Hindawi Publishing Corporation
International Journal of Photoenergy
Volume 2016, Article ID 2734139, 12 pages
http://dx.doi.org/10.1155/2016/2734139

Research Article

Efficacy of 670 nm Light Therapy to Protect against Photoreceptor Cell Death Is Dependent on the Severity of Damage

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Received 23 October 2015; Accepted 26 January 2016

Academic Editor: Zofia Stasicka

Photobiomodulation at a wavelength of 670 nm has been shown to be effective in preventing photoreceptor cell death in the retina. We treated Sprague-Dawley (SD) rats with varying doses of 670 nm light (9; 18; 36; 90 J/cm^2) before exposing them to different intensities of damaging white light (750; 1000; 1500 lux). 670 nm light exhibited a biphasic response in its amelioration of cell death in light-induced degeneration *in vivo*. Lower light damage intensities required lower doses of 670 nm light to reduce TUNEL cell death. At higher damage intensities, the highest dose of 670 nm light showed protection. *In vitro*, the Seahorse XFe96 Extracellular Flux Analyzer revealed that 670 nm light directly influences mitochondrial metabolism by increasing the spare respiratory capacity of mitochondria in 661 W photoreceptor-like cells in light damaged conditions. Our findings further support the use of 670 nm light as an effective treatment against retinal degeneration as well as shedding light on the mechanism of protection through the increase of the mitochondrial spare respiratory capacity.

1. Introduction

Photoreceptors in the retina have high rates of oxygen consumption due to their demanding metabolism, and as the light-sensitive retinal cells they are also exposed to large amounts of visible light [1–3]. These facets render them particularly sensitive to oxidative stress which has been implicated in diseases associated with aging [4, 5], as well as being an important factor in retinal degenerations [6–8] including the pathogenesis of Age-Related Macular Degeneration (AMD) [9–12]. The implication of oxidative stress in AMD and other retinal diseases has led to the study of antioxidants such as dimethylthiourea [13, 14], zinc oxide [15], dietary carotenoids [16, 17], and antioxidant enzymes [18, 19], with the aim of improving photoreceptor survival thereby retarding the progression of the disease (reviewed in [20]).

Photobiomodulation is the modulation of biology with photons of light. Red/near-infrared light (R/NIR) has been reported to reduce the severity of maladies in humans and in various animal models of disease (reviewed in [21]). Using the light-induced model of retinal degeneration, several investigations have shown attenuation of degenerative effects in the retina after treatment with 670 nm light [22–26]. The exact mechanism underlying the protective effect of photobiomodulation is not known, although it is believed that light in the R/NIR range is absorbed by cytochrome c oxidase, promoting a change in the oxidation state of the enzyme [27–30]. This change in the cytochrome c oxidation state has been postulated to increase the efficiency and function of oxidative phosphorylation (OXPHOS), leading to an increase in ATP production through amplified flux of the electron transport chain [28, 31].

In this study we used the light-induced rodent model of retinal degeneration to investigate the dose effects of 670 nm light on retinal damage *in vivo*. We also investigated the direct effects of 670 nm light on mitochondrial function in an immortalized line of photoreceptor (661 W) cells. We found that the dose of 670 nm light needed for a therapeutic effect varies with severity of retinal damage and that excessive exposure to 670 nm light can result in retinal damage. We also found that 670 nm light directly influences mitochondrial metabolism by increasing the spare respiratory capacity of mitochondria in 661 W cells adding further evidence of mitochondrial involvement in photobiomodulation.

2. Methods

2.1. Animal Rearing. All animal procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, as well as The Australian National University (ANU) Animal Experimentation Ethics Committee (ANU Ethics Protocol Number: A2011/029). Animals used for these experiments were adult albino Sprague-Dawley (SD) rats aged between 90 and 120 days. The animals were born and raised in a 12-hour light, 12-hour dark cycle (12 : 12 L/D) in dim light conditions (5 lux).

2.2. 670 nm Light Treatment and Light Damage. Treatment with 670 nm light was conducted using a WARP 75 light source (Quantum Devices Inc., Barneveld, WI) a light emitting diode (LED) array producing a power flux of 60 mW/cm^2. 670 nm light treatments commenced consistently at 9:00 AM every morning. Animals were individually towel-wrapped to aid in handling during treatments. The light source was positioned approximately 2.5 cm away from the eyes of the animal when treated. Animals received one of the following doses of 670 nm light: 9 J/cm^2 (3-minute continuous treatment); 18 J/cm^2 (6-minute continuous treatment); 36 J/cm^2 (12-minute continuous treatment); 90 J/cm^2 (30-minute continuous treatment). Animals were treated once daily on five consecutive days.

Immediately following the fifth day of 670 nm light treatment, animals were placed in individual transparent cages. Animals were exposed for a period of 24 hours to 750-lux ("low" intensity), 1000-lux ("moderate" intensity), or 1500-lux ("high" intensity) white light, as indicated by a digital light meter placed on the cage floor (Digitech, QM1587). Light was generated from cold-white fluorescent bulbs (COLDF2 2 × 36 W IHF, THORN lighting). Control animals were exposed to white light at "low," "moderate," or "high" intensity, with no prior treatment with 670 nm light.

2.3. Tissue Collection and Processing. Animals were euthanized with CO_2 immediately after light damage (LD). A mark was made on the superior surface of the eye before extraction for orientation purposes. Whole eyes were immediately injected with 4% paraformaldehyde before being immersion fixed in 2 mL of 4% paraformaldehyde solution for 4 hours at 4°C and then processed as described previously [32]. Eyes were sectioned in the sagittal plane at a thickness of 16 μm and mounted on poly-L-lysine slides (Thermo Scientific, Waltham, MA).

2.4. Histological Analyses

2.4.1. Cell Death. Cell death was assessed by the TdT-mediated dUTP nick end labelling (TUNEL) technique to identify the fragmentation of DNA characteristic of apoptotic cells, following a previously published protocol [32] using a fluorophore, Alexa 594, for visualization. Counting was done manually by a skilled investigator, who was "blind" to the treatment protocol, using a Zeiss AxioVert 200 (Zeiss Australia, North Ryde, NSW) inverted fluorescence microscope. The retina was divided into eight equal-sized regions, four on the superior and four on the inferior. TUNEL+ cells in the ONL (Outer Nuclear Layer) of the retina were counted in each region; then all counts were added together to give the total amount of TUNEL+ cells in the retina for that respective eye section. Representative TUNEL images were taken on a Nikon A1 Confocal microscope (Nikon, Tokyo, JP).

2.4.2. ONL Thickness Ratio. ONL ratios were analyzed on a Zeiss AxioVert 200 inverted fluorescence microscope. The ONL contains the cell bodies of the photoreceptors and will become thinner as more layers of photoreceptors die through apoptosis, providing a measure of retinal degeneration. The thickness of the ONL layer was measured using ProgRes Capture Pro (Jenoptik AG, Jena, Germany) and divided by the thickness of the overall retina spanning from the inner limiting membrane to the outer limiting membrane in order to generate the ONL thickness ratio. One measurement was taken from each of the eight equal-sized regions of the retina before being averaged together.

2.5. Cell Culture. The 661 W photoreceptor cell line used in these studies was generously provided by Dr. Muayyad Al-Ubaidi (University of Oklahoma). Cells for experimental purposes were used within 5 passages of authentication. Validation of the cells authenticity was performed by Cell Bank Australia for species specificity. They were further validated using RT-PCR for expression of cell specific markers including cone arrestin, opsin pigments, and SV40. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), L-glutamine (3 mM), sodium pyruvate (1 mM), and glucose (25 mM). Cells were allowed to grow to 80–90% confluence before use for experiments. Cells were kept in 37°C and 5% CO_2.

Cells treated with 670 nm light were given two separate 9 J/cm^2 (3-minute) treatments before the running of the respective assay. Cells were treated once before light damage, and once directly after. Treatments were conducted using a WARP 75 light source (Quantum Devices Inc., Barneveld, WI) LED array at 60 mW/cm^2 energy flow emitting light at a wavelength of 670 nm. The LED was placed underneath the cell culture plate so that the cells were directly subjected to the light source.

Table 1: Drug concentrations used for Seahorse XFe96 assays.

Drug compound	Concentration	Source
Oligomycin	0.5 μM	Sigma Aldrich (St. Louis, MO)
Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)	1.0 μM	Sigma Aldrich (St. Louis, MO)
Antimycin A	1.0 μM	Sigma Aldrich (St. Louis, MO)
Rotenone	1.0 μM	Sigma Aldrich (St. Louis, MO)

Light damage in cell culture experiments was done by exposing 661 W cells to 15,000-lux light for 5 hours while maintaining them at 37°C in 5% CO_2. The cell culture plate was placed underneath the lights in a manner that resulted in even distribution of the light across the plate. Temperature was closely monitored using a thermometer placed at the level of the cell culture plate and was not allowed to exceed 37°C.

2.6. Mitochondrial Metabolism. Mitochondrial metabolism was measured using the Seahorse XFe96 Extracellular Flux Analyzer (Seahorse Biosciences, Boston, MA). Cells were plated in the custom Seahorse XFe96 well plate at a seeding density of 7,500 cells/well and were allowed to grow to 80–90% confluence in 10% FBS DMEM (approximately 24 hours). After cells were confluent, the 10% FBS serum was replaced with reduced serum media (1% FBS) for an additional 24 hours. Non-670 nm light treated plates were then placed directly into light damage whereas treated plates underwent 670 nm light irradiation before being placed in light damage.

Custom Seahorse sensor cartridges were hydrated in calibrant solution and kept in a non-CO_2, 37°C environment at least 12 hours before the running of the assay. Seahorse assay media were warmed to 37°C in a non-CO_2 incubator before L-glutamine (6 mM), sodium pyruvate (1 mM), and glucose (5.5 mM) were added. 3 μL (per 50 mL of media) of 10 mM NaOH was then added to the media to bring it to a pH of 7.4. It was then kept at 37°C before use. Assay drugs were made up using the dilutions indicated in Table 1. 25 μL of each drug was injected into the appropriate ports on the cartridge before placing into the Seahorse XFe96 analyzer for plate calibration. The cell culture plate was washed twice in the assay media. A final volume of 175 μL was then added to each well on the plate. Plates were then placed into a 37°C non-CO_2 incubator to be degassed. After the degassing period, the cell culture plate was placed into the Seahorse XFe96 analyzer and the assay allowed to run to its entirety (approximately 1.5 hours). Three baseline measurements of oxygen consumption rate (OCR) were taken before the injections of mitochondrial inhibitors and substrates in the following order: oligomycin; FCCP; antimycin A; and rotenone (Table 1). Three additional readings of OCR were taken following each injection. Due to an "edge effect" on the plate, which typically yielded inconsistent data, wells on the edges of the plate were not included in the analysis. Basal extracellular acidification rates (ECAR) were simultaneously measured in the same manner during the running of the assay.

Hydrogen peroxide (H_2O_2) assay (Promega, Madison, WI) was carried out according to the manufacturer's instructions. The relative luminescence was measured using the Tecan Infinite 200 Pro (Tecan, Mannedorf, Switzerland). The luminescent signal is proportional to the H_2O_2 concentration in the solution. A CellTox kit (Promega, Madison, WI) was used to assess cell toxicity, according to the manufacturer's instruction. The plate was viewed under a Zeiss AxioVert 200 (Zeiss Australia, North Ryde, NSW) fluorescence microscope and labelled cells counted manually.

2.7. Statistical Analysis. Statistical analysis was done using GraphPad Prism software (GraphPad Software v6, La Jolla, CA) using unpaired Student's *t*-test unless indicated otherwise. Graphs were generated using GraphPad Prism software.

3. Results

Exposure to white light at low, moderate, or high intensities induced photoreceptor death such that the intensity of light damage was directly correlated with levels of photoreceptor death. Light damage at 750 lux resulted in 527.8 ± 50.7 dead/dying photoreceptors per retina, while 1000-lux and 1500-lux light damage resulted in an average of 933.4 ± 78.6 and 1405.7 ± 88.9 per retina, respectively (mean ± SE, Figure 1(a), control)

3.1. Effects of 670 nm Light Dose on Low, Medium, and High Intensity Retinal Damage

3.1.1. 670 nm Light Effects in Low Intensity Light Damage. "Low" intensity light damage (750 lux) in animals pretreated with 9 or 18 J/cm^2 red light resulted in significantly lower levels of photoreceptor death compared with controls (Figures 1(b), 2(a)–2(c)). There was no difference in numbers of TUNEL+ cells between control animals and those receiving 36 J/cm^2 670 nm light (Figure 2(d)). However, treatment with 90 J/cm^2 670 nm light resulted in significantly higher levels of photoreceptor death compared to those treated with 9 or 18 J/cm^2 (Figure 2(e)).

Control light damaged retinas (no 670 nm light treatment) had a significantly thinner ONL compared to retinas pretreated with 670 nm light (Figure 3(a)).

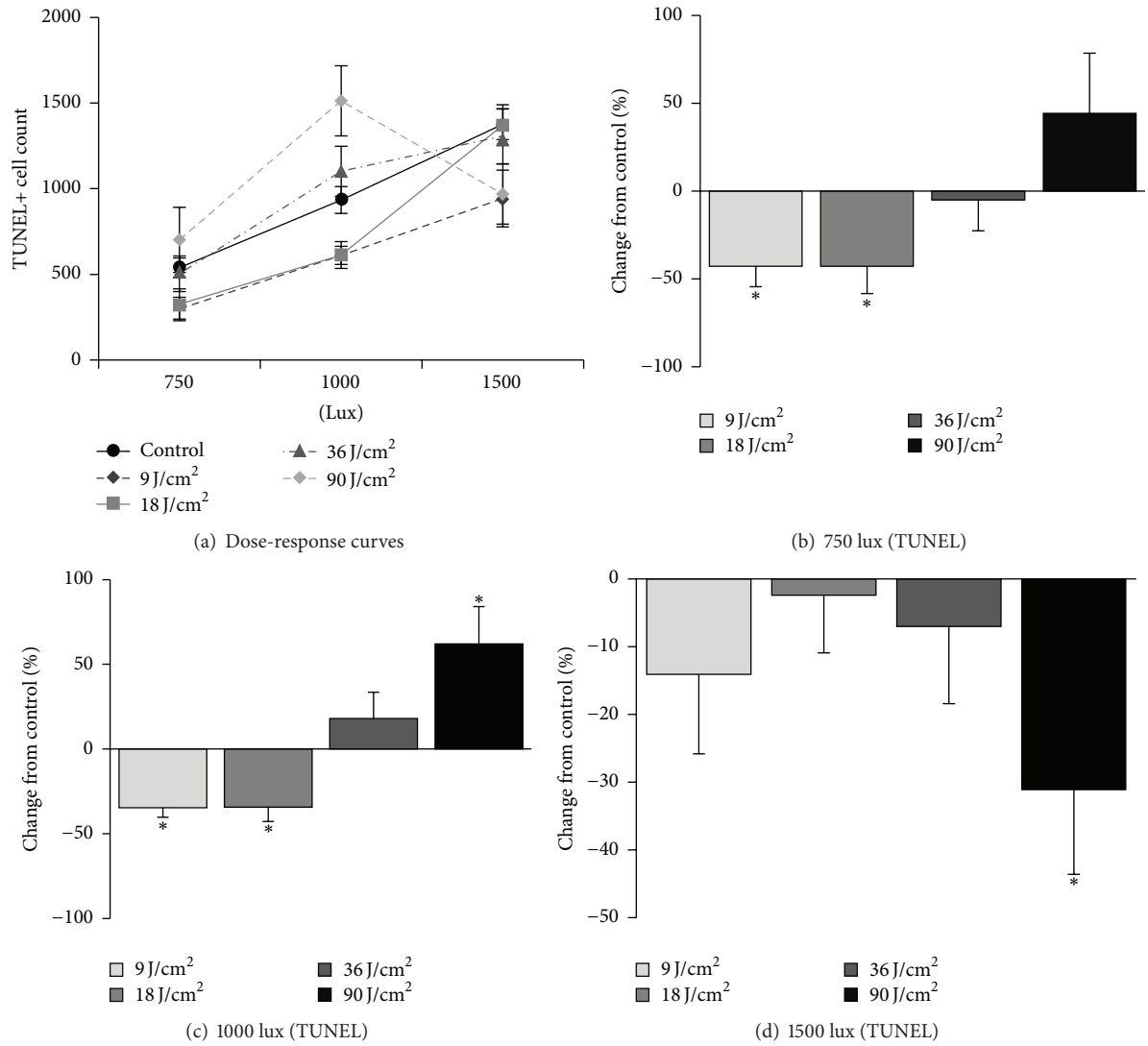


Figure 1: Effects of 670 nm light treatment on photoreceptor cell death induced by exposure to different intensities of white light. (a) Dose-response curves for 670 nm light showing the impact on photoreceptor death in "low" (750 lux), "moderate" (1000 lux), and "high" (1500 lux) intensity light damage (LD) paradigms. The highest dose of red light (90 J/cm^2) increases levels of photoreceptor death in the low and medium intensity light damage paradigms but protects photoreceptors from cell death in high intensity bright light conditions. Panels (b)–(d) depict the percentage change of TUNEL+ cell count in the retina between the four 670 nm light treatment groups when compared to the light damage only controls. (b) 750 lux: doses of 9 and 18 J/cm^2 670 nm light significantly reduced the number of TUNEL+ cells in the ONL compared to the light damaged controls. However, there was no change in the TUNEL+ cell count in retinas treated with 36 or 90 J/cm^2 670 nm light ($n = 5$ for each group). (c) 1000 lux: doses of 9 and 18 J/cm^2 significantly reduced the number of TUNEL+ cells compared to controls, while a dose of 36 J/cm^2 showed no difference in comparison to controls. Animals treated with 90 J/cm^2 had a significant increase in TUNEL+ cells in the ONL compared to the light damaged controls ($n = 4$ for each group). (d) 1500 lux: doses of 9, 18, or 36 J/cm^2 670 nm light had no significant effect on the numbers of TUNEL+ cells, compared to controls, while treatment with 90 J/cm^2 670 nm light significantly reduced the numbers of TUNEL+ cells in the ONL ($n = 6$ for control and 9 J/cm^2; $n = 5$ animals for 18 J/cm^2; $n = 4$ for 36 J/cm^2 and 90 J/cm^2 groups) ("$*$"denotes significance $P < 0.05$; error bars represent SEM).

3.1.2. 670 nm Light Effects in Moderate Intensity Light Damage. "Moderate" intensity light damage (1000 lux) following 9 or 18 J/cm^2 red light treatment resulted in significantly lower levels of cell death, similar to those from the "low" light damage experiments (Figures 1(c), 2(f)–2(h)). Animals treated with 36 J/cm^2 red light were comparable with controls and significantly higher than in animals that received 9 or 18 J/cm^2 before treatment (Figure 2(i)). The highest dose of 90 J/cm^2 resulted in significantly higher levels of TUNEL+ cells compared to the light damaged controls (Figure 2(j)).

Treatment doses of 9 J/cm^2, 18 J/cm^2, and 36 J/cm^2 resulted in the ONL being significantly thicker than light

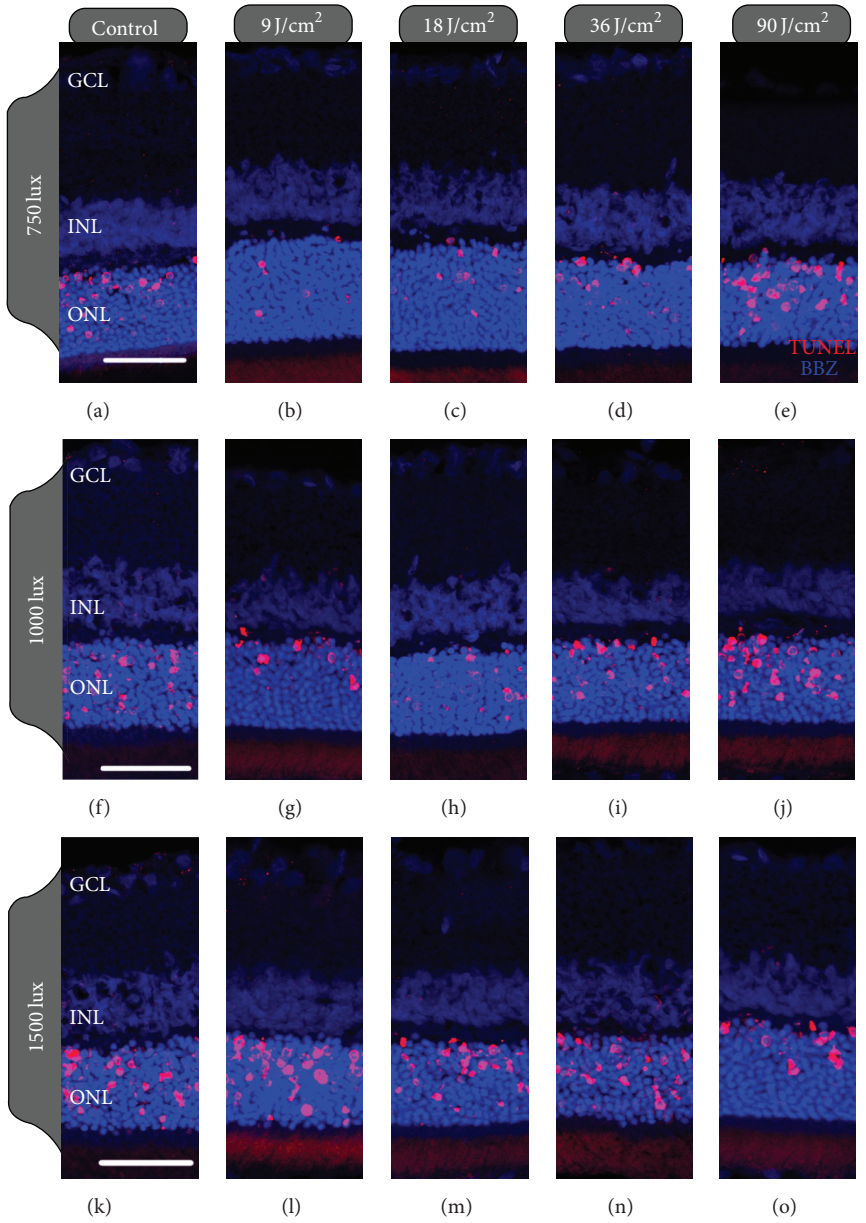


Figure 2: Representative images of the effects of 670 nm light treatment on photoreceptor cell death induced by exposure to different intensities of white light. Images were all taken from the same area in the superior portion of the retina. ((a)–(e)) 750-lux light damage. TUNEL counts were highest in light damage controls with a reduction in TUNEL+ cells seen in retinas that underwent 9 and 18 J/cm^2 670 nm light treatment. There were no visible changes between the control and 36 J/cm^2 treatments but an increase was seen in the 90 J/cm^2 treatments. ((f)–(j)) 1000-lux light damage. Similar to the 750-lux paradigm, TUNEL counts were highest in light damage controls with a reduction in TUNEL+ cells seen in retinas that underwent 9 and 18 J/cm^2 670 nm light treatment. No visible differences were seen between the control and 36 J/cm^2 treatments. Treatments with a dose of 90 J/cm^2 670 nm light appeared to increase the amount of TUNEL+ cells in the ONL. ((k)–(o)) 1500-lux light damage. All retinas stained positive with a high amount of TUNEL cells with only 90 J/cm^2 treatments appearing to reduce TUNEL+ cell counts (scale bars indicate 50 μm; BBZ is a bisbenzimide stain).

damaged controls (Figure 3(a)). Treatment with 90 J/cm^2 resulted in no significant difference in ONL ratios compared with the light damaged controls.

3.1.3. 670 nm Light Effects in High Intensity Light Damage. Treatment doses of 9, 18, and 36 J/cm^2 had no effect on levels of cell death in "high" intensity (1500 lux) light damaged retinas (Figures 1(d), 2(k)–2(n)). However, in this group pretreatment with 90 J/cm^2 significantly reduced the TUNEL+ cell count (Figure 2(o)).

Similarly, there was no significant difference in mean ONL thickness across the retinas of animals treated with 9, 18, or 36 J/cm^2 red light, compared to light damaged controls (Figure 3(a)). However, a 90 J/cm^2 pretreatment dose resulted

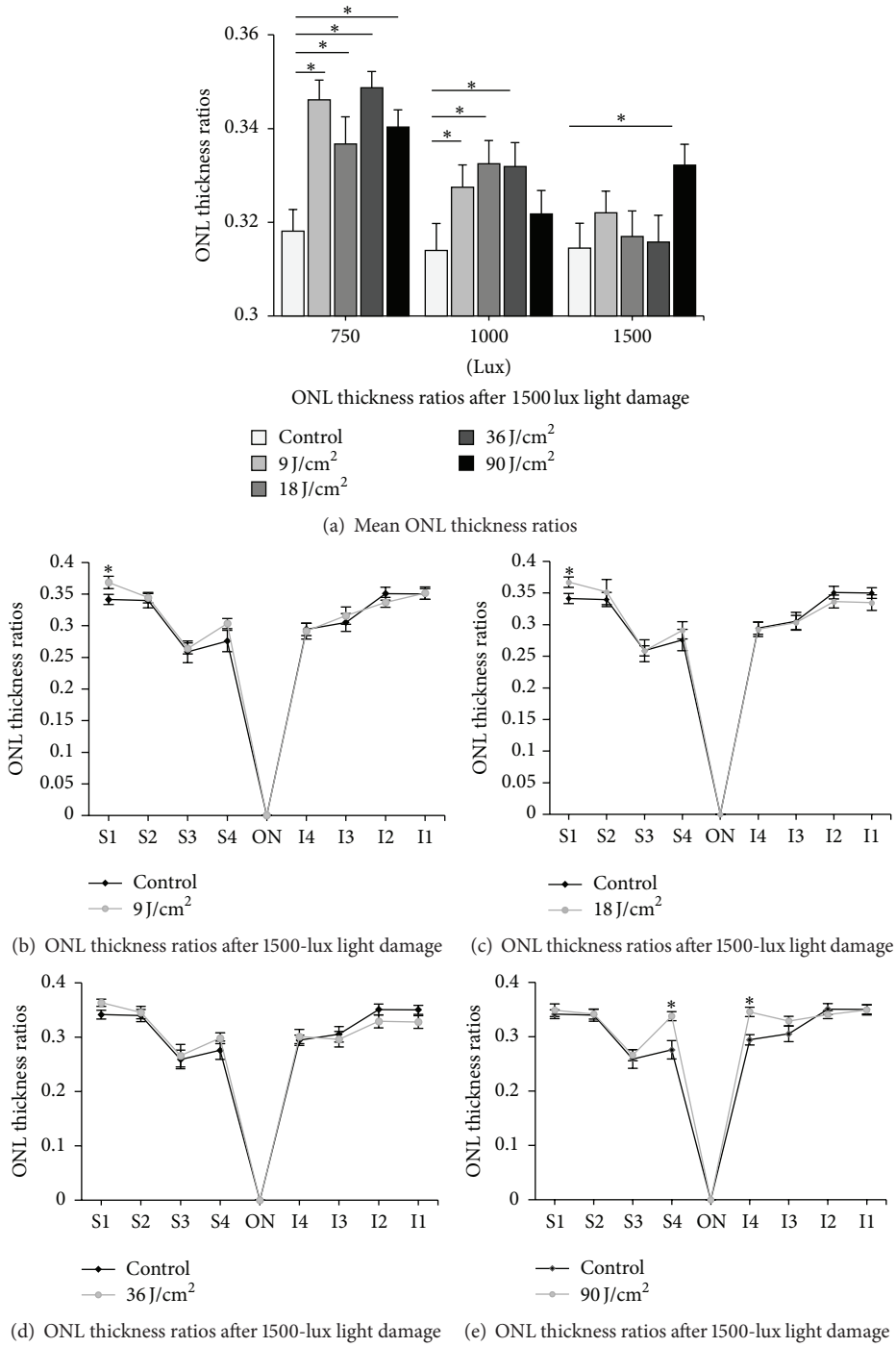


Figure 3: Effect of 670 nm light treatment on ONL thickness ratios. (a) Histograms showing mean ONL : retinal thickness ratios for three light damage protocols (750 lux/"low" intensity; 1000 lux/"moderate" intensity; 1500 lux/"high" intensity), summarizing the effects of four different treatment protocols. Following "low level" light (750 lux) damage ONL thickness is greater in the 670 nm light treated animals compared to controls (light damage only). Following "moderate" intensity (1000 lux) light damage, 9, 18, and 36 J/cm^2 670 nm pretreated eyes have a significantly thicker ONL compared to controls, while those treated with 90 J/cm^2 are not significantly different from untreated controls. Following "high" intensity light damage only treatment with 670 nm light at 90 J/cm^2 preserves ONL thickness. ((b) and (c)) Retinal thickness ratios in animals treated with 9 or 18 J/cm^2 670 nm light are similar to controls, except at the superior edge (sample S1). (d) Retinal thickness ratios in animals treated with 36 J/cm^2 670 nm light demonstrated no significant differences from controls. (e) Retinal thickness ratios in animals treated with 90 J/cm^2 670 nm light were significantly greater in the region surrounding the optic nerve head (S4 and I4) compared with controls ($n = 6$ animals for control and 9 J/cm^2; $n = 5$ animals for 18 J/cm^2; $n = 4$ for 36 J/cm^2 and 90 J/cm^2 groups; "$*$" denotes significance $P < 0.05$; error bars represent SEM). ON: optic nerve.

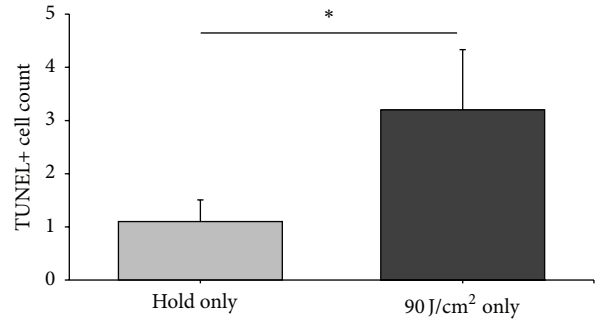
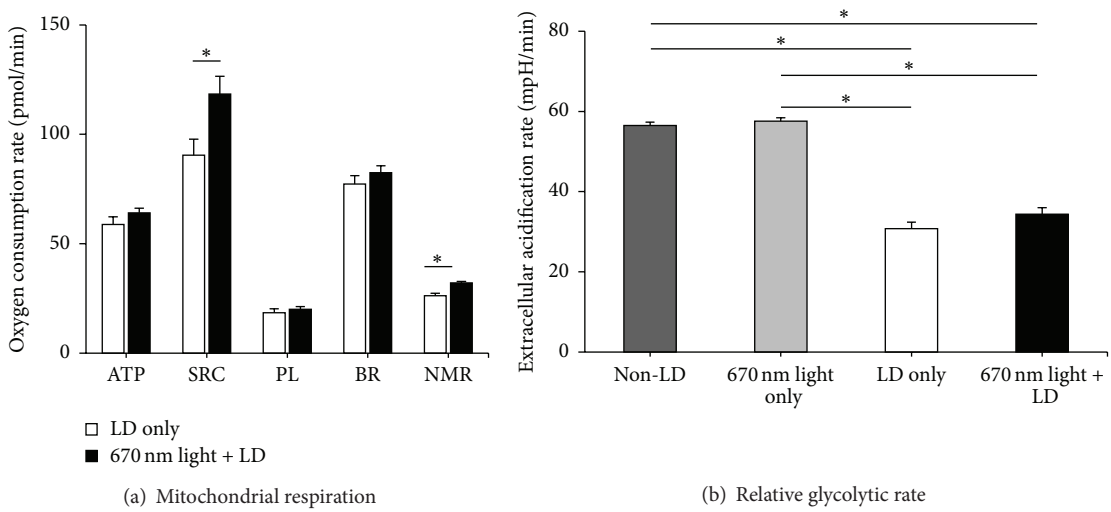


Figure 4: TUNEL+ cell count in dim-reared animals. Animals raised in dim light (no light damage) treated with 90 J/cm^2 670 nm light had three times more TUNEL+ cells compared to control animals that were hand-held only for 30 minutes to mimic treatment conditions ($n = 3$ animals for each group, "$*$"denotes significance $P < 0.05$; error bars represent SEM).



(a) Mitochondrial respiration

(b) Relative glycolytic rate

Figure 5: Effect of 9 J/cm^2 of 670 nm light on the mitochondrial respiration of 661 W photoreceptor cells. (a) Under light damage conditions, the cells exhibited an increase in both spare respiratory capacity (SRC) and nonmitochondrial respiration (NMR) when treated with 670 nm light. ATP production (ATP), proton leak (PL), and basal respiration (BR) did not change between groups ($n = 4$ for both groups; "$*$" denotes significance, $P < 0.05$; error bars represent SEM). (b) 670 nm light did not produce a difference in relative basal ECAR measurements in both normal and light damaged conditions. Light damage (LD) significantly decreased the ECAR measurements in cells, both treated and nontreated, when compared to nonlight damaged cells ($n = 3$ for nonlight damaged cells; $n = 4$ for light damaged cells; "$*$" denotes significance using one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$; error bars represent SEM).

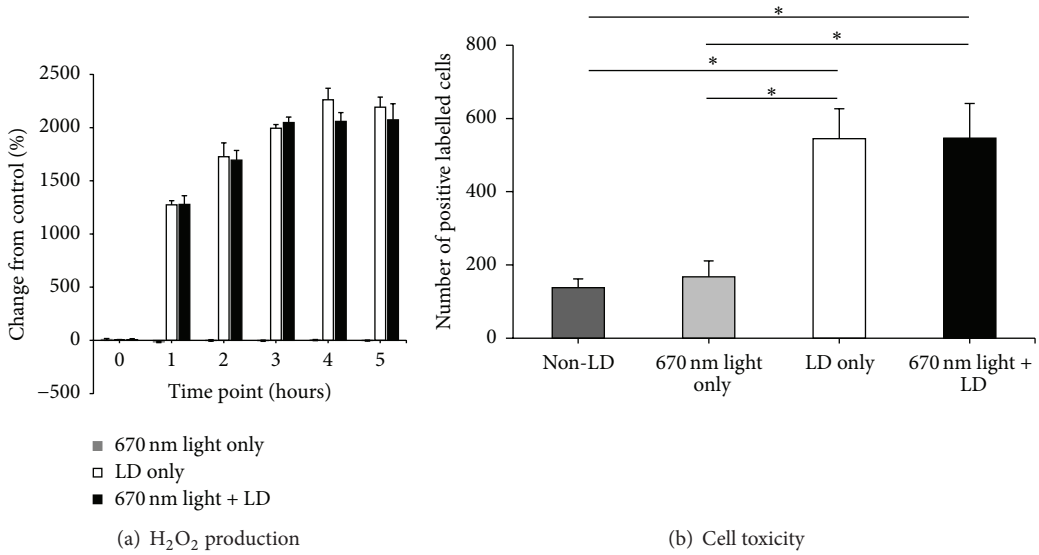
in a thicker ONL, compared to all other treatment groups and controls exposed to high intensity damage.

Figures 3(b)–3(e) show the effects of four different 670 nm light doses in animals exposed to "high" intensity light damage. The data show largely comparable ONL thickness in the light damage controls and animals treated with 9, 18, or 36 J/cm^2 670 nm light (Figures 3(b)–3(d)) but a significantly thicker ONL adjacent to the optic nerve head in animals treated with 90 J/cm^2 red light (asterisks Figure 3(e)).

3.1.4. 90 J/cm^2 670 nm Effect on Normal Retinas. Animals reared in dim light conditions, not subjected to light damage or 670 nm light treatment, had virtually no TUNEL+ cells in the retina (control, Figure 4). Dim-reared animals treated with 90 J/cm^2 670 nm light had low numbers of TUNEL+ cells in the ONL, although these numbers were significantly higher than in the control dim-reared animals (90 J/cm^2 only, Figure 4).

3.2. Effect of 670 nm Light on Mitochondrial Metabolism. Light damaged 661 W photoreceptor cells treated with 670 nm light exhibited a significant increase in spare respiratory capacity, compared to control cells (light damage only) (Figure 5(a)). In addition to a change in spare respiratory capacity, there was also an increase in nonmitochondrial respiration in treated cells compared to control cells. However, there was no change in ATP production, proton leak, or basal respiration.

No changes were seen in the measurements of basal extracellular acidification rate (ECAR) between 670 nm light treated cells and nonlight damaged cells (Figure 5(b)). Light damaged cells that had been treated with 670 nm light



(a) H_2O_2 production (b) Cell toxicity

FIGURE 6: (a) The effect of 9 J/cm^2 of 670 nm light and light damage on H_2O_2 production in 661 W photoreceptor cells is shown here where bars represent the percentage change of H_2O_2 production when compared to nonlight damaged cells. Light damage significantly increased H_2O_2 production in the cells directly after one hour of light exposure. H_2O_2 production steadily increased amongst light damaged cells, both treated and nontreated, with increasing time under bright light conditions ($n = 3$ for all groups; each run consisted of duplicate wells for each group; "$*$" denotes significance using one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$; error bars represent SEM). (b) There was an increase in cell toxicity in light damaged (LD) cells, both treated and nontreated, when compared to nonlight damaged cells. 670 nm light did not significantly decrease cell toxicity in control or light damaged conditions ($n = 3$ for all groups; each run consisted of duplicate wells for each group; "$*$" denotes significance using one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$; error bars represent SEM).

also showed no change in ECAR when compared to light damage only cells. Nonlight damaged cells, both treated and nontreated, demonstrated an increase in ECAR readings when compared to light damaged cells, both treated and nontreated.

3.3. Effect of 670 nm Light on H_2O_2 Production. The production of H_2O_2 was measured as a time series throughout the 5-hour light damage paradigm we used for all of our *in vitro* experiments. H_2O_2 production increased by 1500% after one hour of light damage when compared to nonlight damaged cells (Figure 6(a)). This was followed by a steady increase with each hour in both treated and nontreated cells under the light damage paradigm. There was no significant difference in H_2O_2 production for nonlight damaged cells, regardless of 670 nm treatment or not.

3.4. Effect of 670 nm Light on Cell Toxicity. Cell toxicity and death were measured using a cell toxicity assay (Figure 6(b)). Cells on the plates that were light damaged had a significantly higher amount of cell death when compared to normal nonlight damaged cells. There were no significant differences in the amount of cell death between light damaged, 670 nm light treated cells and nondamaged, 670 nm light treated cells.

4. Discussion

This study has generated three key novel findings on the action of 670 nm in the light damaged model of retinal degeneration. First, our *in vivo* experiments suggest that lower doses of 670 nm light are protective against photoreceptor death, while higher doses promote photoreceptor death. Second, *in vivo* experiments indicate that the protective efficacy of a specific dose of 670 nm light depends on the degree of retinal damage, such that high doses of 670 nm light may be damaging to a healthy retina but may be required to be protective against more severe or progressive retinal damage. Third, our *in vitro* experiments show an increase in spare respiratory capacity of 661 W cells treated with 670 nm light.

4.1. 670 nm Light Dose-Response. Previous studies have typically used a single, often arbitrary dose of 670 nm light to test its efficacy in a simple damage paradigm [24, 26, 33–35]. In others, seemingly arbitrary treatment doses have varied with wavelength, tissue type, and the type of damage [21], leading to a difference in biological response. In this study we varied both the degree of damage and the dose of 670 nm light used to ameliorate that damage, in an attempt to demonstrate the importance of dosage in treatment with 670 nm red light.

Our findings indicate that the *lower* doses of 670 nm light (9 J/cm^2 and 18 J/cm^2) protect photoreceptors from cell death in the low and medium intensity light damage paradigms (750 and 1000 lux). However, *higher* doses of 670 nm light either had no effect on photoreceptor death (36 J/cm^2) or induced additional death (90 J/cm^2), in the low and moderate light damage paradigms (Figure 4). The capacity of 670 nm light alone to induce photoreceptor death was confirmed in

experiments where animals were treated with 90 J/cm^2 in the absence of light damage (Figure 4).

An unexpected finding is that *high* doses of 670 nm light (90 J/cm^2) *reduced* the levels of photoreceptor death when the light damage stimulus is of a high intensity (Figure 1(a) light grey, dotted line, Figure 1(d)). These data indicate that the level of protection afforded by 670 nm light is dependent on the degree of damage done to the retina.

This interplay between retinal damage and effective 670 nm dose has not been identified previously. Favourable outcomes of 670 nm light treatment for retinal degeneration models have typically used a dose of 9 J/cm^2 in a 1000-lux light damage paradigm and show reduced photoreceptor cell death, downmodulation of inflammatory factors, and indicators of oxidative stress [22–24, 33]. Protective effects using 90 J/cm^2 670 nm light have been reported in the retina previously in a higher intensity light damage paradigm (1800 lux) [25]. Those findings are consistent with our present findings and support the concept that higher doses of 670 nm light are required as a therapeutic dose to treat highly stressed/damaged retinas. It is important to note that the high dose of 90 J/cm^2 used might be preconditioning the retina to retinal stress, thereby protecting the retina against further stress.

The dose-response of the retina to 670 nm light is consistent with the concept of *hormesis* (reviewed in [36, 37]), that is, a biphasic dose-response to an environmental agent that is stimulatory at low doses, and inhibitory at high doses.

4.2. Metabolic Effects of 670 nm Light. Evidence of the role of cytochrome c oxidase activity being modulated by red light has been shown by exposing cells to high-fluence, low-power laser irradiation 635 nm lasers, causing photoinactivated respiratory chain oxidase to trigger a fatal mitochondrial superoxide burst [38, 39]. Previous *in vitro* studies have reported increases in cytochrome c oxidase activity, mitochondrial metabolism, and ATP production, after irradiation with red/near-infrared light [27, 28, 30, 40, 41], although the mechanisms generating these effects have not been identified.

Although we did not detect any changes in ATP production by 661 W photoreceptor-like cells using the Seahorse XFe96 Extracellular Flux Analyzer, a change in the spare respiratory capacity of the cells was observed. We detected a 31% increase in spare respiratory capacity in cells treated with 670 nm light, compared with untreated cells. Control, nonlight damaged cells also exhibited an increase in the spare respiratory capacity when treated with 670 nm light.

Spare respiratory capacity of a cell is critical when a sudden burst of energy is required to survive critical stress conditions and is important in the long-term viability of cells [42]. Spare respiratory capacity depletion has been implicated in various disease conditions including heart diseases [43], neurodegenerative disorders [44, 45], and cell death in smooth muscle [46], but its significance in retinal metabolism is yet to be established. The measurable increase in spare respiratory capacity identified in the present study helps substantiate claims of a role for the mitochondria, and specifically cytochrome c oxidase, in 670 nm light therapy [27–31, 47]. We hypothesize that this increase in spare respiratory capacity is the precursor to an increase in ATP production demonstrated in a number of other models [30, 41]; however this increase in ATP is only evident when required by the cells. We suspect that an increase in ATP was not observed here, as the damage stimulus that we had applied was not severe enough to warrant this biological change.

The data also indicated a 23% increase in nonmitochondrial respiration in 670 nm light treated cells, compared to controls. A number of factors can contribute to nonmitochondrial respiration [48]. In the present context, it is possible that nonmitochondrial respiration is reduced by light damage as a result of decreased efficiency of detoxifying and ROS-scavenging enzymes and that treatment with 670 nm light restores those functions, increasing nonmitochondrial respiration. However, we detected no alteration in H_2O_2 output between light damaged 661 W cells, treated *versus* untreated, or any protection against cell death in the 670 nm light treated cells.

The literature includes conflicting reports with regard to changes in ROS levels following red light treatment (reviewed in [21]). In the present study, to our surprise, our *in vitro* experiments detected no changes in cell death levels but did demonstrate an increase in spare respiratory capacity in 661 W cells. Our data indicate that the effects of 670 nm light treatment may be dependent on the experimental environment, involving processes that occur in tissues comprising mixed cell populations (as occurs *in vivo*), as distinct from a monoculture environment (as *in vitro*). This may explain why we see a protective function for 670 nm light treatment *in vivo*, but no changes in cell death rates in 670 nm light treated cells *in vitro*.

4.3. Possible Mechanism of 670 nm Light. Cytochrome c oxidase is complex IV of the mitochondrial electron transport chain and is the immediate regulator of oxidative phosphorylation tightly controlling the production of ATP [49]. Nitric oxide (NO) has the ability to bind to the oxygen-binding site of cytochrome c oxidase, which allows it to inhibit the action of cytochrome c oxidase by competing with molecular oxygen for the respective binding site. This reversible inhibition of complex IV by NO has been shown to occur at nanomolar levels, suggesting that NO may be a regulator of respiration by reducing the rate of ATP production [50–53]. One possibility that has been canvassed previously is that 670 nm light releases NO from cytochrome c oxidase, allowing for an increased rate of oxidative phosphorylation and ATP production [54–56]. Not only will this directly allow for increased respiration due to an increased action of cytochrome c oxidase, but NO may also act as a downstream signaling molecule and vasodilator [54, 57, 58]. Therefore, this release of NO may also allow for a more rapid rate of oxygen delivery for phosphorylation as a further downstream effect [54].

We hypothesize that the increase in spare respiratory capacity detected in cells treated 670 nm light in this study is a result of NO release from cytochrome c oxidase in

the mitochondria. Such release would allow for modulation of metabolic output, by increasing the binding potential of cytochrome c oxidase to oxygen, allowing for increased ATP production. Further studies are needed to provide better insight into the ways that red and near-infrared light interact with NO and cytochrome c oxidase to modify respiratory capacity and generate ATP.

5. Conclusions

Our findings indicate that determining an effective dose for 670 nm light therapy is complex, being dependent on the degree of damage being treated. While low levels of 670 nm light are ineffective, too high a dose has potentially toxic effects. Consistent with this, dose-response curves for 670 nm light appear to trend towards an inverted U-shaped distribution, consistent with hormesis. We also find that 670 nm light induces an increase in spare respiratory capacity in 661 W photoreceptor-like cells, reinforcing the view that red light therapy stimulated mitochondrial function. Such an increase in spare respiratory capacity would provide for increased metabolic output, to maintain retinal homeostasis and protect against the progression of photoreceptor cell death.

Disclosure

Joshua A. Chu-Tan and Matt Rutar are co-first authors.

Conflict of Interests

The authors have no known conflict of interests to disclose.

Acknowledgments

This work is supported by National Health and Medical Research Council project Grant no. 1049990 and the JCSMR Bootes Foundation.

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