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Blue light negatively affects the survival of ARPE19 cells through an action on their mitochondria and blunted by red light

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ABSTRACT.

Purpose: **To ascertain whether red light, known to enhance mitochondrial function, can blunt a blue light insult to ARPE19 cells in culture.**

Methods: **Semi-confluent ARPE19 cells cultured in 10% FBS were subjected to various regimes of treatment with blue (465–475 nm, 800 lux, 26 W/m^2) and red (625–635 nm, 950 lux, 6.5 W/m^2) light, as well as with toxins that inactivate specific enzymes associated with mitochondrial oxidative phosphorylation. Cultures were then analysed for cell viability (MTT assay), mitochondrial status (JC-1), ROS formation, immunocytochemistry and the activation of specific proteins by electrophoresis/Western blotting. In addition, ARPE19 cells were cultured in polycarbonate membrane inserts in culture medium containing 1% FBS. Such cultures were exposed to cycles of red, blue or a combination of red and blue light for up to 6 weeks. Culture medium was changed and the trans-epithelium membrane resistance (TER) of the inserts-containing cells was measured twice weekly.**

Results: **ARPE19 cells in culture are affected negatively when exposed to blue light. This is indicated by a loss of viability, a depolarization of their mitochondria and a stimulation of ROS. Moreover, blue light causes an up-regulation of HO-1 and phospho-p-38-MAPK and a cleavage of apoptosis inhibitory factor, proteins which are all known to be activated during cell death. All of these negative effects of blue light are significantly blunted by the red light administered after the blue light insult in each case. ARPE19 cell loss of viability and mitochondrial potential caused by toxins that inhibit specific mitochondrial enzyme complexes was additive to an insult delivered by blue light in each case. After a time, ARPE19 cells in culture express the tight junction protein ZO-1, which is affected by blue light. The development of tight junctions between ARPE19 cells grown in inserts reached a steady peak of resistance after about 40 days and then increased very slightly over the next 40 days when still in darkness. However, maximum resistance was significantly attenuated, when cultures were treated with cycles of blue light after the initial 40 days in the dark and counteracted significantly when the blue light cycle insult was combined with red light.**

Conclusion: **Blue light affects mitochondrial function and also the development tight junctions between ARPE19 cells, which results in a loss of cell viability. Importantly, red light delivered after a blue light insult is significantly blunted. These findings argue for the therapeutic use of red light as a noninvasive procedure to attenuate insults caused by blue light and other insults to retinal pigment epithelial cell mitochondria that are likely to occur in age-related macular degeneration.**

Key words: ARPE19 cells – blue light toxicity – mitochondria – neuroprotection – red light – trans-epithelial membrane resistance

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Introduction

The mitochondrial theory of ageing argues that the progressive accumulation of mutations or environmental influences on mitochondria or their DNA (mtDNA) results in a reduction in ATP output and an increase in ROS production, thus driving oxidative stress, inflammation and cell loss (Harman 1981; Osborne et al. 2014). This is likely to be the case for age-related

macular degeneration (AMD), where retinal photic injury caused by chronic light exposure and the consequences of oxidative stress may play a part (Beatty et al. 2000; Klein & Klein 2007; Sui et al. 2013). Visible light (400–1000 nm) of defined wavelength might therefore be detrimental to the retina in certain situations over time. In this respect, laboratory studies show short wavelength blue/violet visual light to be particularly damaging to the retina (Organisciak et al. 1998; Algvere et al. 2006; Krigel et al. 2016) with photoreceptors and retinal pigment epithelial (RPE) being mostly affected (Noell et al. 1966; Gorn & Kuwabara 1967; Kuwabara & Gorn 1968; Grignolo et al. 1969; O'Steen & Anderson 1972). The calculated light intensity necessary to cause visual cell damage in nocturnal animals is only two or three times above that of room lighting (Noell et al. 1966; Noell 1979; Organisciak & Vaughan 2010). Such findings have historically been related to the aetiology of debilitating ocular conditions such as AMD, where photoreceptor and RPE dysfunction takes (Sliney 1988; Andley & Chylack 1990; Beatty et al. 2000; Feigl 2009).

Light damage to the retina relates mainly to short wavelength visual blue/violet light and involves the potential activation of a number of retinal chromophores. This inevitably results in the generation of ROS and the activation of a cascade of biochemical events resulting in mitochondrial DNA damage (Godley et al. 2005). Visual pigment chromophores like 11-cis-retinal-protein absorb light across the whole of the visible spectrum. Selectivity is achieved by the different light absorption characteristics of rods and the three different types of cones in the human retina. Rhodopsin is undoubtedly the chromophore responsible for photoreceptor damage in nocturnal rodents but this might not entirely be the case in primates (Rózanowska & Sarna 2005). In some species, photobleaching of rhodopsin and/or other visual pigments can be the cause of the formation of phototoxic products which induce photo-damage in the retina. The chromophore all-trans-retinal (vitamin A aldehyde) is one of the products of photo-bleached rhodopsin and is abundantly present in the retina; it is particularly sensitive to blue light absorbance for cell toxicity (Boulton et al. 1990). Also, retinal chromophores like melanin complexes and lipofuscin in RPE cells (Boulton et al. 1990) increase in content with age (Feeney-Burns et al. 1984) and generate various types of ROS when irradiated with visual light, causing RPE death (Sarna 1992; Boulton et al. 2001; Margrain et al. 2004).

Mitochondria, abundant in photoreceptors and RPE, also absorb visual light. Flavin and cytochrome oxidases associated with mitochondria absorb visual light of different wavelengths in different ways. Proteins like the mitochondrial enzyme cytochrome *c* oxidase (COX), associated with mitochondrial complex IV, absorb blue light maximally at around 400–410 nm (Mellerio 1994). Flavins such as riboflavin (vitamin B2) and flavoprotein nucleotides are essential components of numerous cytosolic and mitochondrial enzyme systems. Absorption spectra of flavins are around 420–520 nm and induce the oxidation of several substances as well as the generation of hydrogen peroxide when activated (García-Silva et al. 1997; Hockberger et al. 1999). Porphyrins are also targets of blue light and exist in the mitochondrial inner membrane; therefore, they are potentially affected by blue light (Gorgidze et al. 1998; Wataha et al. 2004).

Experimental studies on cells that lack pigment show mitochondrial functions to be negatively affected by blue/violet (400–450 nm) light, reducing the formation of ATP and increasing the stimulation of ROS to result in damage to lipids, proteins and DNA (Jung et al. 1990; Egorov et al. 1999; Chen et al. 2003; King et al. 2004; Godley et al. 2005; Wood et al. 2007; Osborne et al. 2008, 2016; Knels et al. 2011). In contrast, long wavelength visual red light (600–1000 nm) has no negative influence on cells in culture and while also absorbed by mitochondrial COX (Karu et al. 2004; Karu & Kolyakov 2005; Wong-Riley et al. 2005; Begum et al. 2013), enhances ATP production, leading to a number of beneficial effects, including attenuating cell death. It is thought that photon absorption-stimulation of COX by red light might involve the photo-dissociation of inhibitory nitric oxide, which binds to the copper and heme centres of COX thus preventing oxygen from gaining access to the active sites (Lane 2006). As a consequence, oxygen consumption and ATP production may increase, thereby raising the mitochondrial membrane potential (Passarella et al. 1984). Red light-induced enhancement of mitochondrial function can therefore result in a spectrum of beneficial effects that include attenuating oxidative stress, inflammation and cell death (Wong-Riley et al. 2005; Liang et al. 2006; Ying et al. 2008; del Olmo-Aguado et al. 2012; Núñez-Á lvarez et al. 2017).

The current experiments involve the use of ARPE19 cells (spontaneously immortalized from human RPE) which are essentially devoid of pigments such as melanin complexes and lipofuscin. The aim of the present studies was to understand more about the manner in which blue light negatively affects mitochondria and to see whether red light can blunt the process. We hypothesize that RPE mitochondrial electron transport dysfunction caused by ageing and the influence of short wavelength blue light is a major cause of their demise in AMD and that long wavelength (red to infrared) light might be a potential noninvasive way to counteract this process (Fitzgerald et al. 2013; Eells et al. 2016; Merry et al. 2017).

Materials and Methods

Human ARPE19 cells (ATCC, Wesel, Germany) were grown in a culture medium that consisted of DMEM-F12 solution (Sigma, St Louis, MO, USA), supplemented with 2% antibiotic penicillin/streptomycin (Sigma) and 10% FBS and in a humidified atmosphere of 5% CO_2, at 37°C. Doubling growth time was approximately 60 hr. About 100 μl, 300 μl or 1.5 ml cultures of cells (approximately 10×10^4 cells/ml) were placed in 96-, 48- or 6-well plates, respectively. In addition, 200 μl of cells was placed in 24-well plates, containing 6.5 mm, 0.4 μm pore polycarbonate membrane inserts (Corning Ref.3413).

After approximately 24 hr, to allow for the cells to settle in 96- or 48-well plates, cultures were generally subjected to one of the following regime treatments. Either, 30 hr in dark, 18 hr blue light followed by 12 hr dark, 18 hr dark followed by 12 red light, 18 hr in blue light followed by 12 red light, 18 hr dark followed by 12 hr curcumin and 18 hr blue light followed by 12 hr curcumin. Blue (465–475 nm, 800 lux, 26 W/m^2) and red light (625–635 nm, 950 lux,

6.5 W/m^2) LEDs (Electro DH SL, Barcelona, Spain) were used to deliver light to the cultures and temperature monitored to always be at 37°C.

Cell viability was assessed by the MTT reduction assay modified from that of (Mosmann 1983). Briefly, cells in 96-well plates were subjected to the appropriate treatment and then MTT was added at a final concentration of 0.5 mg/ml for 75 min at 37°C. Medium was then removed and reduced MTT (blue formazan crystals) was solubilized by adding 100 μl DMSO to each well. After agitation of the plates for 10 min, the optical density of the solubilized crystals was measured using an automated microplate reader at 570 nm wavelength.

For analysis of mitochondrial status, cells were incubated with JC-1 dye (2 μg/ml) for 30 min. JC-1 accumulates in mitochondria to appear as a red/orange fluorescence (590 nm) in healthy organelles and when depolarized, green in fluorescence (530 nm). Fluorescence images of cells were recorded and the relative levels of the intensities of green/red JC-1 fluorescence quantified using ImageJ Software (U.S. National Institutes of Health, Bethesda, MD, USA); statistical analysis was carried out by use of GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, CA, USA).

For the analysis of ROS, culture medium was removed and cells incubated with dihydroethidium (40 μM) for 20 min, the medium was removed and the cells washed twice with fresh medium. Images of the cultures were immediately recorded using phase fluorescence/contrast microscopy. ROS formation was determined by measurement of the ratio fluorescence at 370–420 nm (for cytoplasm of living cells in blue) with that of 535–610 nm (for chromatin of living cells in red) and analysed for significance using GraphPad Prism version 7.00 for Windows.

After approximately 24 hr to allow for cells to settle in 6-well plates, they were subjected to one of the following regime treatments. Either in darkness, 3 hr blue light followed by 12 hr red light, 6 hr blue light followed by 12 hr red light, 12 hr blue light followed by 12 hr red light, 18 hr blue light followed by 12 hr red light. Cell cultures were ultimately then subjected to analysis either by Western blotting or immunocytochemistry. For immunocytochemistry, ARPE19 cell cultures were fixed in cold methanol or 4% paraformaldehyde for 10 min followed by washing in phosphate buffer. After incubation in goat serum (10% in phosphate buffer or PBS) for 60 min and washed in PBS, cultures were then exposed overnight at with either anti-ZO-1 (Thermo Fischer, Waltham, MA, USA, 1:200) or anti-HO-1 (Enzo LS, Farmingdale, NY, USA, 1:500). After washing with PBS, cultures were then exposed for 2 hr to appropriate secondary antibody conjugated either to Alexa Fluor 488 or to Alexa Fluor 594 (1:300), washed in buffer. In some cases, DAPI (0.2 μg/ml) was added to a wash solution. Images were obtained using a Leica DMI6000B fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

For Western blot analysis, cells were collected by scraping them from culture wells, followed by centrifugation and resuspension in a cocktail lysis buffer (approximately 0.1 ml/mg pellet) that contained phosphatase and protease inhibitors (Sigma, Aldrich). After freezing and thawing in combination with sonication, protein content was determined using a bicinchoninic acid protein kit (Sigma-Aldrich). Defined amounts of protein and sample buffer (2 M Tris/HCl, pH 6.8, containing 8% SDS, 40% glycerol, 8% mercaptoethanol and 0.002% bromophenol blue) were then mixed together and immediately heated for 5 min at 95°C. Equal amounts of proteins were fractionated by electrophoresis using 10% polyacrylamide gels containing 0.1% SDS. Proteins were transferred to 0.22 μm nitrocellulose membranes and were incubated overnight at 4°C with one of the following primary antibodies: anti-actin (MAB1501, Millipore, Burlington, MA, USA) 1:4000, anti-p38 (ab31828, Abcam, Cambridge, UK) 1:1000, anti-p38 phosphorylated (ab45381, Abcam) 1:1000, anti-apoptosis inhibitory factor (AIF) (sc-9716, Santa Cruz Biotech, Dallas, TX, USA), 1:500 or Anti-HO-1 (ADI-SPA895, Enzo LS), 1:1000 Detection was then performed with appropriate biotinylated secondary antibodies. The final nitrocellulose blots were developed with a 0.016% w/v solution of 3-amino-9-ethylcarbazole in 50 mM sodium acetate (pH 5.0) containing 0.05% (v/v) Tween-20 and 0.03% (v/v) H_2O_2. The colour colorimetric reaction was stopped with 0.05% sodium azide/PBST solution and the density of the individual bands quantified using ImageJ software.

ARPE19 cells grown in suitable inserts develop tight junctions and have a measured trans-epithelial membrane resistance (TER) potential (Dunn et al. 1996; Qin & Rodrigues 2010). ARPE19 cells were therefore grown in 6.5 mm, 0.4 μm pore polycarbonate membrane inserts (Corning Ref.3413) in 24-well plates for up to 80 days. In the initial 7 days, the culture medium contained 10% FBS and maintained in the dark. Thereafter, the culture medium only contained 1% FBS. Treatment regime began after week 2 and continued for a total period of 80 days. At after week 2, cultures were either maintained in darkness or subjected to 48 hr cycles of light: (1) 12 hr blue light followed by 36 hr in darkness (2) 12 hr red light followed by 36 hr darkness or (3) 12 hr blue light followed by 12 hr darkness, followed by 12 hr red light and 12 hr darkness. Blue (465–475 nm, 400 lux, 13.6 W/m^2) and red light (625–635 nm, 950 lux, 6.5 W/m^2) LEDs were used to deliver light to the cultures and temperature monitored to always be at 37°C. Trans-epithelial membrane resistance (TER) measurements of ARPE19 cells grown in polycarbonate membrane inserts were made with the Volt-Ohm Meter (EVOM) from Millipore (MERS00002) using a STX2 probe. Trans-epithelial membrane resistance (TER) values were recorded twice a week, and each measurement was the average from three recordings 20 min apart. Trans-epithelial membrane resistance (TER) values for each well of cells were the media of the results in $\Omega \cdot cm^2$.

Results

Initially, we determined the concentration of the mitochondrial toxins rotenone (inhibitor of complex I), 3-nitropropionic acid (3-NP or inhibitor of complex II), actinomycin A (inhibitor of complex III) and sodium azide (inhibitor of complex IV) required and the intensity of blue light necessary to cause 30–35% loss of ARPE19 cell viability over a period of 12 hr (Fig. 1). Surprisingly, when cells were first exposed to blue light and then immediately to the complex inhibitors, each for 12 hr, cell loss was more-or-less additive in all

cases (Fig. 1). However, when exposed to a defined intensity of red light that had no influence on its own, it significantly blunted the toxic effect of the various mitochondrial toxins (Fig. 1). Cell viability results were mirrored by the staining of cells with the mitochondrial dye JC-1 (Fig. 2). JC-1 is taken up by mitochondria and appears green in colour in cells exposed to blue light or mitochondria toxins that also reduce cell viability (Figs 1 and 2). In contrast, JC-1 staining in healthy mitochondria is red in colour and this redness is elevated when cells are exposed to red light (Fig. 2). Moreover, and importantly, red light blunts the effects of blue light in terms of the conversion of JC-1 red stain into a green-coloured complex.

Figure 3 shows the viability of ARPE19 cells in culture subjected to an 18 hr insult with blue light followed by exposure to no light or red light for a further period of 12 hr. In such conditions, blue light causes a 50% loss of cell viability and red light clearly attenuates this influence. The blunting of cell viability caused by blue light is not specific for red light but can also be attenuated by the use of curcumin (1 μM). Figures 4–7 show that the loss of cell viability caused by a blue light insult (Fig. 3) is associated with the

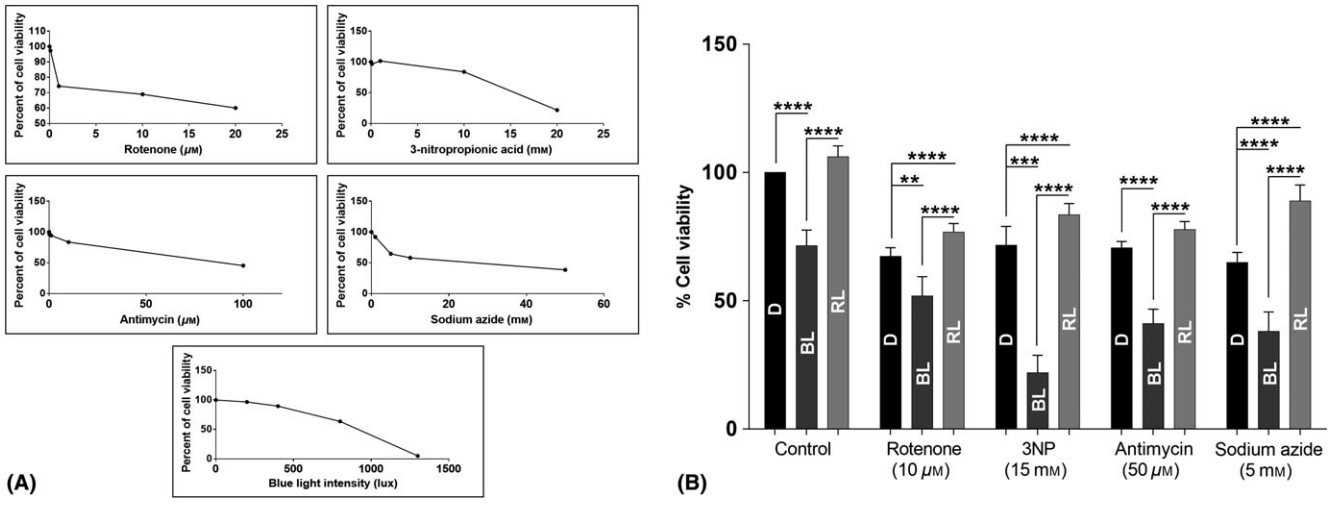

Fig. 1. Left panel shows the effect of various mitochondrial toxins and blue light on the viability of ARPE19 cell cultures. Each value is the mean from six independent experiments. Right panel shows the negative effect of specific amounts of mitochondrial toxins and blue light (BL) to cause a loss of approximately 30–35% ARPE19 cell viability compared with untreated control cells in the dark (D). Blue light in each case exacerbated cell death significantly while red light (RL) had the opposite influence. Results are expressed as mean ± SEM where $n = 6$. Statistics was determined by a two-way ANOVA with Tukey's multiple comparisons test where ***$p < 0.001$, **$p < 0.01$.

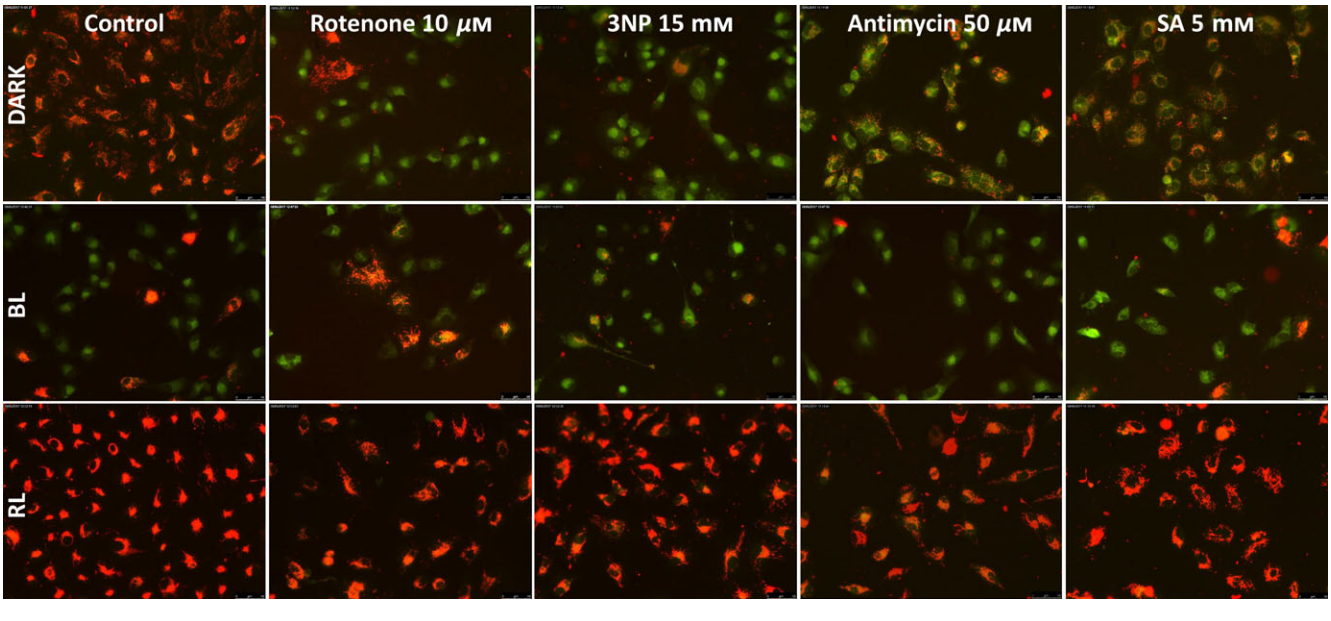

Fig. 2. ARPE19 cell cultures stained with JC-1 dye following exposure to different mitochondrial toxins in the dark or in the presence of blue (BL, 26 W/m^2) or red (RL, 6.5 W/m^2) light. The upper panels show that in comparison with the control cells (Dark), where mitochondria containing JC-1 appear red in fluorescence, they are green in fluorescence following their exposure to the toxins. Blue light also causes mitochondria to appear green and exacerbates the intensity of the green fluorescence in cells already exposed to toxins (middle panels). The bottom panels show that the negative effect of blue light or toxins on cell mitochondria is blunted by red light, where the dye appears as a red fluorescence.

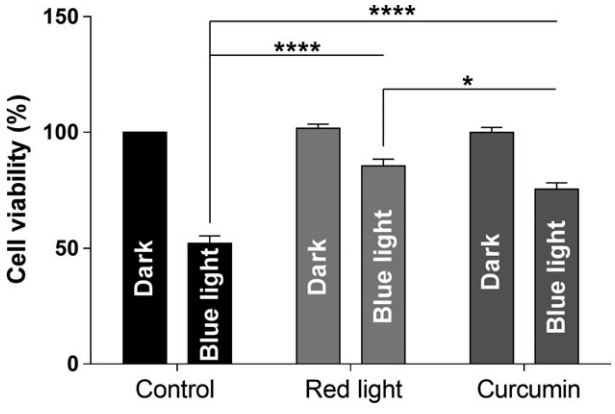

Fig. 3. This figure shows that the negative effect of a blue light (26 W/m^2, 18 hr) insult on the viability of ARPE19 cells compared with cells in the dark is blunted by subsequent exposure to either red light (6.5 W/m^2, 12 hr) or curcumin (1 μM, 12 hr). Neither red light nor curcumin caused loss of cell viability on their own. Results are expressed as mean ± SEM where $n = 6$. After two-way ANOVA statistical analyses, Sidak's multiple comparison test shows significant differences (***$p < 0.001$, *$p < 0.1$).

depolarization of mitochondria (Figs 4 and 5) and the generation of ROS (Figs 6 and 7). Thus, both visual and semi-quantitative analyses show that blue light causes the depolarization of mitochondria (Figs 5 and 7) and an elevation of ROS (Figs 6 and 7) which is significantly blunted by red light.

The analysis of Western blots of protein extracts derived from cells exposed to blue light for various times followed by 12 hr of red light is shown in Fig. 8–10. Three hours of blue light caused no significant effect on AIF (Fig. 8) and a slight stimulation of phospho-p-38-MAPK production (Fig. 9) without HO-1 appearing to be affected (Fig. 10). However, with increasing exposure to blue light, AIF is fragmented to produce a 57 kDa product and a greater up-regulation of phospho-p-38-MAPK and HO-1 occurs. Significantly, the degree of fragmentation of AIF and the up-regulation of phospho-p-38-MAPK and HO-1 when they occur are attenuated by red light. This can be demonstrated histologically for HO-1, where the elevated HO-1 immunoreactivity caused by blue light is blunted by red light (Fig. 11).

As shown in Fig. 12, confluent ARPE-19 cells in culture after about a week develop the clear staining of ZO-1 tight junction proteins between each other. Although we did not attempt to quantify the amount of ZO-1 immunoreactivity in confluent ZO-1 cultures, after 7 days, it was clearly evident that the exposure of such cells to blue light resulted in a loss of ZO-1 immunoreactivity (Fig. 12), as well as cell numbers (not quantified). Significantly, the loss of ZO-1 immunoreactivity caused by blue light (especially after 12 and 18 hr) administration was attenuated when red light (950 lux) was present (Fig. 12).

ARPE-19 cultures grown in 24-well plate inserts (Corning, Ref.3413) over 80 days show an increase in the

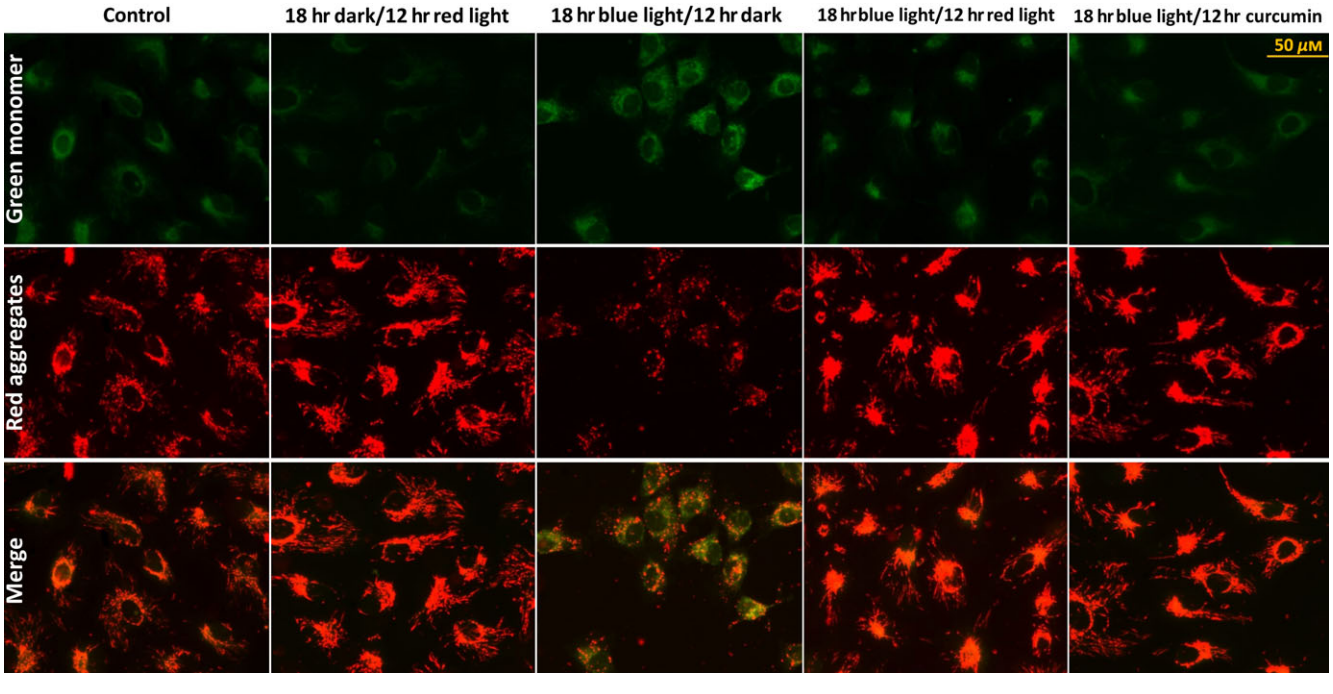

Fig. 4. Cultures of ARPE19 cells exposed for 12 hr to red light (6.5 W/m^2) or curcumin (1 μM) alone or after a blue light (26 W/m^2) insult (18 hr) and stained immediately thereafter with JC-1 dye. The upper two rows of panels show that mitochondria in control cells (dark) alone or exposed to red light exhibited intense red fluorescence JC-1 aggregates and low level green fluorescence JC-1 monomer suggesting their healthy status. In contrast, the negative influence of blue light caused an enhancement of green fluorescence JC-1 monomer and a reduction of red JC-1 aggregates. This effect caused by blue light is clearly reversed following exposure to red light or curcumin. The lower panels show merged images of green/red fluorescence of the JC-1 dye.

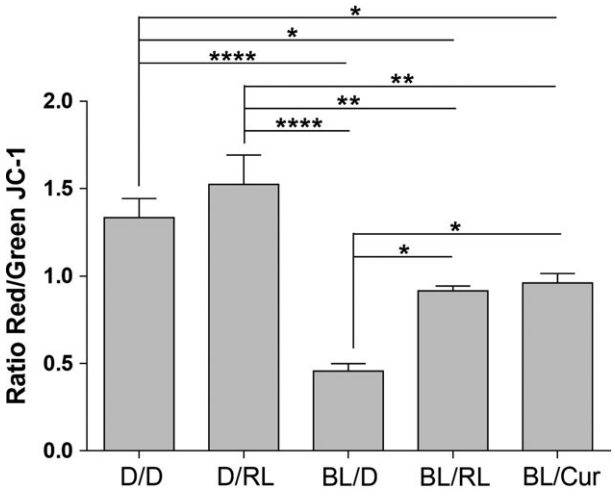

Fig. 5. Quantitative analysis of red (590 nm) and green (530 nm) fluorescence intensities in images as shown in Fig. 4. D/D, experiment in dark; D/RL, 18 hr in dark followed by 12 hr in red light; BL/D, 18 hr in blue light followed by 12 hr in dark; BL/RL, 18 hr in blue light followed by 12 hr in red light; BL/Cur, 18 hr in blue light followed by 12 hr in curcumin. The results show that a blue light insult (26 W/m^2) has a negative effect on mitochondrial activity (ratio between red and green JC-1 is reduced compared with cells in the dark or exposed to red light) and blunted by a subsequent exposure to red light (6.5 W/m^2) or curcumin (1 μM). Results are expressed as mean value ± SEM, n = 4; One-way ANOVA with Holm–Sidak's multiple comparison test (***p < 0.001, **p < 0.01, *p < 0.1).

measurement of TER over time (Fig. 13), which reflects the continuous development of tight junction formation between cells. Such cells were either maintained in darkness or repeatedly exposed after 40 days to 48 hr cycles of light. Peak resistance of TER was more-or-less established to be approximately 130 Ωcm^2, (±SEM, n = 3) after 40 days and increased slightly thereafter over the next 40 days. This increase in TER was elevated clearly when cells were instead exposed to cycles of red light appearing to reach a peak resistance of 140 Ωcm^2 after 80 days. However, TER was significantly reduced in cells in cultures treated after day 40 with cycles of blue light (26 W/m^2) and reached a low of 30–40 Ωcm^2 after day 80. Importantly, use of cycles of blue and red (6.5 W/m^2) light ligated the negative influence on TER elicited solely by blue light with the resistance after 80 days being around 100 Ωcm^2.

Discussion

Considerable evidence supports a role for mitochondrial dysfunction, oxidative stress and inflammation in the onset and development of AMD (Beckman & Ames 1998; Gouras et al. 2016; Datta et al. 2017). Oxidative stress to the retina can be caused by exposure to blue light (King et al. 2004; Lin et al. 2011; Yacout & Gaillard 2017) which results in altered mitochondrial bioenergetics (Jarrett & Boulton 2007) and RPE mtDNA damage (King et al. 2004; Lin et al. 2011; Yacout & Gaillard 2017) all of which are features associated with AMD. Importantly, studies on AMD tissue show specific mtDNA haplogroup variants occurring within the mitochondrial region that encodes for proteins of the electron transport chain (Canter et al. 2008; SanGiovanni et al. 2009), a decreased number of mitochondria (Feher et al. 2006) and greater damage to their mtDNA (Karunadharma et al. 2010). The present study was therefore focused on understanding more about the biochemical events related to blue light mitochondrial-induced toxicity to RPE cells and to determine whether red light blunts this process. The overall aim of the study was to support the premise that red or infrared light can

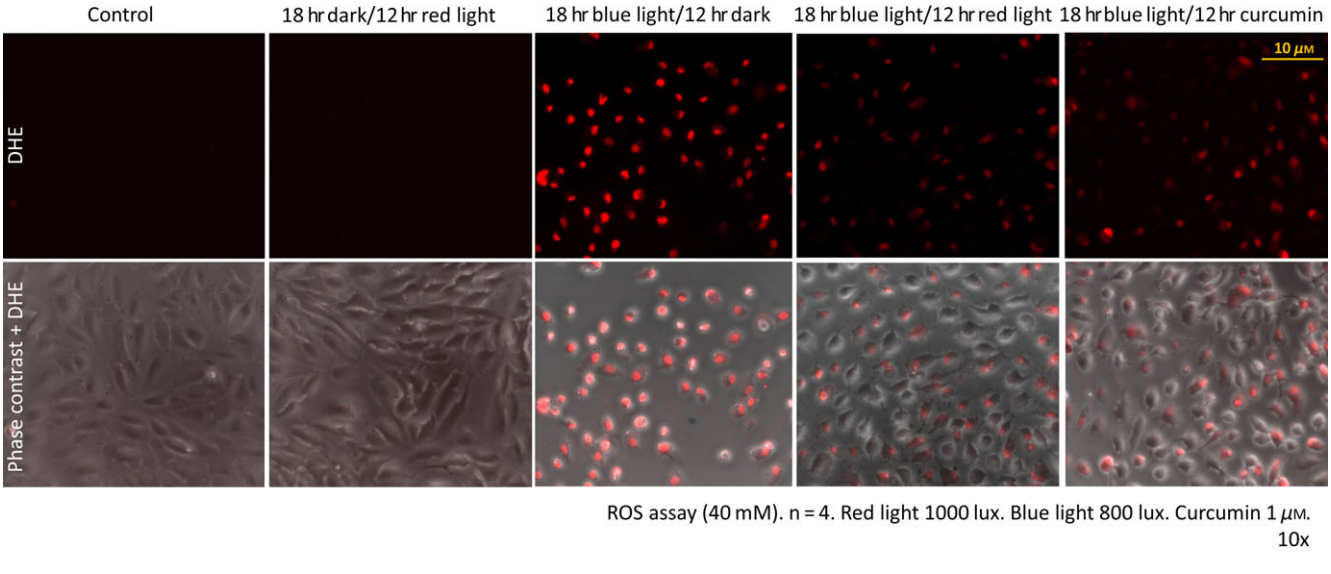

Fig. 6. Exposure of ARPE19 cells to blue light for 18 hr caused a strong stimulation of ROS, indicated by red DHE fluorescence, that was absent from cells in the dark (control) or following exposure to red light. The intensity of ROS production caused by blue light is clearly attenuated following a subsequent exposure to red light (6.5 W/m^2) or curcumin (1 μM). The lower panels show the same fluorescent images as the upper panels but with addition of phase contrast microscopy.

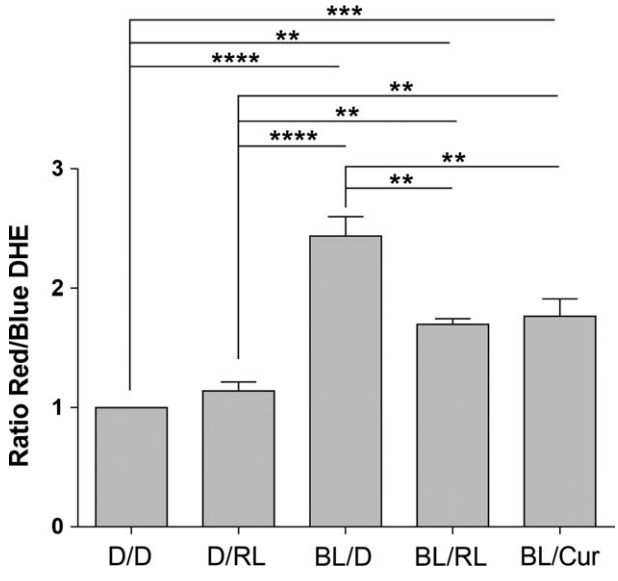

Fig. 7. Quantitative analysis of DHE fluorescence as shown in Fig. 6. Accumulated cytoplasmic DHE in healthy cells has a blue fluorescence (λex 370 nm-λem 420 nm) and converted to a red fluorescence (λex 535 nm-λem 610 nm) when ROS are induced. The ratio between red/blue fluorescence provides a measure for ROS generation. D/D, experiment in dark; D/RL, 18 hr in dark followed by 12 hr in red light; BL/D, 18 hr in blue light followed by 12 hr in dark; BL/RL, 18 hr in blue light followed by 12 hr in red light; BL/Cur, 18 hr in blue light followed by 12 hr in curcumin. The results clearly show that the blue light (26 W/m^2) causes an increase in ROS and blunted following exposure to red light (6.5 W/m^2) or curcumin (1 μM). Results are expressed as mean value ± SEM, $n = 4$; One-way ANOVA with Holm-Sidak's multiple comparison test (***$p < 0.001$, **$p < 0.01$).

be used as a noninvasive procedure to target dysfunctional RPE cells caused by blue light in AMD.

ARPE19 cells lack significant amounts of pigments like melanin and lipofuscin that are located outside the mitochondria (Boulton et al. 1990). Our studies on ARPE19 thus probably relate more to an action of blue light effects on mitochondrial pigments. This is in line with reports showing that blue light causes cell death and structural damage to lipofuscin-free RPE cells (Pang et al. 1998; King et al. 2004) and also causes the generation of ROS in isolated RPE mitochondria (Chen et al. 1993). A direct negative effect of blue light on mitochondrial enzyme complexes has been demonstrated in a study on fibroblasts devoid of mtDNA and unable to synthesize normal mitochondrial enzyme complexes. It was shown that unlike healthy fibroblasts, such fibroblasts were unaffected by light (Osborne et al. 2008). We therefore suggest that the present studies on ARPE19 cells, like that conducted by others (Roehlecke et al. 2009; Youn et al. 2009), provide a means to

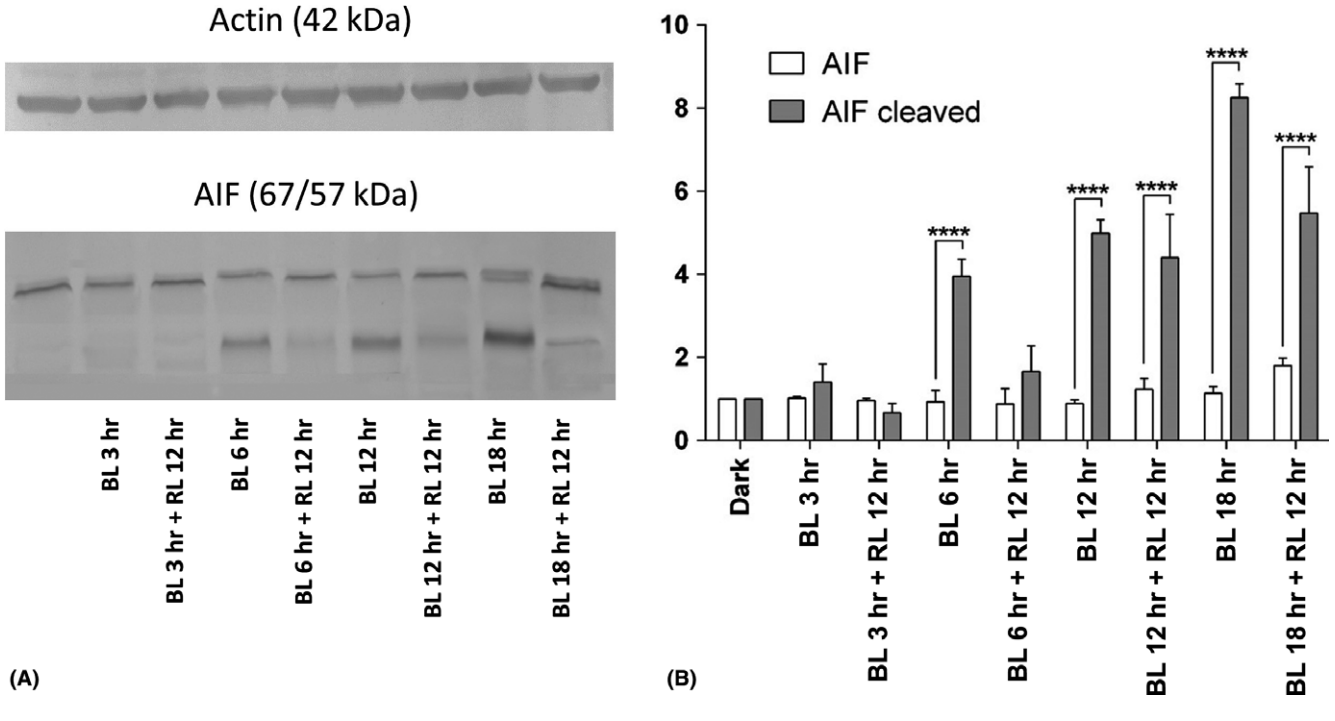

Fig. 8. Western blots where equivalent amounts of extracts from ARPE19 cells cultured under different conditions were fractionated and samples processed for the detection of apoptosis inhibitory factor (AIF) and actin proteins (A). AIF appears as a single band with a molecular weight of 67 kDa in control cultures (Dark). Exposure of such cultures to blue light (26 W/m^2) shows AIF to be gradually cleaved to produce a 57 kDa species in a time-dependent manner. The cleavage of AIF is completely blocked by 12 hr of red light (6.5 W/m^2) delivered after 6 hr of blue light but only partially when the blue light insult was for 12 or 18 hr. B shows the quantitative analysis of the ratio of intensities of the 57 kDa and 67 kDa bands for AIF relative to actin, derived from three experiments as shown in A, ±SEM. Significant differences were determined using a two-way ANOVA with Sidak's multiple comparison test (***$p < 0.001$).

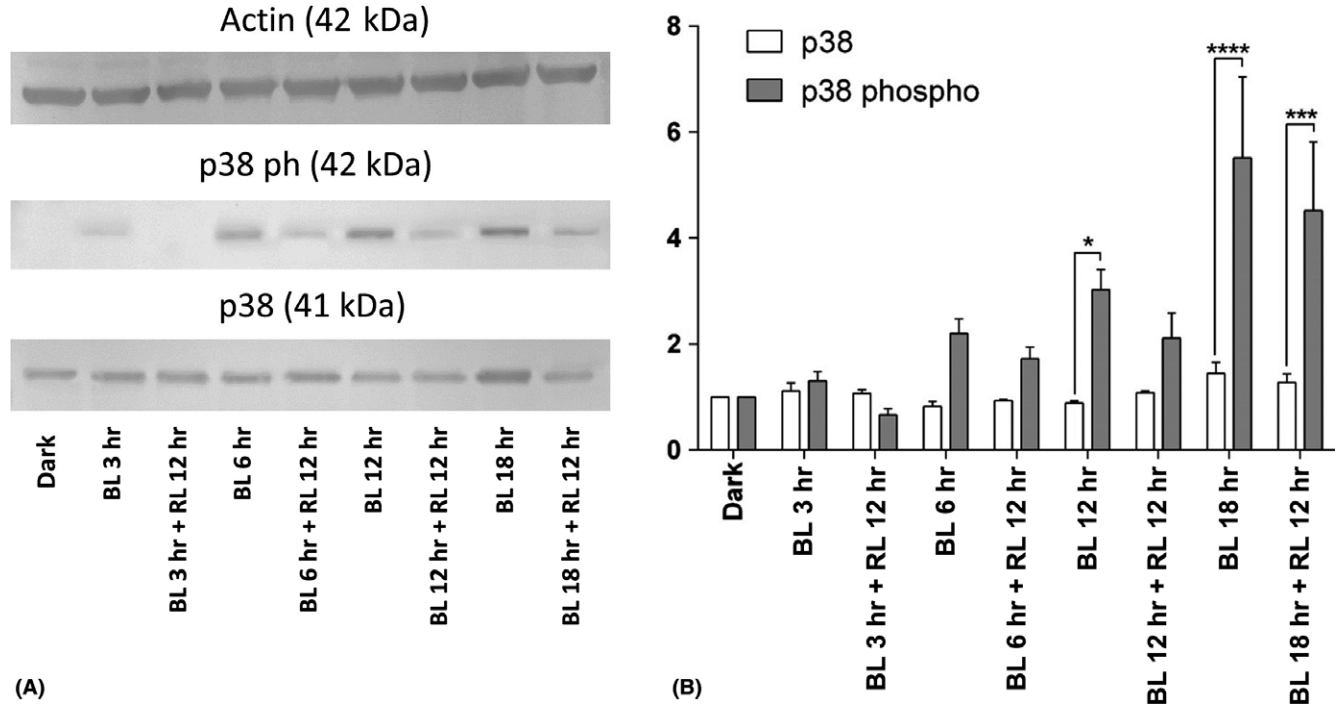

Fig. 9. Western blots where equivalent amounts of extracts from ARPE19 cells cultured under different conditions were fractionated and samples processed for the detection of p38, phosphorylated p38 (p38 ph) and actin proteins (A). Phosphorylated p38 (42 kDa) is not present in control (Dark) cultures but is gradually expressed following exposure of blue (26 W/m^2) between 3 and 18 hr. In contrast, p38 protein (41 kDa) appears to be unaffected by blue light and being of similar amounts in untreated cultures in the dark. The gradual increase in phosphorylated p38 protein is clearly blunted by 12 hr of red light (6.5 W/m^2) delivered after 6, 12 or 18 hr of blue light. B shows the quantitative analysis of the ratio of intensities of the p-38 (42 kDa) and phosphorylated p-38 (41 kDa) proteins relative to actin, derived from three experiments as shown in A, ±SEM. Significant differences were determined using a two-way ANOVA with Sidak's multiple comparison test (***$p < 0.001$).

determine how blue light affects mitochondrial chromophores in RPE cells *in situ*. As shown in Figs 4–7, the exposure of ARPE19 cells to blue light causes mitochondrial depolarization and an elevation of ROS, which are known to be linked to cell death (Heiskanen et al. 1999; Orrenius et al. 2007; Ott et al. 2007). Mitochondrial dysfunction/depolarization was demonstrated by use of the stain JC-1, which accumulates in mitochondria, appearing red/orange in fluorescence (590 nm) in healthy activated organelles and green in fluorescence (530 nm) when mitochondria are depolarized (Perelman et al. 2012).

To determine whether one or another of the mitochondrial enzyme complexes are specifically affected by blue light, we exposed ARPE19 cells to defined mitochondrial enzyme complex toxins – rotenone (inhibitor of complex I), 3-nitropropionic acid (3-NP or inhibitor of complex II), actinomycin A (inhibitor of complex III) and sodium azide (inhibitor of complex IV), in the presence or absence of blue light (Fig. 2). In each case, cell death caused by blue light and toxins was more-or-less additive. These results therefore suggest that blue light toxicity might result from an effect on no single mitochondrial enzyme complex. A similar conclusion was reached in a study by Ji et al. (2011) who compared the biochemical modes of cell death induced by blue light with that of sodium azide. The study was based on the idea that mitochondrial complex IV is specifically affected by sodium azide (Leary et al. 2002; Safiulina et al. 2006) and absorbed by blue light (García-Silva et al. 1997; Hockberger et al. 1999). Blue light-induced cell death activated AIF, c-Jun, JNK and HO-1 without influencing α-fodrin and caspase-3, and cell death caused by sodium azide resulted in the activation of α-fodrin and caspase-3 but had no effect on AIF, c-Jun, JNK or HO-1 (Ji et al. 2011). The studies by Ji et al. (2011) and our group argue for the view that blue light toxicity probably results from an effect on no single mitochondrial enzyme complex. However, both studies rely on assuming specificity for the mitochondrial enzyme complex inhibitors used. This is certainly not the case. For example, sodium azide has been reported to stimulate nitric oxide production (Bennett et al. 1996) and affect the functions of potassium (Qamirani et al. 2006) and calcium (Marino et al. 2007) channels.

Consistent with studies of other cell-types (Li & Osborne 2008; Osborne et al. 2008, 2014; Ji et al. 2011; del Olmo-Aguado et al. 2012; Del Olmo-Aguado et al. 2016), blue light-induced toxicity to ARPE19 cells causes the up-regulation of HO-1 and the cleavage of AIF in a dose-dependent manner. Apoptosis inhibitory factor (AIF) is a flavin-binding mitochondrial intermembrane protein that has been implicated in maintenance of the electron transport chain function, ROS regulation and cell death (Delavallée et al. 2011; Polster 2013; Sosna et al. 2014). It has also been shown that when AIF is cleaved within mitochondria, the 57 kDa product migrates to the nucleus to cause alkylation and DNA damage, resulting in a caspase-independent cell death, termed necroptosis (Artus et al.

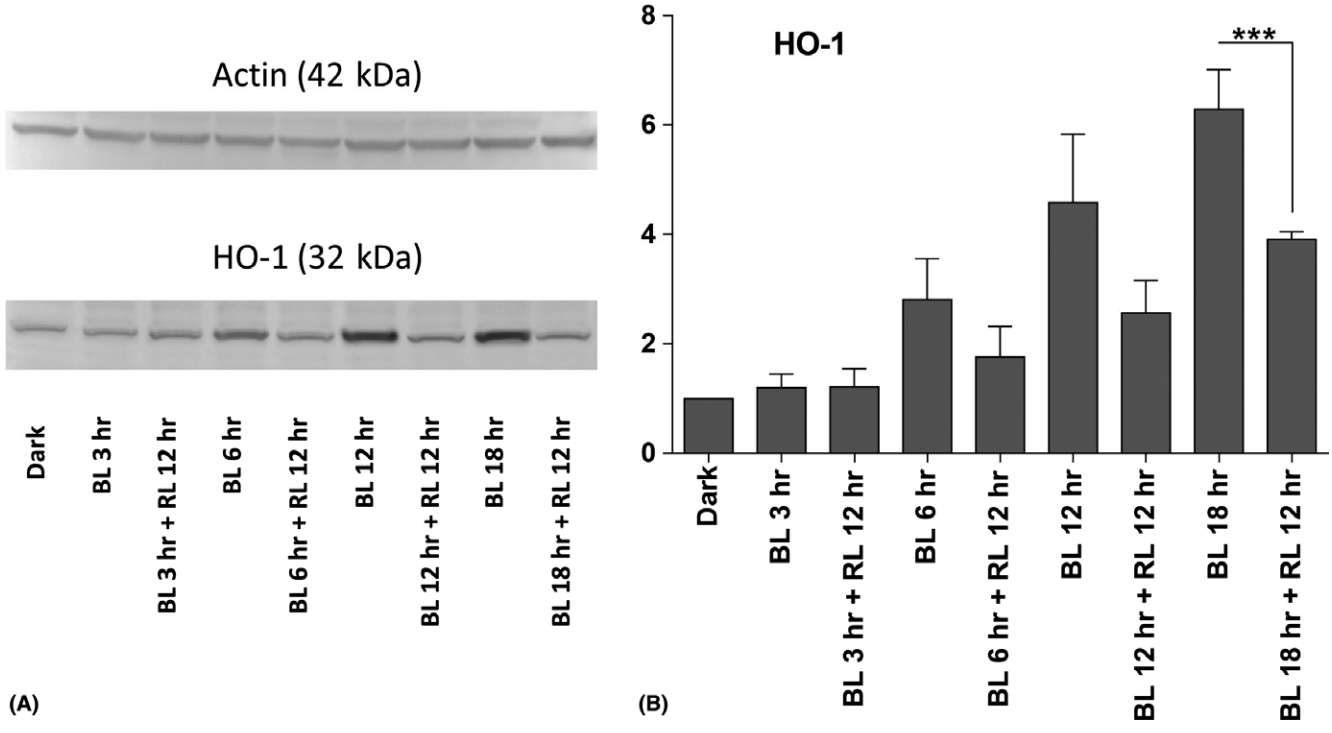

Fig. 10. Western blot analyses of equivalent amounts of extracts from ARPE19 cells cultured under different conditions were fractionated and samples processed to detect HO-1 and actin proteins. The amount of HO-1 protein (32 kDa) increases in cultures exposed to blue light (26 W/m^2) in a time-dependent manner and blunted by red light (6.5 W/m^2) delivered 12 hr after a blue light insult (A). B shows the quantitative analysis (±SEM) of the ratio of intensities of HO-1 (32 kDa) and relative to actin, derived from three experiments where it can be seen that red light blocks the increase in HO-1 caused by blue light at all time intervals. Significant differences were determined using a one-way ANOVA with Tukey's multiple comparison test (***$p < 0.0001$).

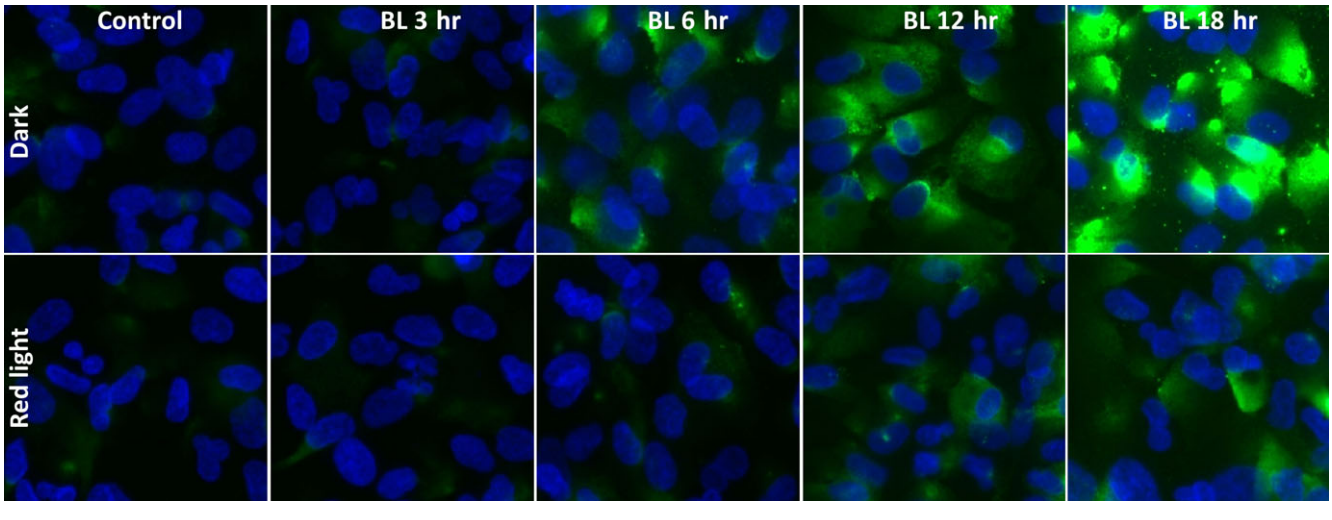

Fig. 11. HO-1 immunoreactivity in ARPE19 cell cultures exposed to different amounts of blue light (26 W/m^2) followed by exposure of 12 hr of red light (6.5 W/m^2). HO-1 immunoreactivity is absent in cultures maintained in the dark (Control) or when exposed to red light. It is clear that blue light delivered between 6 and 18 hr dose-dependently increased HO-1 immunoreactivity and that in each case, red light given thereafter blunted the stimulation of HO-1.

2010; Polster 2013; Hanus et al. 2015). Significantly, red light, which stimulates mitochondrial complex IV or COX (Karu et al. 2004; Karu & Kolyakov 2005; Wong-Riley et al. 2005; Lane 2006; Begum et al. 2013), attenuates blue light-induced AIF cleavage (Fig. 9) and enhances cell viability as a consequence (Fig. 3). These results show that negative actions on flavin-containing nucleotides within mitochondria (as might be caused by blue light) can be blunted through the stimulation of COX (Lane 2006).

The up-regulation of HO-1 protein caused by blue light might reflect its role in endogenous neuroprotection. HO-1 is a stress-response protein that plays a vital function in maintaining cellular homeostasis and is known to be overexpressed in, for example,

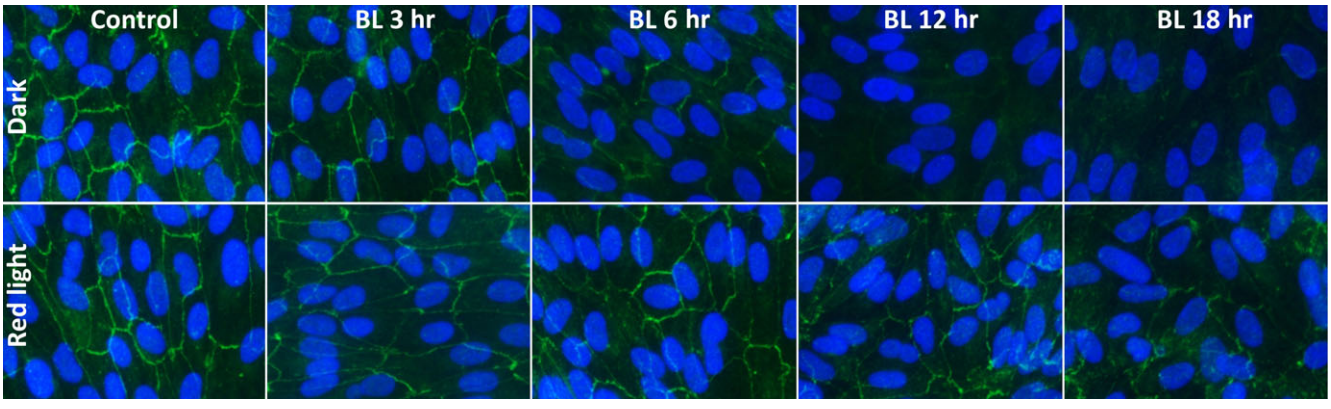

Fig. 12. This figure shows that confluent ARPE cell cultures in the dark (Control) develop tight junctions indexed by the staining for ZO-1 immunoreactivity and is unaffected by exposure to red light. However, with increasing amounts of exposure to blue light (26 W/m^2), tight junction formation is decreased being practically absent after 12–18 hr. Exposure of such cultures to 12 hr red light (6.5 W/m^2) showed enhancement of ZO-1 immunoreactivity now being evident in cultures that had been previously exposed to 12–18 hr of blue light.

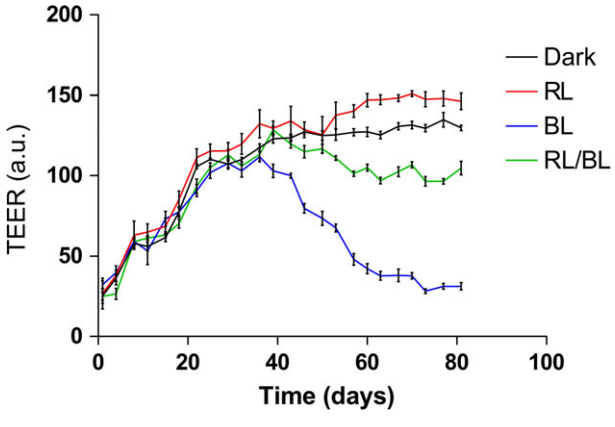

Fig. 13. ARPE-19 cultures grown in 24-well plate inserts gradually allow for an increase in the measurement of their trans-epithelial resistance or TER, which reflects the continuous development of tight junction formation between cells. Such cells were either maintained in darkness for 80 days or repeatedly exposed after 2 weeks to 48 hr cycles of light. Peak resistance of TER was more-or-less established to be approximately 130 Ωcm^2, (±SEM, $n = 4$) after 40 days and increased slightly thereafter over the next 40 days. This increase in TER was elevated clearly when cells were instead exposed to cycles of red light appearing to reach a peak resistance of 140 Ωcm^2 after 80 days. However, TER was significantly reduced in cells in cultures treated after 2 weeks with cycles of blue light (26 W/m^2) and reached a low of 30–40 Ωcm^2 after day 80. Importantly, use of cycles of blue and red (6.5 W/m^2) light ligated the negative influence on TER elicited solely by blue light with the resistance after 80 days being around 100 Ωcm2.

pathological iron deposition, oxidative stress and mitochondrial damage (Schipper et al. 2000; Himori et al. 2014). The magnitude of HO-1 induction therefore might be important in the defence of organisms against oxidative stress-mediated injuries and other diverse factors (Amersi et al. 1999; Ursu et al. 2014). HO-1 gene expression can be induced in different ways, one being the Nrf2 and antioxidant-responsive-element or Nrf2-ARE pathway. This pathway can be activated by pharmacological agents like curcumin to cause the up-regulation of HO-1, which participates in a reduction of various forms of stress (Balogun et al. 2003; He et al. 2014; Dai et al. 2016). Significantly, blue light oxidative-induced toxicity to ARPE19 cells is blunted by both curcumin and red light (Fig. 3). However, red light, as shown in Figs. 10 and 11, seems to cause the down-regulation of the HO-1 protein. As a consequence, we interpret this observation as support for the notion that the beneficial findings associated with red light (Karu et al. 2004; Karu & Kolyakov 2005; Wong-Riley et al. 2005; Begum et al. 2013) occur via the action on COX and that the Nrf2-ARE pathway may not be involved. However, more detailed studies are necessary to demonstrate this to be the case unequivocally. The present data also support the view that the blue light-induced up-regulation of HO-1 reflects an endogenous protective process to counteract injury caused by blue light.

Mitogen-activated protein kinases (MAPKs), including extracellular signal regulators kinase (ERK), c-Jun, N-terminal kinase (JNK) and p38-MAPK, are linked with a diverse range of biological activities including the regulation of cell (Ono & Han 2000; Cuadrado & Nebreda 2010). Activation of ERKs typically contributes to cell differentiation, proliferation and survival, whereas JNK and p38-MAPK are typically activated by pro-inflammatory cytokines and environmental stress (such as UV radiation and osmotic stress; Cuadrado & Nebreda 2010; Davies et al. 2000; Ono & Han 2000). The present studies show that the exposure of ARPE19 cells to blue light resulted in the occurrence of the phosphorylated product of p38-MAPK which was increasingly elevated when the exposure time of blue light was increased (Fig. 9). Stimulation of the formation of phospho-p38-MAPK by blue light has also been reported to occur in photoreceptor (Kuse et al. 2015) and retinal-derived cells (Ji et al. 2011). Thus, it appears that a common feature associated with a blue light insult involves the activation of p38-MAPK and for cell death to occur by necroptosis. Importantly, these negative influences of blue light to ARPE19 cells are attenuated by the use of red

light. Red light decreases the up-regulation of phospho-p38-MAPK caused by blue light (Fig. 9), which is correlated with increased cell survival (Fig. 2).

Retinal pigment epithelial (RPE) cells are separated from each other by tight junctions *in situ* and can be visualized by staining for the localization of proteins like ZO-1, claudin-19 and occludin. Tight junctions retard the trans-epithelial diffusion of solutes via the spaces that lie between neighbouring cells (Rizzolo et al. 2011) and are probably affected by low grade, subclinical inflammatory processes in AMD (Yang et al. 2007; Peng et al. 2012; Perez & Caspi 2015). Our studies, and reports by others (Dunn et al. 1996; Qin & Rodrigues 2010), show that ARPE19 cells can be grown in 24-well plate inserts to gradually develop tight junctions to allow for a trans-epithelial membrane resistance (TER) to be recorded. As shown in Figure 12, the tight junction protein ZO-1 is gradually expressed in ARPE19 cultures. The TER results recorded for ARPE19 cells grown in 24-well plate inserts (Fig. 13) clearly reveal that the maximum TER values recorded for cultures maintained in the dark for 80 days and increased slightly when exposed to cycles of red light after an initial period in blue light. However, cycles of blue light given to cultures at day 40 steadily reduced TER values reached after 40 days in the dark but if the blue light cycle is combined with red light the TER values remained more-or-less constant. We interpret these findings to suggest the potential for blue light to affect the tight junction function of RPE cells *in situ,* which ultimately leads to cell dysfunction, and that red light can prevent this from happening.

In conclusion, our studies show that the exposure of RPE cell mitochondria to blue light causes mitochondrial malfunction and oxidative stress and suggest that such a chronic influence is associated with the pathogenesis of AMD. Importantly, we show that red light delivered after a negative effect of blue light attenuates this process, thereby supporting the mass of other experimental data for the use of red or infrared light therapy, also known as photomodulation, as a noninvasive procedure to target RPE dysfunction in AMD. Various preclinical research studies have reported the beneficial influence of red light therapy in ocular diseases such as in AMD (Ivandic & Ivandic 2008; Merry et al. 2017; the TORPA (NCT00940407) and LIGHT-SIGHT (NCT02725762) trials) and for diabetic retinopathy (Tang et al. 2014). However, the use of red light in the treatment of human eye disorders remains to be established in mainstream medicine (Huang et al. 2012; Fitzgerald et al. 2013). This is because the data from existing randomized clinical trials are controversial and unequivocal details of the underlying biochemical mechanism remain to be established. Moreover, a biphasic dose–response has been observed and low levels of red light seem to have a better effect than higher levels. In addition, experimental results are often difficult to replicate, being hampered by the undisclosed nature of the irradiation source (laser or light-emitting diode), the mode of delivery (pulsed or continuous), stimulation wavelengths, as well as the rate, duration and frequency of treatments (Quirk & Whelan 2011; Fitzgerald et al. 2013). In addition, little attention has been paid to the potential negative effects of red light therapy which require detailed analysis.

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