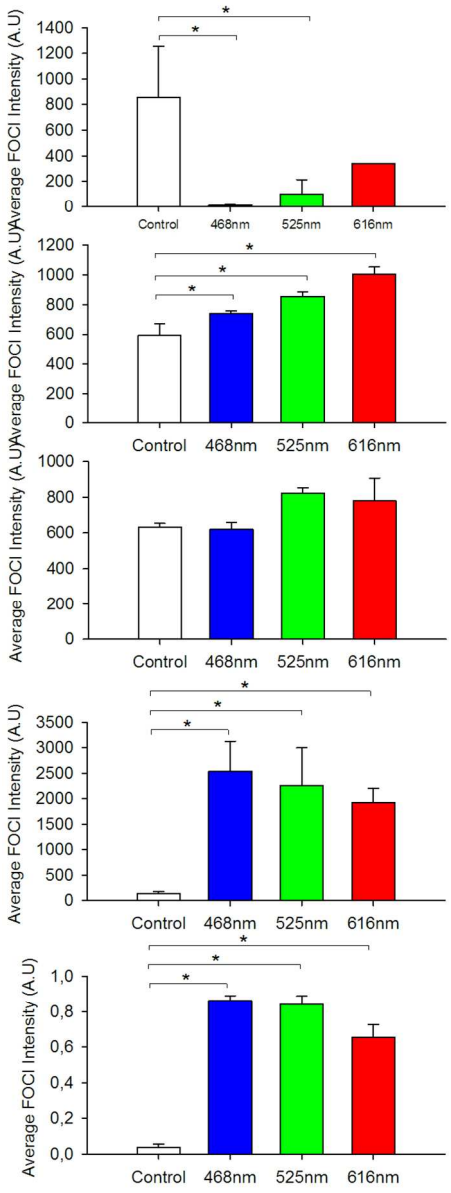

Effects of monochromatic LED lighting on human retinal pigment epithelial cells in vitro. HRPEpiC cells were exposed to blue, green and red LED lighting (irradiated cells) or maintained in the dark (control) for 3 light-darkness cycles (12hours/12hours). The graph displays mean fluorescence intensity radios of irradiated cells versus unirradiated controls. Bars represent mean ± SD from n=3-5 experiments. The asterisk (*) indicates significant differences as compared to controls ($p<0.05$, t-student test). A) Cellular viability of HRPEpic cells determined by labeling all nuclei with DAPI. B) ROS production determined by the H2DCFDA staining and fluorescence microscopy. C) Mitochondrial membrane potential determined by the TMRM staining and fluorescence microscopy. D) DNA damage determined by the activation of H2AX histone. E) Apoptosis determined by the activation of caspases-3, -7 is observed as a pink coloration around DAPI stained cells.
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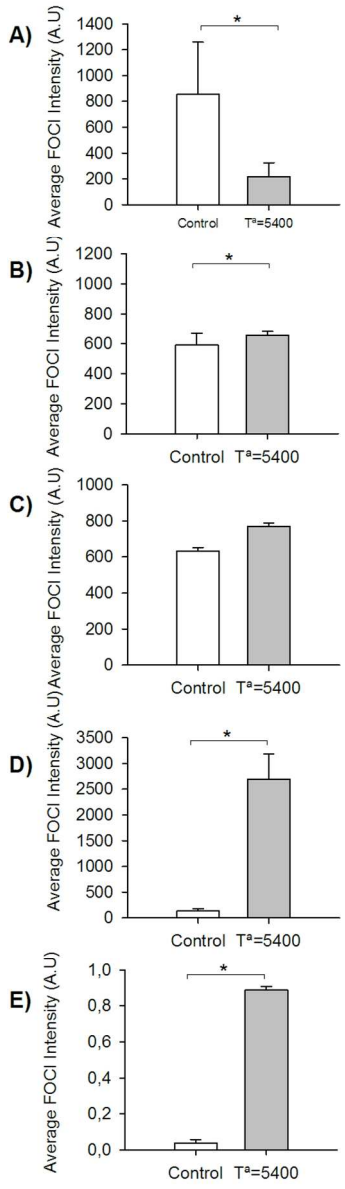

Effects of white LED lighting on human retinal pigment epithelial cells in vitro. HRPEpiC cells were exposed to white LED lighting (irradiated cells) or maintained in the dark (control) for 3 light-darkness cycles (12hours/12hours). The graph displays mean fluorescence intensity radios of irradiated cells versus unirradiated controls. Bars represent mean ± SD from n=3-5 experiments. The asterisk (*) indicates significant differences as compared to controls ($p<0.05$, t-student test). A) Cellular viability of HRPEpic cells determined by labeling all nuclei with DAPI. B) ROS production determined by the H2DCFDA staining and fluorescence microscopy. C) Mitochondrial membrane potential determined by the TMRM staining and fluorescence microscopy. D) DNA damage determined by the activation of H2AX histone. E) Apoptosis determined by the activation of caspases-3, -7 is observed as a pink coloration around DAPI stained cells.
103x297mm (150 x 150 DPI)