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Age-related retinal inflammation is reduced by 670 nm light via increased mitochondrial membrane potential

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Abstract

The mitochondrial theory of aging argues that oxidative stress, caused by mitochondrial DNA mutations, is associated with decreased adenosine triphosphate (ATP) production leading to cellular degeneration. The rate of this degradation is linked to metabolic demand, with the outer retina having the greatest in the body, showing progressive inflammation, macrophage invasion, and cell loss, resulting in visual decline. Mitochondrial function shifts *in vitro* after 670-nm light exposure, reducing oxidative stress and increasing ATP production. *In vivo*, it ameliorates induced pathology. Here, we ask whether 670 nm light shifts mitochondrial function and reduces age-related retinal inflammation. Aged mice were exposed to only five 90-second exposures over 35 hours. This significantly increased mitochondrial membrane polarization and significantly reduced macrophage numbers and tumor necrosis factor (TNF)-alpha levels, a key proinflammatory cytokine. Three additional inflammatory markers were assessed; complement component 3d (C3d), a marker of chronic inflammation and calcitonin, and a systemic inflammatory biomarker were significantly reduced. Complement component 3b (C3b), a marker of acute inflammation, was not significantly altered. These results provide a simple route to combating inflammation in an aging population with declining visual function and may be applicable to clinical conditions where retinal inflammation is a key feature.
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Keywords: RPE; Retina; Mitochondria; Photobiomodulation; 670nm; NIR; JC-9; Aging; Inflammation

1. Introduction

The mitochondrial theory of aging assumes that oxidative stress, caused by mitochondrial DNA mutations, results in age-related degenerative changes (Harman, 1972). Mitochondria are intracellular structures acting as primary providers of energy via adenosine triphosphate (ATP) production. They also generate progressively detrimental free radicals associated with oxidative stress. A shift in the balance between the production of these two is thought to lead to aging and disease (Barot et al., 2011; Feher et al., 2006; Green et al., 2011; Green and Amarante-Mendes, 1998; Harman, 1972; Jarrett et al., 2008; Kroemer and Reed, 2000). Metabolically demanding tissues, including the outer retina with its large photoreceptor population, have elevated oxidative stress that may be associated with accelerated age-related inflammation resulting in retinal degeneration (Jarrett et al., 2008, 2010; Liang and Godley, 2003).

There is evidence that mitochondrial function shifts with selective absorption of red light resulting in increased ATP production (Lim et al., 2009; Wong-Riley et al., 2001). It has been documented that 660–680-nm irradiation increased electron transfer in Cytochrome C oxidase, leading to augmented ATP synthesis *in vitro* (Liang et al., 2008; Passarella et al., 1984; Pastore et al., 2000). This can ame-

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liorate experimentally induced pathology and has been shown to have cytoprotective abilities in a range of tissues including the brain and the retina (Albarracin et al., 2011; Eells et al., 2003; Fitzgerald et al., 2010; Whelan et al., 2001). Here, we ask whether brief exposure to light generated by 670-nm light emitting diodes (LED) measurably alters mitochondrial function and reduces age-related retinal inflammation in old mice.

2. Methods

2.1. Experimental overview

Two experiments were undertaken, and forty-six 12-month-old C57BL/6 mice were used. First, the impact of 670-nm light exposure on mitochondrial membrane polarization in the retinal pigmented epithelium (RPE) was assessed using a specific mitochondrial dye. Mitochondria are composed of two compartments; the matrix, surrounded by the inner membrane, and the intermediate space, surrounded by the outer membrane. The near-infrared light contains the key elements for ATP production and has selective permeability. In proapoptotic conditions, inner membrane-permeability alteration is manifested as a dissipation of the proton gradient in the mitochondrial transmembrane potential (Smiley et al., 1991). Second, having demonstrated manipulability of this, the impact of 670 nm light was determined on age-related inflammation in the retina using a range of independent markers. The parameters used in the experiments have been chosen as they reflect those used effectively by other investigators (Shaw et al., 2010; Wong-Riley et al., 2001). However, it is possible that these are not the optimal, but a very large number of variables need to be manipulated to refine these, which is beyond the scope of this study. Further, although higher doses have been used, these have commonly been applied to significant induced pathology, rather than simply aging (Albarracin et al., 2011; Eells et al., 2003). The dose used will not be filtered by the lens, cornea, or optic media because they transmit close to 100% at 670 nm (Lei and Yao, 2006).

In the first experiment, six mice were exposed to a 670 nm LED light (Quantum Devices Warp 10, Barneveld, WI, USA), five times for 90 seconds, each spaced evenly over 35 hours. Ninety seconds is the fixed duration of one delivery of this device. This was delivered at 40 mW/cm^2. The primary aim was to assess the impact of light exposure on mitochondrial membrane polarization.

Mice were lifted by the scruff of the neck, and the LED device was held approximately 1 cm vertically above the head over the eyes. Care was taken to maintain the position over the midline so that both eyes were exposed equally. The LED provides radiation over an area of ~10 cm^2 at this distance. The animals appeared to relax during the exposure, and after the exposure they resumed normal behavior. Three days after the final exposure, the animals were killed with CO_2, and the eyes were rapidly removed.

Twenty-four whole eyecups were incubated in JC-9 mitochondrial dye (Invitrogen, Paisley, UK, 0.9 mM) at 37 °C/5% CO_2 for 45 minutes. Eyecups were then washed ×3 in phosphate-buffered saline (PBS) prior to fixation for 1 hour and 15 minutes in 4% paraformaldehyde in PBS. Eyes were dissected and flatmounted, as described previously (Kokkinopoulos et al., 2011). When membrane depolarization occurs, the cationic dye shifts toward 525 nm (green) emission. When membrane potential increases, the JC-9 monomers form aggregates that shift the light emission toward 590 nm (red) (Smiley et al., 1991) RPE flatmounts were mounted under glass coverslips and imaged confocally. The absolute intensity was measured using a dual laser 488/543 beam splitter using a C-Apochromat 40×/1.20 W Korr M27 lens, using 493–534 filters. For each RPE flatmount, absolute intensity ratios were taken from the periphery and center of the RPE (four per region), and the mean absolute intensity ratio was analyzed using the ZEN 2009 software.

In the second series of experiments, the same patterns of exposure were used on 17 mice, with an additional 17 animals used as controls. However, these animals were allowed to survive for 6 days after the last exposure. Here, the primary aim was to assess the impact of light exposure on levels of age-related retinal inflammation once mitochondrial membrane polarization had been shifted. Twenty-two eyes were used for flatmounting, for macrophage labeling in the subretinal space, whereas 12 eyes were sectioned for immunolabeling for markers of inflammation. Mice were sacrificed with CO_2, and eyes were rapidly removed and fixed as mentioned earlier in the article.

2.2. Immunohistochemistry

Eyes were dissected and flatmounted, as described previously (Hoh Kam et al., 2010; Kokkinopoulos et al., 2011). Flatmounts were washed ×3 in PBS for 5 minutes, before blocking with 5% normal donkey serum (NDS) in 3% Triton X-100 in PBS for 2 hours. Flatmounts were briefly washed in PBS and incubated with ionized calcium-binding adaptor molecule 1 (IBA-1) (rabbit polyclonal; 1:1000; A. Menarini Diagnostics, Wokingham, UK) antibody overnight. The following day, these were washed in PBS before the appropriate conjugated Alexa-Fluor secondary antibody (1:2000; Invitrogen, Paisley, UK) was added in 2% NDS with 0.3% Triton X-100 for 2 hours. Flatmounts were then washed in PBS followed by washing in Tris-buffered saline (TBS), before mounting with Vectashield (Vector Laboratories, Peterborough, UK).

Eyes for sectioning were removed and fixed as aforementioned for 1 hour and cryo-preserved in 30% sucrose in PBS and embedded in optimal cutting temperature compound (OCT, Agar Scientific, Stanstead, UK). Cryostat sections were cut at 10 μm and thaw-mounted onto charged slides. Fluorescence immunohistochemistry was performed as described previously (Hoh Kam et al., 2010), using com-

plement component 3b (C3b) (rat monoclonal; 1:100; Hycult Biotechnology, Uden, The Netherlands), complement component 3d (C3d) (goat polyclonal; 1:100; R&D Systems, Abingdon, UK), and tumor necrosis factor (TNF)-alpha (rabbit polyclonal; 1:200; Abcam, Cambridge, UK), and the appropriate conjugated Alexa-Fluor secondary antibodies (1:2000; Invitrogen, Paisley, UK).

Retinal sections were processed for immunohistochemistry using the streptavidin horseradish peroxidase complex. The sections were incubated for 1 hour in a 5% NDS in 0.3% Triton X-100 in PBS, pH 7.4, followed by an overnight incubation with calcitonin (rabbit polyclonal; 1:100; Abcam, Cambridge, UK) made in 1% NDS in 0.3% Triton X-100 in PBS. Sections were washed ×3 in PBS, then placed in 0.3% hydrogen peroxide in PBS. Sections were incubated with a biotin–conjugated secondary antibody against rabbit (1:1000; Jackson ImmunoResearch Laboratories, Newmarket, UK) made in 2% NDS in 0.3% Triton X-100 in PBS, for 1 hour. The primary antibody was omitted in −ve controls. Sections were washed several times and incubated with horseradish peroxidase-streptavidin solution (Vector Laboratories, Peterborough, UK) for 30 minutes, followed by a peroxidase substrate solution, 3,3-diaminobenzidine (DAB), for 10 minutes. Slides were mounted in glycerol.

2.3. Analysis

Fluorescence images were taken close to the optic nerve head in JPEG format at 400×, using an epi-fluorescence bright-field microscope with a 24-bit color images at 3840 × 3072 pixel resolution using Nikon DXM1200 (Nikon, Tokyo, Japan) camera. Pictures were put together, and the integrated density, which is the product of the area chosen (in pixels) and the mean gray value (the measurement of the brightness), was measured using Adobe Photoshop CS4 extended edition. The lasso tool was used to draw a line around Bruch's membrane and regions of the photoreceptors, specifically the inner and outer segments (Hoh Kam et al., 2010). Results are presented as the mean ± SD. Where appropriate, n is the number of eyes used. Statistical analysis was performed using GraphPad Prism 5 using a Mann–Whitney's *U* test.

3. Results

3.1. 670 nm light alters mitochondrial membrane polarization in the aged RPE

The RPE was examined after 670-nm light exposure, using the JC-9 mitochondrial membrane dye (Fig. 1A). The dye shifts toward longer wavelengths with the polarization of the mitochondrial membrane, with wavelengths around 525 nm (green; depolarized) representing relatively depolarized membranes and wavelengths around 590 nm (red; polarized) representing more polarized membranes (overlay) (Smiley et al., 1991). After 670-nm light exposure, the inner membrane potential in the majority of RPE mitochondria showed increased polarization, compared with controls whose label was of a shorter wavelength (Fig. 1B). This is consistent with the notion that mitochondrial inner membrane in the RPE show increased polarization after 670-nm light exposure. This is associated with increased ATP production (Liang et al., 2008; Passarella et al., 1984; Pastore et al., 2000).

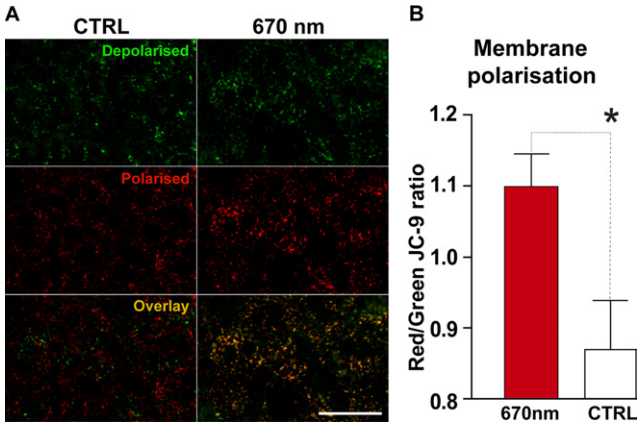

Fig. 1. 670 nm LED increases mitochondrial membrane potential in aged murine RPE. (A) Representative photomicrographs of RPE flatmounts labeled with JC-9 in control (CTRL) and after exposure to 670 nm light. Images (top and middle) represent the depolarized (green) and polarized (red) mitochondrial inner membrane. The bottom represents the superimposition of both images giving a visible ratio of the two, indicating the overall degree of mitochondrial polarization. (B) The absolute Red/Green JC-9 intensity ratio was measured ($\Delta\Psi_M$) (Smiley et al., 1991). After exposure to 670 nm LED, $\Delta\Psi_M$ increased significantly, in comparison with controls (n = 6). Mann–Whitney's *U* Test; * $p = 0.04$, error bars = SD. Scale bars = 5 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

3.2. 670 nm light decreases outer retinal inflammation

Age-related inflammation is a common outer retinal feature in normally aged animals (Bhutto et al., 2011; Xu et al., 2009). The complement cascade is active in the aged controls, with C3b and C3d proteins expressed along Bruch's membrane (Fig. 2A and 2B) (Chen et al., 2011; Seth et al., 2008). However, after 670-nm light exposure, the inflammatory factor C3d showed a significant decrease (Fig. 2A), whereas C3b, another marker of inflammation, was not statistically different, compared with controls (Fig. 2B).

Calcitonin is an acute-phase protein that increases with age in the retina and is a complement-independent biomarker of inflammation (Chen et al., 2010). Both control and 670-nm light mice showed clear calcitonin expression patterns in the retina (Fig. 2C). Expression was present in the plexiform layers and in photoreceptor inner segments, which are mitochondrial-rich regions of the cell. Mice exposed to 670 nm light had significantly lower calcitonin levels than their controls in inner segments (Berger, 1964). Hence, reduced inflammation resulting from 670-nm light

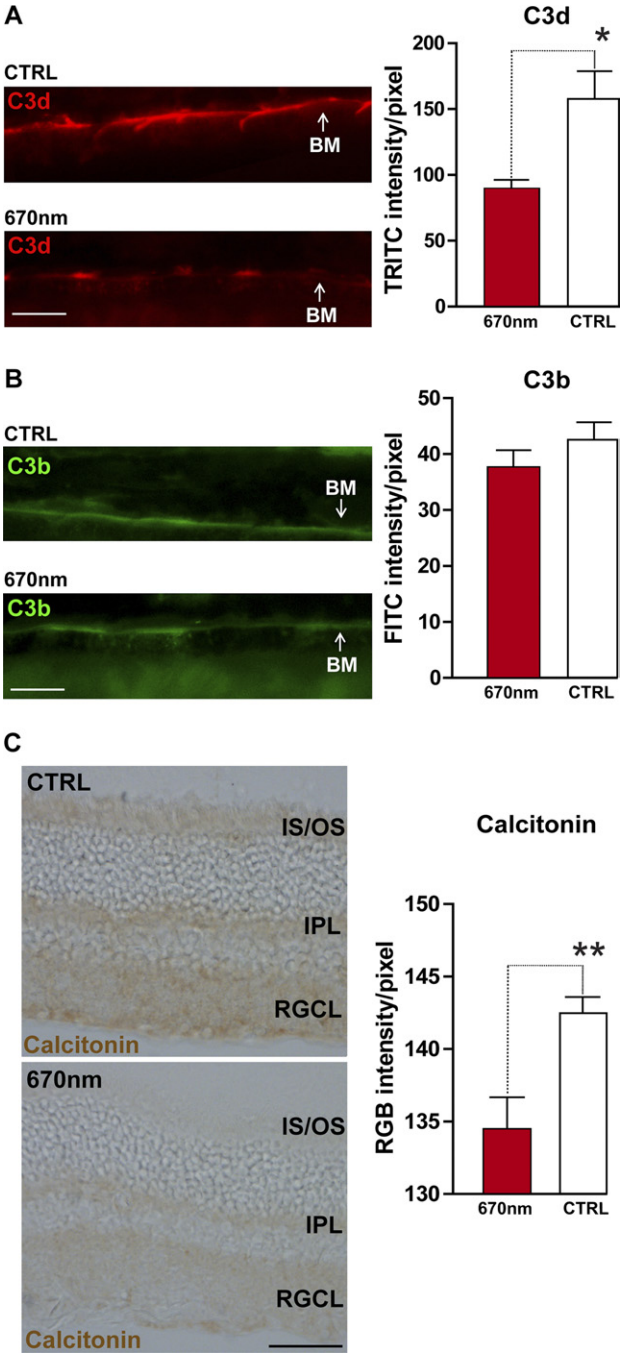

Fig. 2. 670 nm LED decreases inflammation in the aged RPE. (A) Complement component C3d (red) showed decreased expression on Bruch's membrane 7 days after 670-nm light treatment in comparison with the controls (CTRL) (n = 6 experimental and n = 6 controls). (B) Complement component C3b (green) did not show a statistically significant reduction 7 days after 670-nm light treatment in comparison with controls on Bruch's membrane. Tissues derived from the same animals as in (A). (C) Calcitonin expression was downregulated significantly in the inner/outer segments of the 670-nm light mice, in comparison with controls (n = 4 experimental and n = 4 controls). Here, the protein expression is brown. Mann–Whitney's *U* Test C3; * $p = 0.032$, calcitonin; ** $p = 0.028$, error bars = SD. Scale bars = 5 μm. Abbreviations: IS/OS, inner/outer segments; IPL, inner plexiform layer; RGCL, retinal ganglion cell layer. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

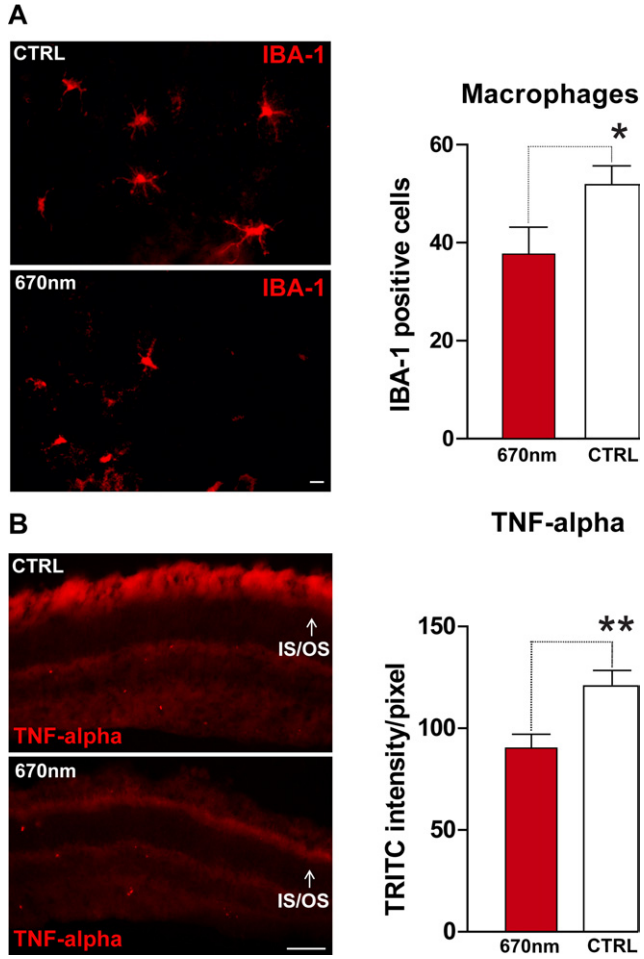

Fig. 3. 670 nm LED modulates macrophage numbers and TNF-alpha expression. (A) Whole mounted eye cups were labeled with IBA-1 to identify macrophages (red) in the subretinal space, which were subsequently counted. These accumulate with age. IBA-1$^+$ macrophage numbers were significantly reduced a week after 670-nm light treatment (n = 11, 670 nm and n = 11, CTRL). (B) TNF-alpha expression was significantly reduced 7 days after 670-nm light treatment, in comparison with control (n = 4, 670 nm; n = 4, CTRL). Mann–Whitney's *U* Test. Macrophage numbers; * $p = 0.04$, TNF-alpha; ** $p = 0.0032$. Error bars = SD. Scale bars = 5 μm. Abbreviations: IS/OS, inner/outer segments; IBA-1, ionized calcium-binding adaptor molecule 1; TNF-alpha, tumor necrosis factor alpha. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

exposure is not confined to Bruch's membrane alone but is also present in the neural retina.

3.3. 670 nm light affects macrophage number and cytokine secretion in the aged outer retina

Macrophage infiltration is common in the aged mouse subretinal space (Hoh Kam et al., 2010). Exposure to 670 nm light resulted in a significant 15% decrease of macrophage numbers in the subretinal space when compared with controls. Further, TNF-alpha, a proinflammatory cytokine produced by macrophages, was also significantly reduced in the outer retina at the level of the outer segments (Fig. 3A).

Table 1
Differing treatment regimes and the effect of 670 nm used on organs or tissues

Tissue	670-nm treatment regimes	Impact	Publication(s)
Retina	180 seconds/day for 5 days (Quantum Devices Warp 10)	Protection of the retina from light-induced photoreceptor degeneration	(Albarracin et al., 2011)
Retina	Three 144-second exposures; each 5, 25, and 50 hours after methanol treatment (Quantum Devices Warp 10)	Methanol-induced retinal dysfunction is attenuated by 670-nm LED treatment	(Eells et al., 2003)
Optic nerve	30 minutes/day for 10 days (Quantum Devices Warp 10)	Reduce oxidative stress and improve function in the CNS after traumatic injury	(Fitzgerald et al., 2010)
Brain	3 times/week for 6 months; various doses	Reduction in amyloid-β load and reduced inflammation	(De Taboada et al., 2011)
Brain	Four 90-second exposure over 30 hours (Quantum Devices Warp 10)	Neuroprotection of midbrain dopaminergic cells in MPTP-treated mice	(Shaw et al., 2010)
Primary neuronal cultures	80 seconds twice a day for 5 days (Quantum Devices Warp 10)	Increased energy metabolism in neurons functionally inactivated by toxins	(Wong-Riley et al., 2001, 2005; Liang et al., 2008)
Kidney	Once/day for 14 days (Quantum Devices Warp 10)	Renal function and antioxidant defense capabilities improvement in the kidney of Type I diabetic rats	(Lim et al., 2010)
Liver	300 seconds/day for 18 days (14 treatments in total) for the acute study and for 98 days (70 treatments in total) for the chronic study (Quantum Devices Warp 10)	Treatment increased ATP production but did not significantly affect the state of oxidative and energy stress in livers of acute and chronic diabetic rats	(Lim et al., 2009)
Skin	Various doses *in vitro* and *in vivo*	Accelerated wound healing	(Whelan et al., 2001)
Oral epithelium	71 seconds/day	Decrease in ulcerative oral mucositis	(Whelan et al., 2001)

This is not an exhaustive list. However, it does reflect the divergent systems in which 670 nm has been used to ameliorate induced pathology. It also shows the different treatment regimes that have been used.
Key: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

4. Discussion

Aging is the progressive accumulation of changes with time, associated with increased susceptibility to disease and eventually death. The outer retina has the greatest metabolic demand in the body (Graymore, 1969), resulting in elevated oxidative stress (Ballinger et al., 1999; Liang and Godley, 2003).

Mitochondria play a key role in aging and age-related pathogenesis (Barot et al., 2011; Feher et al., 2006; Harman, 1956, 1972; Jarrett et al., 2008; Kroemer and Reed, 2000; Lin et al., 2011; Nordgaard et al., 2008). They provide the energy for many subcellular reactions via ATP production, but with age they also generate increasing levels of reactive oxygen species, which, if unchecked, compromise the efficiency of ATP production (Barot et al., 2011; Feher et al., 2006; Green et al., 2011; Green and Amarante-Mendes, 1998; Harman, 1972; Jarrett et al., 2008; Kroemer and Reed, 2000).

Mitochondria selectively absorb 670 and 830 nm light, which may influence their efficiency (Greco et al., 1989). Cytochrome C oxidase, a key mitochondrial photoacceptor molecule, has been shown to be activated by 670 nm light (Karu, 1999; Wong-Riley et al., 2005). Further, exposure to 670 nm light has cytoprotective properties after light-induced retinal degeneration and methanol-induced retinal cytotoxicity (Albarracin et al., 2011; Eells et al., 2003). Ninety seconds has been commonly used because it is the fixed timer setting on some models of the delivery device (Quantum Devices Warp 10, Berneveld, WI, USA) and has proved to be largely effective. However, other studies have used different times and cycles of exposure. We provide details of some of these in Table 1. Irrespective of differences between these, the outcomes are largely effective.

Although the exact mechanism of 670-nm illumination and its precise time course remain unknown, here, we have correlated changes in mitochondrial inner membrane potential with reduced inflammation along Bruch's membrane/RPE and in the outer retina. The Bruch's membrane/RPE was the chosen target for a read out of mitochondrial changes because of its critical role in outer retinal function (Boulton et al., 2004; Boulton and Dayhaw-Barker, 2001; Del Priore et al., 2002; Feng et al., 2003; Fleming et al.,

1996; Gao and Hollyfield, 1992; Harman et al., 1997; Ida et al., 2003; Sauvé et al., 1998). It was predicted that 670-nm light exposure in aged Bruch's membrane/RPE would initiate a change in mitochondrial inner membrane potential, which was revealed with the JC-9 dye that has been widely used as a marker of mitochondrial inner membrane potentials (Chazotte, 2011; Cossarizza et al., 1993; Keil et al., 2011; Kuhnel et al., 1997; Legrand et al., 2001; Reers et al., 1995; Smiley et al., 1991). Convincing demonstrations of direct links between 670 nm light and shifts in mitochondrial inner membrane potential, resulting in increased ATP production and cell rescue, have not been made *in vivo*. However, they have been demonstrated *in vitro* (Liang et al., 2008; Passarella et al., 1984; Pastore et al., 2000), and as such are a platform for this study.

Reduced ATP and increased reactive oxygen species probably drives aging and age-related inflammation, which are likely to be of significance in the metabolically demanding environment of the outer retina (Chen et al., 2011; Feher et al., 2006; Green et al., 2011; Jarrett et al., 2010; Kroemer and Reed, 2000; Lin et al., 2011). We assessed inflammation levels firstly by labeling for complement components C3b and C3d on the Bruch's membrane/RPE interface, a key site of age-related inflammation (Buschini et al., 2011; Hoh Kam et al., 2010). C3 is one of the major proteins involved in the formation of the membrane attack complex (Walport, 2001b, 2001a). Upon activation, it is broken down into C3a and C3b by C3 convertase. C3b binds to host cell surface where it is inactivated by Factor H. This inactivation causes further cleavage, eventually leading to C3d. If the latter is not cleared effectively then extensive accumulation leads to further complement activation. Here, we found a significant decrease in C3d expression, but not in C3b, in response to 670-nm light exposure.

RPE cells are phagocytes, ingesting photoreceptor outer segments. Their phagocytic ability may be affected by alterations in their microenvironment (Karl et al., 2007). As macrophage numbers were reduced significantly, it is possible that RPE phagocytic capability was upregulated following 670-nm light exposure, initiating clearance of C3d deposits from Bruch's membrane. However, we did not observe a significant difference for C3b. This could be because C3d clearance precedes that of C3b.

Calcitonin expression occurs in the normal aged retina. It is a systemic biomarker of inflammation independent of complement and a key gene upregulated in the aging mouse eye (Chen et al., 2010). Calcitonin expression is marked in the aged outer retina and prominent in inner and outer segments. However, we found significant decreases in calcitonin deposition at these locations after 670-nm light exposure.

Macrophages accumulate with age in the subretinal region and are associated with elevated inflammation (Hoh Kam et al., 2010; Luhmann et al., 2009; Xu et al., 2008). Our results demonstrate that 670-nm light exposure significantly reduced macrophage numbers. Here, we have used IBA-1, which may be a selective marker of a subpopulation of macrophages. It has been argued that this marker is specifically expressed in macrophages/microglia, and it is upregulated during their activation (Sasaki et al., 2001). Reduction of inflammation on Bruch's membrane/RPE interface may reduce the need for the presence of these cells. Further, their removal may be associated with reductions in TNF-alpha, a key inflammatory cytokine that they produce and which was also significantly reduced by 670-nm light exposure. These data indicate that 670 nm light alleviates inflammation, possibly via mitochondrial inner membrane potential changes. The latter are key targets in retinal inflammation, as they play an important role in photoreceptor homeostasis and regulating the local innate immune response (Feng et al., 2003; Forrester et al., 1990; Zamiri et al., 2007). Hence, there are multiple independent windows on the impact of 670-nm light exposure in the outer retina, all consistently indicating that a key mode of action is reduced inflammation.

This study has shown an association between 670 nm light, a shift in mitochondrial inner membrane potential, and reduced inflammation in the outer retina. This region is susceptible to damage because of its high metabolic stress (Graymore, 1969). Although 670 nm light may play a significant role in reducing age-related inflammation, many key factors remain obscure. To date there has been no clear physiological demonstration of the relationship between exposure to this light and actual changes in oxidative phosphorylation levels *in vivo* although this has been established *in vitro* (Passarella et al., 1984; Pastore et al., 2000). This fundamental demonstration is necessary to reveal the mechanism of action. However, data presented here are consistent with a potentially important therapeutic role for this agent in the aging retina.

Disclosure statement

The authors declare no conflicts of interest. All animals were used with University College London ethics committee approval and under a UK Home Office animal project license. All animal procedures conformed to the United Kingdom Animals Licence Act 1986.

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