Photobiomodulation Reduces Photoreceptor Death and Regulates Cytoprotection in Early States of P23H Retinal Dystrophy

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ABSTRACT

Irradiation by light in the far-red to near-infrared (NIR) region of the spectrum (photobiomodulation, PBM) has been demonstrated to attenuate the severity of neurodegenerative disease in experimental and clinical studies. The purpose of this study was to test the hypothesis that 670 nm PBM would protect against the loss of retinal function and improve photoreceptor survival in a rodent model of retinitis pigmentosa, the P23H transgenic rat. P23H rat pups were treated once per day with a 670 nm LED array (180 sec treatments at 50 mW/cm^2; fluence 9 joules/cm^2) (Quantum Devices Inc., Barneveld WI) from postnatal day (p) 16-20 or from p10-20. Sham-treated rats were restrained, but not exposed to NIR light. The status of the retina was determined at p22 by assessment of mitochondrial function, oxidative stress and cell death. In a second series of studies, retinal status was assessed at p30 by measuring photoreceptor function by ERG and retinal morphology by Spectral Domain Optical Coherence Tomography (SD-OCT). 670 nm PBM increased retinal mitochondrial cytochrome oxidase activity and upregulated the retina's production of the key mitochondrial antioxidant enzyme, MnSOD. PBM also attenuated photoreceptor cell loss and improved photoreceptor function. PBM protects photoreceptors in the developing P23H retina, by augmenting mitochondrial function and stimulating antioxidant protective pathways. Photobiomodulation may have therapeutic potential, where mitochondrial damage is a step in the death of photoreceptors.

Keywords: retinal degeneration, photobiomodulation, mitochondrial dysfunction, electroretinogram, Spectral Domain Optical Coherence Tomography, cytochrome oxidase, retinitis pigmentosa.

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1. INTRODUCTION

The retinal dystrophies, or retinitis pigmentosa (RP), are a heterogeneous group of blinding retinal diseases, with the common feature of photoreceptor degeneration, commonly associated with single-gene mutations[1,2]. Numerous investigations have provided considerable information regarding the genetic bases of these degenerations, and several previous studies have provided evidence that, in many forms of dystrophy, oxidative damage to mitochondria is a step in the death of photoreceptors[1-3]. Photoreceptors have a dense concentration of mitochondria in their inner segments to provide ATP for the energy-intensive processes of outer segment renewal and maintenance of dark current[3].

Evidence is growing that exposure of tissue to photons in the far-red (FR) to near-infrared (NIR) range of the spectrum (630-900 nm) can restore the function of damaged mitochondria[4-6]. Using low energy lasers or light-emitting diode (LED) arrays, FR/NIR light treatment (also termed termed photobiomodulation [PBM]) has been applied clinically to accelerate healing of lesions and wounds in soft tissues, for more than 40 years[7]. NIR penetrates tissues including heart muscle, spinal cord and brain and has been documented to improve recovery from ischemic heart injury[8] to attenuate damage in the injured spinal cord, retina and optic nerve[9-11] to reduce the oral mucositis in patients undergoing bone marrow transplants[12] and to improve functional recovery from stroke[13].

The therapeutic effects of NIR light have been hypothesized to result from intracellular signaling pathways triggered when NIR photons are interact with the mitochondrial photoacceptor molecule, cytochrome c oxidase[5,6]. This interaction results in the production of transcription factors, changes in gene transcription and culminating in improved cellular mitochondrial energy metabolism and antioxidant production[5-7] as shown in Figure 1. Importantly**,** the cytoprotective action spectrum of FR/NIR light corresponds with the cytochrome oxidase absorption spectrum[5-7]. Gene discovery studies conducted using microarray technology documented a significant upregulation of gene expression in pathways involved in mitochondrial energy production and antioxidant cellular protection[9].

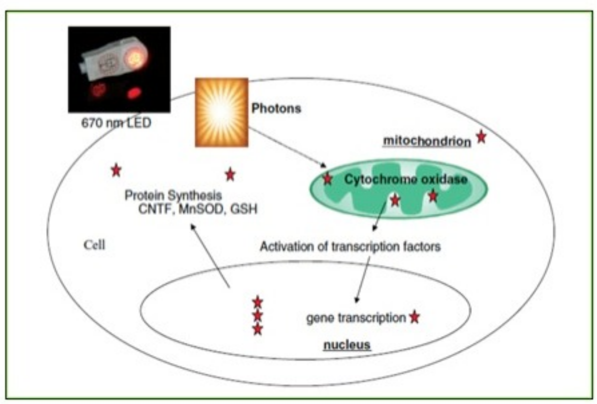

Figure 1: Hypothesized Mechanism of Action of Photobiomodulation

This study tests the effect of PBM on the death of photoreceptors in the P23H rat, a model of human disease, in which the transgene is a rhodopsin gene engineered to mimic a mutation which causes an autosomal dominant form of human RP common in North America. PBM was applied during the critical period of photoreceptor development, from p16-20 or p10-25[13]. Many photoreceptor degenerations begin during this period, which lasts from p16-25 in the non-degenerative SD rat, and from p12-22 in the C57BL/6 mouse; documented examples include the degenerations observed in the RCS rat [13,14], and the P23H rat[15]. We report that photobiomodulation increases the expression of cytochrome c oxidase and MnSOD and significantly reduces photoreceptor death during the critical period of photoreceptor death in the P23H rat.

2. METHODS

2.1 Animals studied

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and were conducted in accordance with the ARVO (The Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research, with the requirements of The Australian National University Animal Experimentation Ethics Committee and with the National Institutes of Health regulations. Observations were made in transgenic rats containing the P23H mutation on the rhodopsin gene (Line 1 and Line 3, Retinal Degeneration Rat Model Resource [LaVail Laboratory], University of California, San Francisco). P23H homozygous animals were established as a breeding colony. The animals used in the present experiments were heterozygotes, the offspring of mating P23H homozygotes with Sprague-Dawley (SD) albinos. All rats were raised in dim cyclic light (12h at 5 lux, 12 hours dark), with food and water available *ad libitum*.

2.2 Experimental Design

In the first series of experiments, rat pups were randomly assigned to two groups, treated and sham-treated control. The treated group were removed briefly from the mother each day for 5d from p16-20 and exposed for 180 seconds to light with a wavelength of 670nm. The light was generated by a LED array engineered to eliminate heat (WARP 10, Quantum Devices, Barneveld, Wisconsin). The array was positioned 2.5cm above the dorsum of the pup, generating light with a power intensity of 50mW/ cm^2 and an energy density of 9 joules/cm^2 at the dorsal surface of the pup. These parameters have been previously demonstrated to stimulate cellular proliferation and cytochrome oxidase activity in cultured neurons[6] to promote wound healing clinically[7,12] and in experimental animal models[7] and to promote the survival and functional recovery of the retina and optic nerve *in vivo* after acute injury by a mitochondrial toxin[10]. Sham-treated control animals were handled in the same way, except that they were not exposed to the 670nm light. Eyes were harvested at p22 and prepared for immunohistochemical analysis. In the second series of experiments, rat pups were treated once per day from p10-25 (180 s, 50 mW/cm2, 9 J/cm2). Spectral Density Optical Coherence Tomography (SD-OCT) and electrophysiology (ERG) measurements were made at p30.

2.3 Tissue Collection and Processing

At p22, animals were euthanized with an overdose of sodium pentobarbital (>60 mg/kg, intraperitoneal). Eyes were marked with an insoluble projection pen at the superior aspect of the limbus for orientation, enucleated and immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4 for 1-3 h. For cryosectioning, eyes were rinsed three times in 0.1 M PBS and left in a 15% sucrose solution overnight to provide cryoprotection. Eyes were embedded in Tissue-Tek OCT Compound (ProSciTech, IA018), snap frozen in liquid nitrogen and cryosectioned at 14 µm. Sections were mounted on gelatin and poly-L-lysine-coated slides and dried overnight at 50ºC before being stored at -20ºC.

2.4 Immunohistochemistry

Antibodies against the following proteins were used: cytochrome oxidase IV subunit I (COX) (Mitosciences, ms404, 1/200) and manganese superoxide dismutase (MnSOD) (Upstate, 06-984, 1/200). Sections were processed following protocols published previously[14,15] . Briefly, sections were washed in 70% ethanol for 15 min, followed by a 5 min wash in distilled H_2O and two 5 min washes in 0.1 M PBS. With the antibodies against COX and MnSOD, slides were incubated for 3h at 37°C in Immuno Reveal It™(diluted 50% in PBS). Sections were washed twice in 0.1 M PBS for 5 min, then incubated with an antibody to rabbit IgG conjugated with Alexa Fluor 488, or with an antibody to mouse IgG conjugated to Alexa Fluor 594 (1:1000, Molecular probes, Eugene, OR) for 24 h at 4ºC. After washing twice in 0.1 M PBS, sections were incubated for 2 min with the DNA-specific dye bisbenzamide (1:10,000) in 0.1 M PBS, before being coverslipped with a glycerol/gelatin medium. Levels of fluorescent intensity were assessed using confocal microscopy (Zeiss Pascal) and optical densitometry (ImageJ).

2.5 Dying and surviving photoreceptors

To assess the number of dying photoreceptors, sections were labeled with the TUNEL technique as described previously[14,15]. Eyes from 9 animals from each group were examined. Sections which passed through or close to the optic nerve head were selected for counting. Sections were cut and scanned from the superior to the inferior edge of the retina, in 1 mm steps using a 20x objective. The number of cells observed in the outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) were all counted and recorded separately for each 1mm step.

2.6. Electroretinography

Photoreceptor function was assessed by full-field flash-evoked electroretinography (ERG) at p30. Rats were dark-adapted overnight and prepared under red dim light. Animals were anesthetized with a ketamine and xylazine cocktail (100 mg/kg and 5 mg/kg respectively, ip) and placed on a heating pad at 37°C during recordings. Pupils were dilated with 0.1% atropine and mild topical anesthesia (proparacaine 0.5%). Full-field ERGs were obtained in a Ganzfeld dome (76 mm diameter Flash Dome with 55 mm aperture) using nylon coated gold thread electrode placed on the corneal surface, overlaid with 1% methylcellulose and a contact lens. A sub dermal needle reference electrode and a ground needle electrode were placed in the cheek and tail respectively. A high-intensity flash unit (HMsERG instrument, Retvet Corp, MO) was used to provide flash intensities (ranging from 10 $mcd.s/m^2$ to 25000 $cd.s/m^2$) for scotopic intensity series response measurements. The ISCEV protocol was also employed to examine rod-mediated and rod and cone-mediated responses. Signals were amplified, digitized and averaged using ERG view 2.5 software (RetVet Corp. Inc., Columbia, MO). A custom-made Faraday cage was employed to block 60 cycle electrical noise. The amplitudes and latencies of the a-waves (photoreceptor responses) and b-waves (bipolar cell and Muller cell responses) were fit to a computational model to determine transduction parameters for photoreceptor responses.

2.7 Spectral Domain Optical Coherence Tomography (SD-OCT) imaging

High-resolution scans were performed at p30 to assess retinal morphology using SD-OCT (Bioptigen Inc, Research Triangle Park, NC). Animals were anesthetized with a ketamine and xylazine cocktail (i.p., 50:5 mg/kg body weight) and were placed on a custom made six-axis animal alignment system. Both eyes were imaged in a single session after dilation of pupil (1% Atropine sulphate) and hydration of cornea (Systane ultra, polyethylene glycol 400, 0. 4%). The fundus imaging camera in the optical head of the OCT provided initial alignment for the light source during the real time aiming. Final positioning was guided by monitoring and calibrating the real-time OCT image of the retina formed. The optic nerve head (ONH) was used as a landmark (Figure 1a and Figure 1b) and linear (1000 A-scans/B-scan, 80 B-scans) and volume scans (750 A-scans/B-scan, 250 B-scans/volume) per eye were captured and saved using the Bioptigen's InVivoVue™ software. For the linear scans, the B-scans were registered and averaged as previously described[16] using ImageJ [17] (National Institutes of Health, Bethesda, MD) and segmentation of retinal layers for total thickness were determined using an algorithm generated in MatLab (The MathWorks, Inc., Natick, MA). The difference in reflectivity of retinal layers was used in generating longitudinal reflectivity profile[18]

3. RESULTS

3. 1 Attenuation of photoreceptor loss

At P22, TUNEL+ cells were frequent in the ONL (Figure 2); that is, photoreceptor cells were undergoing apoptotic death, at an abnormally high rate, confirming Yu et al. 2004. This high frequency of TUNEL+ cells in the ONL of the development P23H-3 retina was reduced 70% by PBM from P16-20.

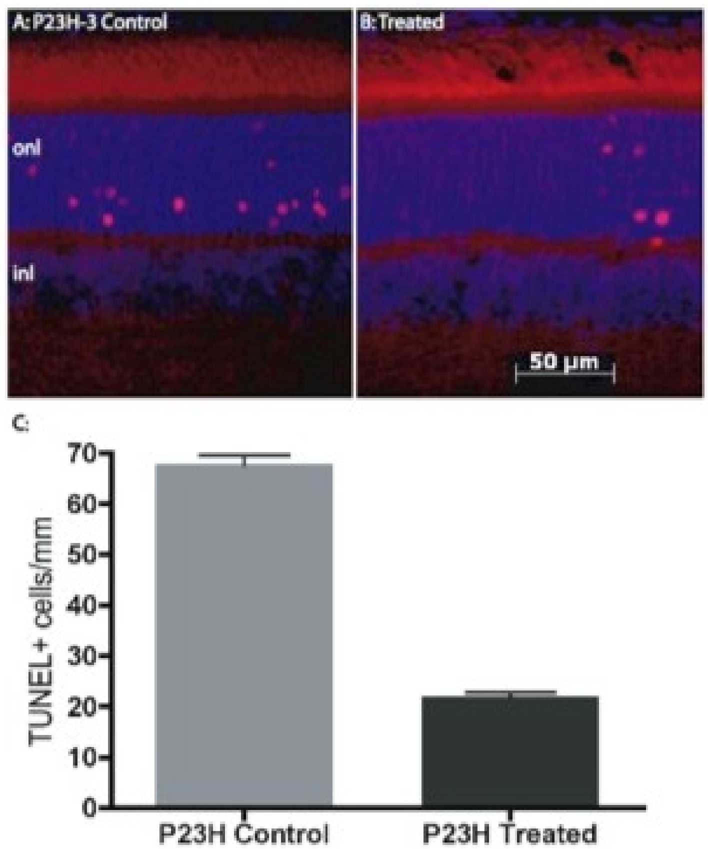

Figure 2: PBM attenuates photoreceptor cell death. TUNEL positive somas are labelled red; nuclear DNA is labelled blue, with bisbenzamide. onl – the ONL; inl – the INL Right Panel: TUNEL+ cells in the control retina were frequent, and confined to the (outer nuclear layer (ONL). Left Panel: The frequency of TUNEL positive ONL cells after photobiomodulation C: Quantitative results, summarising data from 6 animals, with 5 sections measured per animal. The error bars represent the standard error of the mean.

3.2 Upregulation of cytochrome oxidase (CO)

CO is a key mitochondrial enzyme and, in non-degenerative retina, is expressed strongly where mitochondria concentrate, particularly in inner segments, but also in the OPL, IPL and GCL. As shown in the left panel of Figure 3 in the untreated (control) P23H-3 retina at P22 (Fig 3A) CO is not strongly expressed in inner segments. Following PBM, CO expression was increased (Fig 3 B), most prominently in the inner segments. The effect is shown layer by layer in Figure 3C. Increased CO expression was apparent in the GCL and, most prominently, in the layer of inner segments. The rise in inner segment expression of CO is shown quantitatively in Fig 3D.

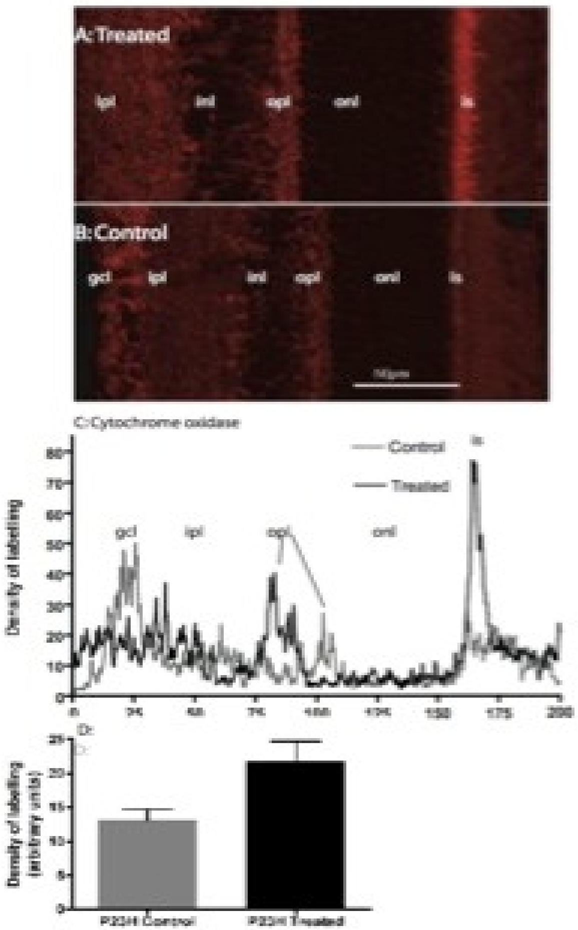

Figure 3: PBM upregulates of CO expression. A,B: Sections of control and NIR-treated P23H-3 retinas, at P22, labeled for CO. Ganglion cell layer (glc); inner plexiform layer (ipl); inner nuclear layer (inl); outer plexiform layer (opl); outer nuclear layer (onl) ; inner segments (is)C: Optical densitometry of A and B, showing the density of labeling in transects across each retina. D: For in inner segments, mean and standard errors for CO labelling for 5 animals, 5 sections from each animal. $P < 0.05$ on an unpaired, 2-tailed t-test.

3.3. Upregulation of manganese superoxide dismutase

MnSOD is the mitochondrial form of superoxide dismutase, an antioxidant enzyme responsible for the conversion of the highly reactive superoxide anion to the less reactive hydrogen peroxide. Photobiomodulation increased the expression of MsSOD in the P23H retina, most markedly in the layer of inner segments as shown in the right panel of Figure 3. (compare A and B). The difference is shown more quantitatively in Figure 3C; the increase in the treated retina is most marked in the layer of inner segments, although some increase is apparent in the ganglion cell layer.

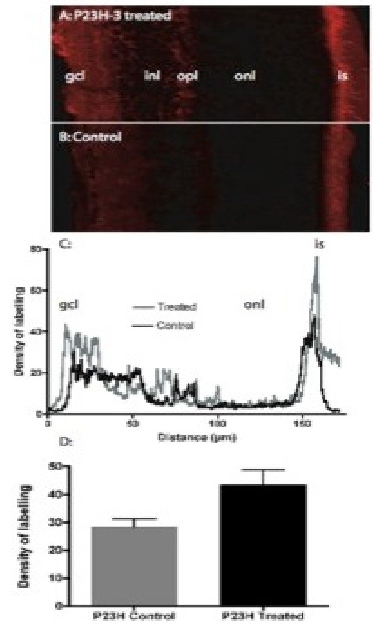

Figure 4: Upregulation of MnSOD expression A,B: Sections of control and NIR-treated P23H retinas, at P22, labeled for MnSOD. Ganglion cell layer (glc); inner plexiform layer (ipl); inner nuclear layer (inl); outer plexiform layer (opl); outer nuclear layer (onl) ; inner segments (is). C: Optical densitometry of A and B, showing the density of labelling in transects across each retina. D: Labeling density for inner segments, mean and standard errors for CO labeling for 5 animals, 5 sections from each animal. $P < 0.05$ on an unpaired, 2-tailed t-test.

3.4 Protection of Photoreceptor Function

Figure 5 shows the differences in retinal function between Sprague Dawley (SD) non-dystrophic, sham-treated P23H and NIR-treated P23H rats. Rats were treated during the critical period of photoreceptor cell loss (p10-p25) and the ERG was measured at p30. The function of the photoreceptors (a-wave amplitude; Figure 5 right panel) was assessed by full-field flash ERG in dark-adapted rats (scotopic intensity series protocol) at p30.

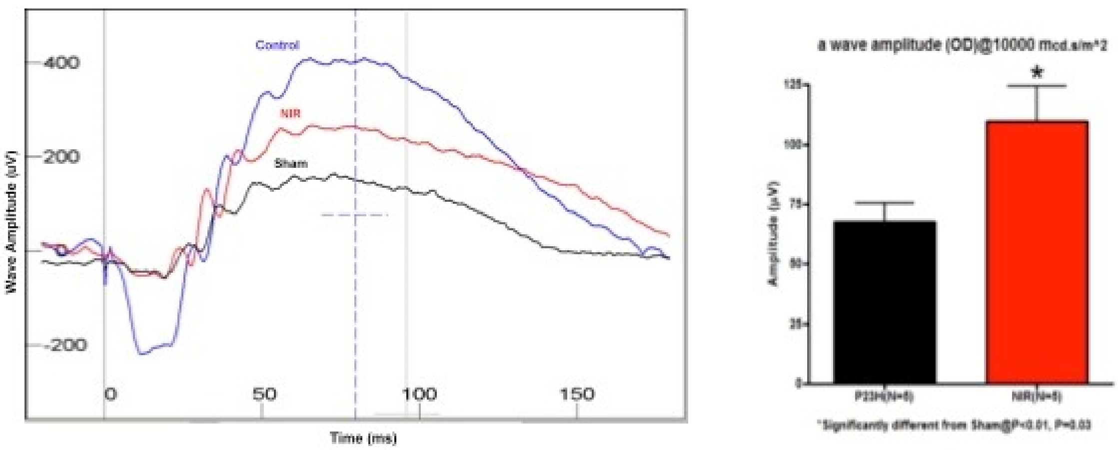

Figure 5: PBM protects photoreceptor function: Mean a-wave amplitude assessed at 1000 mcd:s/m^2 in sham and NIR-treated P23H rats at p30. $P < 0.05$ obtained from an unpaired Student's t-test.

3.6 Protection of Retinal Structure

Figure 6 shows a comparison of the total retinal thickness of sham-treated P23H rats and NIR-treated P23H rats using SD-OCT at p30. In our studies the preservation of total retinal thickness was evident in the in NIR-treated group compared to the non-treated group. The mean total retinal thickness was significantly (*p*<0.0001) higher in the NIR- treated group compared to the sham-treated group.

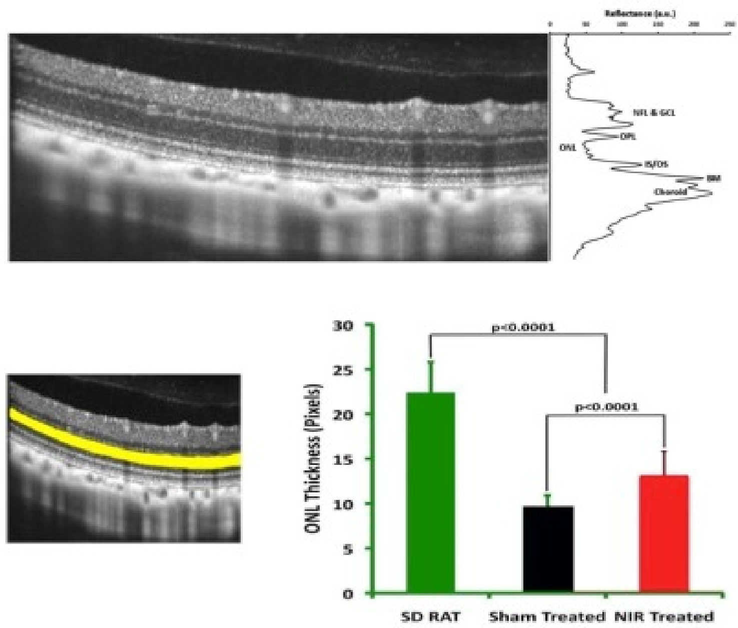

Figure 6: PBM protects retinal structure: The ONL thickness of the PBM-treated group derived from Longitudinal Reflectivity Profile (LRP) [left panel] was significantly greater compared to the sham-treated group across the entire scan. Error bars: SEM.

4. DISCUSSION

The present study has demonstrated that photobiomodulation (low intensity far-red light, 670nm) administered during the critical period of photoreceptor development in the P23H rat increases retinal mitochondrial cytochrome oxidase activity, upregulates the production of the antioxidant protective enzyme, MnSOD and prevents apoptotic photoreceptor cell death. PBM also preserved retinal structure and protected against the loss of photoreceptor function.

4.1 The case for testing NIR

Near-infrared radiation (NIR) from light-emitting diode sources or from low intensity laser sources, is a newly developed method for accelerating wound healing, and is being tested in animal models of wound healing and neural injury[7]. The effective wavelengths range from 630 – 900nm, corresponding to the absorption spectrum of cytochrome c oxidase (CO)[5,6]. Radiation at 670nm has been shown to be particularly effective in increasing ATP production and CO activity cultured neurons[6], and has been used to accelerate wound healing in diabetic mice[9], to aid in the healing of oral mucositis during bone marrow replacement in pediatric patients[12] and protect cultured rodent primary visual cortex neurons from the effects of KCN exposure[6]. In a photoreceptor toxicity model, Eells *et al*[10], demonstrated photobiomodulation reduces the damage to mitochondria in photoreceptors induced by methanol poisoning, and greatly reduces photoreceptor death. In a human clinical trial, photobiomodulation per cranium has been show to reduced the size of the damaged area caused by the vessel blockage, and to reduce the associated loss of cognitive function[13].

4.2 Mechanisms

In vitro studies in our laboratory, and by other investigators, have shown that that near-infrared radiation interacts with the enzyme cytochrome oxidase (CO, in mitochondria, triggering signaling mechanisms which result in improved energy metabolism, antioxidant production and cell survival[4-6]. A key outcome of this signaling pathway is the up-regulation of CO activity. CO is the rate-limiting step in mitochondrial respiration and increased cytochrome oxidase activity improves respiratory efficiency under conditions of oxidative stress, thus attenuating the production of reactive oxygen species[19].

4.3 Therapeutic potential

These findings have significant implications for the use of photobiomodulation in the treatment of retinal degenerative diseases. They document the therapeutic potential of 670 nm PBM in an established rodent model of retinitis pigmentosa, establishing a basis for clinical trials of photobiomodulation in human disease. The present results build on our previous findings *in vitro* and *in vivo,* and strongly support the hypothesis that photobiomodulation augments mitochondrial function and stimulates antioxidant protective pathways in the neural retina to protect against retinal degeneration. By harnessing the potential of damaged cells to self-repair, photobiomodulation provides an innovative and non-invasive therapeutic approach for the prevention and treatment of retinal dystrophies and degenerative disease.

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