High energy blue light induces oxidative stress and retinal cell apoptosis

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Abstract

Blue light (BL) is a high energy, short wavelength spanning 400 to 500 nm. Found in technological and environmental forms, BL has been shown to induce photochemical damage of the retina by reactive oxygen species (ROS) production. Excess ROS leads to oxidative stress, which disrupts retinal mitochondrial structure and function. As mitochondria amply occupy photoreceptors, they also contribute to oxidative stress due to their selectively significant absorption of BL at 400 to 500 nm. ROS generation that induces oxidative stress subsequently promotes retinal mitochondrial apoptosis. BL filtering and preventative mechanisms have been suggested to improve or repair BL-induced retinal damage, including BL blocking lenses and the low light therapy called photobiomodulation (PBM). The mechanism behind ROS leading to apoptosis when stimulated by red light (RL) and near-infrared light (NIRL), or PBM, remains poorly understood. However, previous studies have shown PBM to improve mitochondrial apoptotic conditions and respiration, while also decreasing ROS production and protecting photoreceptor gene expression. This thesis evaluates the ameliorative effect of PBM on BL-induced retinal mitochondrial apoptosis, oxidative stress, and metabolism instability. A study conducted by Heinig et al. (2020) observed that PBM increased oxidative phosphorylation complex activity, improved apoptotic conditions, and regulated mRNA gene expression following BL exposure. Additionally, Heinig et al. determined that complexes I and II are also PBM targets besides complex IV in the mitochondrial respiratory chain. A study by Kaynezhad et al. (2022) found that environmental BL significantly disrupts retinal cell hemodynamics and mitochondrial metabolism. PBM has illustrated the ability to improve retinal damage caused by BL, solidifying its revolutionary application in effective treatments against BL exposure.

Overview

Blue light (BL), in forms of technology such as LEDs and environment such as sunlight, obtains a high energy and short wavelength. Its wavelength can expose the retina in the eye to damage that involves stress and inflammation. Absorbed high energy BL leads to the generation of reactive oxygen species (ROS), which are unstable molecules that eventually lead to oxidative stress. Oxidative stress occurs when there are too many ROS to protect the retina against. This injury that results due to oxidative stress leads to retinal cell death. In order to prevent BL-induced damage, BL filtering glasses were created to relieve stress and irritation on the eye. In addition, other treatments such as the low intensity laser therapy called photobiomodulation (PBM) were developed for improving injurious eye conditions caused by BL. PBM effectively uses red light (RL) and near-infrared light (NIRL) stimulation to ease the BL-induced effects such as increased ROS production, decreased oxygenation, interrupted metabolism, and increased cell death. This thesis closely looks at how PBM is able to enhance retinal function, following the instability due to oxidative damage and cell death that BL can bring. Also, the thesis explores how BL exposure plays a significant role in the retina by affecting metabolism, which involves the regulation of energy.

Two studies are used to support the observation of BL's harmful effects on the eyes. The first study done by Heinig et al. in 2020 used PBM to improve eye conditions in mice. After being exposed to BL, the eyes of the mice experienced improvement when exposed to RL and NIRL. PBM notably decreased ROS levels, increased oxygenation, and decreased cell death. The expression for the molecule mRNA, which creates protein for the body, was also restored by PBM. The amount of ATP, which is an energy source in the mitochondria, increased when exposed to RL and NIRL. This correlates to knowing whether the metabolism is interrupted or

regulated. In this case, the metabolism was regulated following RL and NIRL exposure. The second study by Kaynezhad et al. looks at BL found in the environment and its effects on the eyes of mice. Continuous exposure to environmental BL increased changes in hemoglobin and oxidase proteins that are involved in regulating metabolism. Importantly, deoxygenated hemoglobin experienced the greatest change in instability. BL was shown to disrupt metabolism in the mitochondria, which is where energy is produced and respiration occurs.

Even though BL can cause the retina to worsen its function, BL can also help in other areas of research such as cancer cells, umbilical cord cells, and wound healing. In addition, it has been found that the shorter the wavelength of BL, the more detrimental its effect can be on the eye. Therefore, blue-turquoise light can act as a beneficial light source rather than blue-violet, which has an even shorter wavelength and much higher energy. However, BL hazard concerns are more prevalent than ever, since increased technology use is causing a plethora of eye problems especially in children who have doubled their blue LED intake since the 1990s. Furthermore, the importance of monitoring BL exposure is crucial in preserving and improving eye health.

Introduction

Blue light (BL) is a universally experienced short wavelength and high energy traversing species of animals as well as plants. The wavelength, ranging from 400 to 500 nm, was first documented to have an impact in how plants move in correspondence with sun exposure. This heliotropism was preliminarily recorded by Charles Darwin in 1881, in which he tested a filtration method using potassium dichromate to remove BL illuminating from the sun (Cashmore et al., 1999). Darwin observed that BL wavelengths were particularly recognized by plants in how they function. This experiment led to the eventual understanding that BL selective perception and feedback are omnipresent across the seven kingdoms in biology (Cashmore et al., 1999).

In addition to environmental BL, technological BL in the form of light emitting diodes (LED) revolutionized BL utilization and influence in therapies, lamps, televisions, phones, and more. Notably, scientist Isamu Akasaki led the invention of blue LEDs in 1989. Alongside Akasaki from 1995 to 1996, engineers Hiroshi Amano and Shuji Nakamura created an alloy device composed of semiconductors gallium nitride (GaN) and indium gallium nitride (InN) to develop efficient blue LEDs (Maruska and Rhines, 2015). While BL has proved to be a groundbreaking tool in light emissions in science, its radiation transmittance can significantly impact the eyes.

The significance surrounding BL exposure hazard is relevant as the rise in technology overwhelms populations on a global scale, with increased focus on children. Blue LED exposure has doubled with children under two years of age from 1997 to 2014, which poses safety concerns and draws attention to further research needing to be conducted on a long-term basis (Munsamy et al., 2022). As schools shift to utilizing more online educational approaches and

resources, children increase their screen time in addition to their home technology. Children are facing visual acuity issues as they start school, including myopia or nearsightedness. In addition to children, all age groups are experiencing increased ocular disturbances especially since the start of COVID-19. A study conducted in 2020 surveying people older than 18 years of age about their symptoms from digital device use resulted in a consensus that screen time significantly increased since the pandemic began and lockdown occurred. It was reported that 93.6% of participants noted that they increased their technology usage and 62.41% stated that they were unable to sleep efficiently (Bahkir and Grandee, 2020). Common symptoms included eye strain, vision blurring, headaches, dry eye, and increased sensitivity to light (Bahkir and Grandee, 2020). As symptoms like these frequented most people's eyes, the way in which BL enters the eye has remained a prevalently studied concept.

Light passes through the eye by the refractive system starting with the cornea, traveling through the aqueous humor, proceeding through the lens, and progressing through the vitreous in order to lastly reach the retina (Fan et al., 2022). The outermost neuronal cell layer of the retina contains specialized neuroepithelial cells called photoreceptors, which are responsible for transforming light into electrical signals (Fan et al., 2022). Moreover, the photoreceptors in the retina include rods and cones, which control low and high light levels of vision respectively. BL that enters the eye can cause photochemical retinal damage by photooxidation and further ROS production, where absorbed BL from chromophores can break down molecular bonds to possibly create hydrogen peroxide, hydroxyl, and superoxide radicals (Fan et al., 2022). Superfluous ROS that form in the retina from BL exposure can disrupt the cell and nuclear membrane leading to protein dysfunction and gene mutations and attack polyunsaturated fatty acids leading to lipid peroxidation (Fan et al., 2022).

Retinal phototoxicity or injury induced by high energy BL was earliest documented in 1978 through a study using rhesus monkeys. Scientists reported that continual exposure at 1000 seconds to BL at 441 nm led to photochemical damage of the retina. This was concluded following postexposure examinations, ranging from one hour to 90 days after BL exposure (Ham et al., 1978). Results including cellular proliferation and disruption in retinal pigment epithelium (RPE) and choroid as well as hypopigmentation in RPE aided insight in ocular conditions such as eclipse blindness and solar retinitis (Ham et al., 1978). Most notably, Ham et al. suggested for there to be BL filtering mechanisms for retinal damage prevention.

Experiments like these provided further insight into the mechanisms for BL hazard due to excessive exposure. In particular, oxidative stress shapes the molecular mechanism for BL damage, involving reactive oxygen species (ROS) damaging cells by photoactivation (Touitou and Point, 2020). A chromophore relocates absorbed light energy to $^3O_2$ which leads to ROS production (Touitou and Point, 2020). When the volume of ROS surpasses the extent to which protection can occur through defense utilization of antioxidants and BL filters such as lutein and zeaxanthin, oxidative stress to the retina transpires due to capacity overload (Touitou and Point, 2020). Moreover, mitochondria contribute to oxidative stress exhibited through ROS, as they abundantly occupy photoreceptors. Mitochondrial chromophores absorb the greatest at 400 to 500 nm. Therefore, mitochondria selectively absorb significantly more BL energy which leads to increased photochemical retinal damage (Fan et al., 2022). ROS production that leads to oxidative stress damage impacts the structure of retinal cell proteins, DNA, and lipids, which cumulatively leads to retinal mitochondrial apoptosis, or programmed cell death.

Apoptosis is cell death that involves two main pathways, including extrinsic and intrinsic. The extrinsic pathway uses extracellular conditions as signals to trigger caspase, an enzyme

involved in apoptosis, while the intrinsic pathway uses intracellular signals to obtain the apoptotic activator from mitochondria in order to trigger caspase (Fan et al., 2022). More specifically, the extrinsic pathway is activated by death receptors such as the tumor necrosis factor (TNF) and the intrinsic pathway is activated by mitochondrial cytochrome c (Fan et al., 2022). The process of apoptosis is involved in maintenance of normal cell function and homeostasis. However, external triggers such as BL can excessively induce apoptosis.

Following BL exposure, various genes are differentially regulated involving apoptotic pathways. Blue LEDs trigger proteins caspase-3 and caspase-9 in apoptosis, which can correlate to making the lysosomal membrane more permeable (Ouyang et al., 2020). In addition, genes Bcl-2 and Bax inhibit and aid in regulation of mitochondrial apoptosis. Bcl-2 prevents the release of cytochrome c and is found in the mitochondrial outer membrane (Ouyang et al., 2020). While Bcl-2 is an anti-apoptotic protein, Bax on the other hand is pro-apoptotic, encourages the release of cytochrome c, and is found in the cytoplasm (Ouyang et al., 2020). Continual BL exposure considerably increases Bax expression while decreasing Bcl-2 expression. In order to prevent apoptosis, BL filtering mechanisms must inhibit oxidative stress from occurring.

A previous study by Theruveethi et al. (2022) tested whether BL-induced transformations in retinal and cortical histology could be improved with blue light blocking lenses (BBLs). Commercially accessible BBLs, particularly Crizal Prevencia (CP) lenses and Duravision Blue (DB) lenses, were investigated in whether their advertisement of effectively blocking BL was truthful. The hypothesis tested the effect neural and retinal structures in the visual cortex experienced following sustained mild BL exposure (Theruveethi et al., 2022). A second hypothesis analyzed that the commercial BBLs would improve any retinal damage (Theruveethi et al., 2022). Apoptosis in the outer nuclear layer, accompanied with retinal pigment epithelial

and photoreceptor injury, has been established to occur from short term exposure to BL intensity levels of 1000 illumination lengths (lux) (Theruveethi et al., 2022).

Groups of Wistar rats were exposed to blue LED light of 400 to 500 lux ranging from wavelengths of 400 to 490 nm. Results obtained through the study indicated that continued exposure to higher levels of BL led to retinal damage and ongoing apoptosis in retinal ganglion cells. Without BBLs, BL disrupted the outer retina leading to damaged outer plexiform layers and the losses of photoreceptor nuclei and outer segments (Theruveethi et al., 2022). Additionally, BL damaged the inner nuclear layer and ganglion cell layer, documented through clustered and disorganized cell nuclei and vacuoles (Theruveethi et al., 2022). In comparison, the groups with CP and DB lenses endured less nuclear injury, especially to outer retinal layers. Prior studies exhibited results consistent with the study run by Theruveethi et al., in which BBLs were able to lower retinal photochemical injury by 80% (Theruveethi et al., 2022). In summation, the study illustrated that BL exposure leads to reactive oxygen species retinal apoptosis, neuron remodeling in the visual cortex, and mitochondrial injury (Theruveethi et al., 2022). BBLs considerably enhanced apoptotic conditions of the retinal pigment epithelium, inner retina, and photoreceptors, suggesting possible retinal amelioration against moderate BL.

While BL filtering lenses may serve as ameliorative tools for retinal injury, photobiomodulation (PBM) can also play a significant role in improving oxidative stress conditions. Low intensity laser and LED therapy, or PBM, is known to improve inflammatory and damaged cells and tissues. Majorily, PBM has been deemed successful through use at home and in healthcare offices. Although its mechanism is unclear, one hypothesis suggests that mitochondrial chromophores absorb light energy from PBM, leading to ATP and ROS production. Cytochrome c oxidase, present in complex IV of the mitochondrial respiratory chain,

primarily functions as a chromophore to absorb the red light (RL) to near-infrared light (NIRL) stimulated by PBM. Moreover, electron carriers including cytochrome c transfer electrons activated from PBM from higher energy levels to the final electron acceptors, which leads to proton gradient production and ATP synthesis (Dompe et al., 2020). PBM promotes ROS production due to the low intensity light radiation but utilizes specific enzymes to regulate sufficient mitochondrial metabolic conditions. For instance, cyclooxygenase enzymes (COX) function to maintain and restore mitochondrial membrane potential especially following disruption by inhibiting excess ROS production, which may be increased due to oxidative stress (Dompe et al., 2020). ROS are also able to maintain metabolism through ATP production and restore tissue with the help of growth factors and cytokines, which promotes stem cell and fibroblast differentiation and proliferation (Dompe et al., 2020).

As studies surrounding PBM have been on the forefront of scientific research in recent years, research has mainly centered around enhancements in skin, dental, and systemic conditions such as diabetes. Also heavily researched was the promotion of human fibroblast proliferation from PBM. The mechanism behind how PBM serves as an ameliorative tool in BL-induced retinal injury still remains fully unclear as to its full potential spanning across all mitochondrial respiratory chain complexes, in correlation to oxidative stress and respiration functionality. In this thesis, I will evaluate the protective and ameliorative effect of PBM, or RL to NIRL stimulation, on BL-induced retinal mitochondrial oxidative damage, apoptosis, and metabolism instability.

Current Investigations

BL has been evidently observed to induce mitochondrial apoptosis and oxidative stress leading to mitochondrial metabolic instability. The underlying mechanism for the role reactive oxygen species (ROS) play in giving rise to cell death when stimulated by RL to NIRL remains poorly understood. Recent studies illustrate that RL and NIRL are able to assist in photoreceptor defense and improvement of apoptotic conditions due to BL-induced retinal damage and oxidative stress. Two studies have analyzed the association between BL and disruption in oxygenation and retinal mitochondrial metabolism. It was discovered that RL and NIRL stimulation through low intensity laser therapy, also known as PBM, is able to reduce mitochondrial apoptosis, enhance respiration and oxygenation, decrease ROS intensities, and protect regulated photoreceptor gene expression.

Oxidative stress and apoptosis induced by BL contribute to instability in retinal mitochondrial metabolism. Heinig et al. (2020) examined RL and NIRL molecular mechanisms in order to determine whether PBM is successfully able to provide neuronal cell death protection. In addition, they wanted to observe oxidative stress to see if PBM indeed provided photoreceptor protection and improved respiration. Interestingly, a majority of previous studies they found concerning PBM only centered on complex IV in the mitochondrial respiratory chain. A narrow scope of understanding regarding the effect of PBM on respiratory complexes led them to finally examine whether additional complexes could describe the full extent of PBM, more specifically RL and NIRL, pertaining to enhanced respiration. Kaynezhad et al. (2022) used broadband near-infrared spectroscopy (bNIRS) to observe how BL impacts retinal mitochondrial oxygenation and metabolism. In particular, they measured instability changes in metabolism regulator

proteins such as deoxygenated hemoglobin (HHb), oxygenated hemoglobin (HbO2), and oxidized cytochrome-c-oxidase (oxCCO).

Ex-Vivo Cultivation of the Retina

Through utilization of an established ex-vivo BL retina tissue damaging model, Heinig et al. examined the beneficial effects of photoreceptors upon PBM against BL-induced retinal cell disruption. Photoreceptors that endure oxidative stress principally characterize this particular BL model (Heinig et al., 2020). Hematoxylin and eosin (H&E) staining, which is a method to visualize cells and tissues, of the cultivated retina was conducted in order to lessen the amount of retinal damage following BL exposure (Heinig et al., 2020). This staining allowed for the total cultivation period to be nine hours (Heinig et al., 2020). In particular, this study used C57BL/6 mice as the model organisms and applied 405 nm BL, 670 nm RL, and 810 nm NIRL. RL and NIRL accompanied BL being applied to the eyes of the mice in order to analyze the effect RL and NIRL stimulation had on BL damaged photoreceptors. Figure 1 shows that mice were arranged into groups including the control or non-irradiated eyes, BL, BL+NIRL 810 nm diode laser, and BL+RL 670 nm LED. They conducted an early event analysis after 40 minutes and a late event analysis after nine hours of cultivation.

Protein Regulation Expression by Immunohistochemistry and Western Blots

Secondly, Heinig et al. analyzed Caspase-9, Bcl-2, and Bax protein regulation expressions in relation to mitochondria-induced apoptotic pathways by using immunohistochemical staining and western blot analysis. Immunohistochemical staining uses a fluorescent dye or enzyme to identify antibodies that bind to antigens in tissue. In this

experiment, a blue-fluorescent dye called DAPI, which stands for 4′,6-diamidino-2-phenylindole, was used to visualize and bind to adenine-thymine (A-T) regions of DNA. Staining displayed that Caspase-9 and Bax pro-apoptotic protein expressions were amplified in the inner segments (IS) of photoreceptors after BL exposure. After exposure to BL+RL and BL+NIRL, Bcl-2 anti-apoptotic protein expression increased, as seen in Figure 2A. Western blots detect individual proteins in tissue by use of gel electrophoresis. In this experiment, gel electrophoresis separates proteins by molecular weight found in the sample of retinal tissue. The western blot analysis, included in Figure 2B, shows increased Bcl-2 protein expression but decreased Caspase-9 and Bax protein expression after BL+RL and BL+NIRL exposure. These results suggest that RL and NIRL exposure leads to reduced apoptosis and more photoreceptor continuity in comparison to only BL.

Oxidative Phosphorylation by Histochemical Staining and Western Blots

After nine hours, the late event analysis was conducted. Next, Heinig et al. assessed photoreceptor respiration in order to determine the effect of RL and NIRL on oxidative phosphorylation (OXPHOS). They selected Complex I, or NADH-CoQ oxidoreductase (NADH), and Complex II, or Succinate dehydrogenase (SDH), for histochemical assay analysis of OXPHOS performance (Figure 3A). These complexes indicate beginnings for two OXPHOS pathways, including Complex I+III+IV and Complex II+III+IV (Heinig et al., 2020). Histochemical staining utilized the enzymes NADH and SDH to visualize retinal cells exposed to BL in comparison to the control, RL and NIRL. After BL was applied, Complex I activity significantly reduced in the photoreceptor IS by 0.40 and outer segments (OS) by 0.42. Complex II activity also decreased in IS by 0.40 and in OS by 0.52. The photoreceptors that were exposed

to BL+RL and BL+NIRL regained their enzyme activity, particularly NADH for Complex I and SDH for Complex II. Moreover, complex activity increased when exposed to RL and NIRL in IS and OS. Complex I activity increased to 0.95 in IS and 0.97 in OS when exposed to the 670 nm RL. When exposed to the 810 nm NIRL, Complex I activity grew to 0.94 in IS and 0.88 in OS. Consistent with Complex I, Complex II activity was regained increasing in IS to 0.89 and 0.88 in OS when exposed to RL and 0.88 in IS and 0.86 in OS when exposed to NIRL.

OXPHOS magnitudes were measured by isolating 0.04 mg protein of purified OS and analyzing how much oxygen was expended. The two OXPHOS pathways, I+III+IV and II+III+IV, were analyzed through stimulation of 20 mM fumarate and 20 mM succinate respectively. In the Krebs cycle, which derives energy through aerobic respiration, SDH contributes electrons to the electron transport chain by oxidation of succinate to fumarate. Fumarate is the final electron acceptor for this cycle. Upon RL exposure, Complex I increased oxygen consumption by 84% and Complex II experienced an increase by 94% (Figure 3B). An ATP bioluminescence assay was conducted to measure the relative ATP content of retina lysates using ATP Bioluminescence Assay Kit HS II. This assay is used to quantify cell viability and amount of intracellular ATP through measurement by the Tecan infinite M200 plate reader. RL and NIRL both significantly improved ATP content in comparison to only BL (Figure 3C). The retina lysates and OS were further examined by western blot analysis, utilizing an OXPHOS antibody mixture in which Complex I tested the NDUFB8 antibody, Complex II tested the SDHB antibody, Complex III tested the UQCRC2 antibody, Complex IV tested the MTCO1 antibody, and Complex V tested the ATP5A antibody. Notably, protein expression appears to be consistent regardless of exposure to BL, RL, or NIRL in Complex I, Complex II, Complex III,

Complex IV, and Complex V for the OS and retina lysates (Figure 3D). Complex IV bands of protein expression are visibly thicker than the rest of the complexes.

ROS Assessment by CM-H2DCFDA Staining and Western Blots

ROS were subsequently examined utilizing CM-H_2DCFDA dye and NADPH oxidases Nox2 and Nox4. CM-H_2DCFDA dye was used to indicate oxidative stress and ROS production in singlet oxygen, radical, peroxide, superoxide, or anion expression (Heinig et al., 2020). This dye also fluoresces when ROS cause dihydrofluorescein to oxidize to fluorescein to produce emittance shown in Figure A. Retinal cells were stained for 10 minutes with 25 µM CM-H2DCFDA at 37°C following forty minutes of cultivation. Upon BL exposure, ROS fluorescence intensity levels rose from 1 to 1.89 in IS and to 2.66 in OS (Figure 4A). ROS levels significantly decreased by 0.63 upon NIRL exposure and 0.50 upon RL exposure in IS. Furthermore, ROS levels were reduced by 1.43 and 1.49 in OS upon NIRL and RL exposure respectively. Nox2 and Nox4 can significantly produce ROS. Therefore, Nox2 and Nox4 protein expression in IS and OS in photoreceptor cells increased upon BL irradiation and decreased upon RL and NIRL exposure (Figure 4B). According to western blot analysis, Nox4 protein expression decreased in isolated OS when exposed to RL and NIRL (Figure 4C). This western blot utilized antibodies against Nox4.

MRNA Expression by Laser Capture Microdissection and In-Situ Hybridization

Heinig et al. further conducted analysis of mRNA gene expression of photoreceptors in order to evaluate the efficacy of RL and NIRL in protection against BL. Laser Capture Microdissection (LCM) was conducted using the confocal laser scanning microscope called

Zeiss LSM 510. This method utilizes a microscope to directly visualize the separation of cells from tissue with a laser. Retinal cell tissue samples were divided and cut using a razor blade into 40 μm thick vibratome components (Heinig et al., 2020). LCM displayed that transformations in gene expression were not able to be changed by alternate retinal cell types (Figure 5A). They analyzed upregulated and downregulated genes split among three groups, including control vs. BL, BL vs. BL+NIRL, and BL vs. BL+RL (Heinig et al., 2020). RNA sequencing recognized 35,169 annotated genes. Notably, differential gene expression analysis illustrated in Figure 5B described significant transformation in twenty two genes for control vs. BL, seven genes for BL vs. BL+NIRL, and lastly nine genes for BL vs. BL+RL (Heinig et al., 2020). Eight differentially expressed genes (DEGs) overlapped between the three groups, which exhibited significant fold change (Figure 5C). In comparison to BL damaged photoreceptors, crystallin genes exposed to RL and NIRL experienced upregulation by 1.1 to 2.2-fold.

They more closely examined αA-crystallin and αB-crystallin, or cryaa and cryab respectively, due to their activities against apoptosis and oxidative stress. In-situ hybridization and immunohistochemistry (Figures 5D and 5E) showed that NIRL and RL were able to restore gene expression in cryaa and cryab mRNA in retinal cells, similar to the state of gene expression for the control. Moreover, the method of in-situ hybridization isolates DNA and RNA gene expression in tissue samples. In this experiment, cryaa and cryab were accompanied by two probes established through PCR, also known as polymerase chain reaction which amplifies DNA. These nuclear probes were diluted in hybridization solution consisting of 0.1% CHAPS, 1× Denhardts, 0.01 M EDTA, 50% formamide, 0.1 mg/mL Heparin, 5× SSC, 0.4% Tween-20, and 0.2 mg/mL Yeast RNA. Afterwards, these hybridized probes were incubated along with slides of retinal cell sections overnight at 70°C.

Mitochondrial Metabolism and Oxygenation Observed by bNIRS

While RL and NIRL prove to be sufficient ameliorative tools against BL retinal damage leading to improvement of mitochondrial metabolism, Kaynezhad et al. more closely look at how constant exposure to 420 nm environmental BL impacts retinal hemodynamic and mitochondrial behavior. In particular, they utilize lights that exist in environmental daylight in-vivo. They used bNIRS, which is a method that utilizes NIRL that reflects from the retina to analyze in-vivo blood oxygenation and mitochondrial activity. Specifically, the model organisms were seven C57BL mice, ranging from six to seven months old. These mice were measured for their concentrations in oxygen and hemoglobin in light ranging from 780 to 900 nm through the bNIRS device miniCYRIL. The experimental setup depicted in Figure 6 first incorporated filtered light from a halogen white light source. This light was accompanied with a yellow filter that blocked short wavelengths under 500 nm, which passed through an optical fiber to irradiate the dilated pupils of the mice. This first optical fiber carried light to the retina, while a second optical fiber passed the reflected light to a spectrometer to determine metabolism and oxygenation based on spectral data from 780 to 900 nm (Kaynezhad et al., 2022). The modified Beer-Lambert law, which supports continuous-wave near-infrared tissue spectroscopy (cwNIRS), reveals that fluctuations in light intensity are proportional to changes in hemoglobin concentrations of tissue (Kocsis et al., 2006).

The spectral signals that were recorded filtered out wavelengths less than 695 nm in order to prevent 420 nm light disturbance to spectral data taken during blue LED application. Changes in deoxygenated hemoglobin (HHb), oxygenated hemoglobin (HbO_2), and oxidized cytochrome-c-oxidase (oxCCO) were shown during a one hour baseline, one hour during 420 nm blue LED exposure, and around one hour following blue LED exposure (Figure 7A). All three signals

uniformly changed in the first hour as seen in the baseline. Upon 420 nm BL exposure, the consistency in the signals diverged and there was a rise in variation during BL exposure and recovery in comparison to the baseline (Kaynezhad et al., 2022). During the recovery period following BL exposure, the concentration of HbO_2 increased into a positive slope compared to the two other signals which decreased into a negative slope in concentration. Next, MATLAB 2018b was used to determine bNIRS signal instability. Absolute mean changes in blood and mitochondrial signals were calculated during the three periods of baseline, blue LED exposure, and time following exposure. These periods were distributed evenly into 60 minute increments (Figure 7B). Furthermore, it is shown that during the recovery period there is an increased variation in concentrations in HHb, HbO_2, and oxCCO. However, it is more evident that there are significant differences in each signal, with HHb having the most significant change in instability. There appears to be a gradual increase in BL-induced instability in these signals, indicating that effects are not abrupt but are due to a progressive exposure that does not experience amelioration until 60 minutes later following blue LED exposure (Kaynezhad et al., 2022).

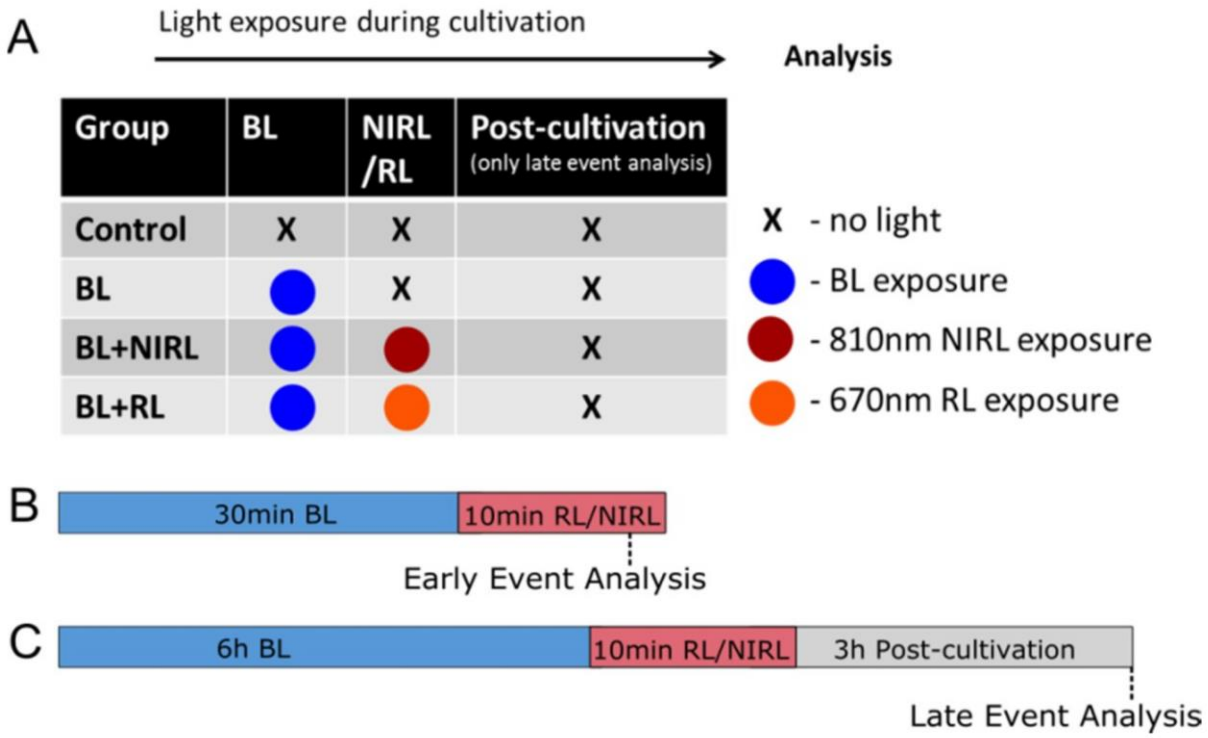

Figure 1. Schematic light exposure arrangement. (A) Grouping of eyes to their light irradiation, including the control, BL, BL+NIRL, and BL+RL. (B) Early event analysis conducted following 30 minutes of BL and 10 minutes of BL+RL and BL+NIRL exposure. (C) Late event analysis conducted following six hours exposed to BL, ten minutes exposed to BL+RL and BL+NIRL, and three hours after cultivation. Figure 1 is reproduced from Heinig et al. (2020), Figure 1.

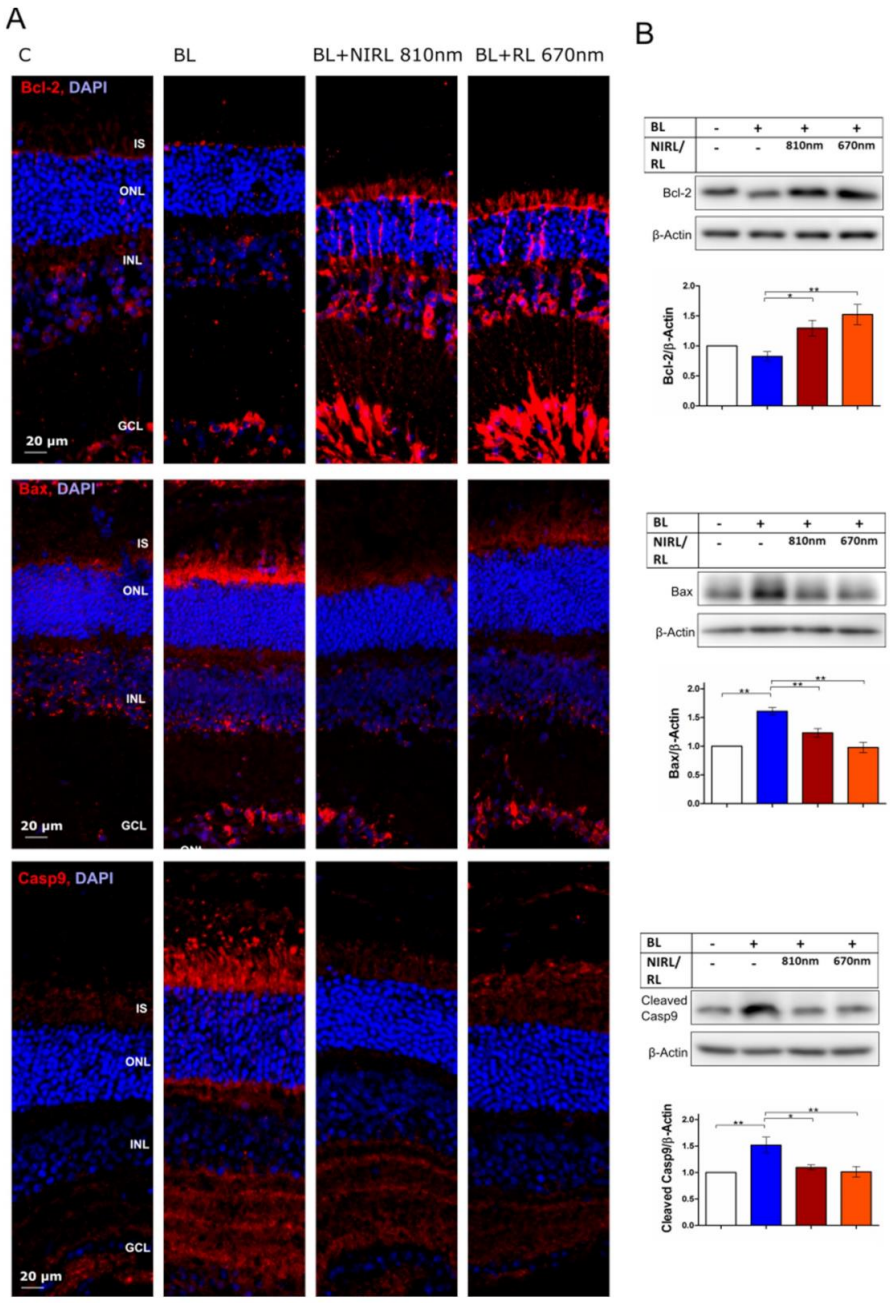

Figure 2. Late event analysis following nine hours of cultivation, in which mitochondria-induced apoptotic pathways are analyzed with the Bcl-2, Bax, and Casp9 protein expressions shown. (A) Bcl-2, Bax, and Casp9 in retinal sections were immunohistochemically stained with DAPI. (B) Retinal lysates depicted through western blot analysis. β-Actin served as the loading control and antibodies were utilized against the proteins Bcl-2, Bax, and Casp9. Figure 2 is reproduced from Heinig et al. (2020), Figure 2.

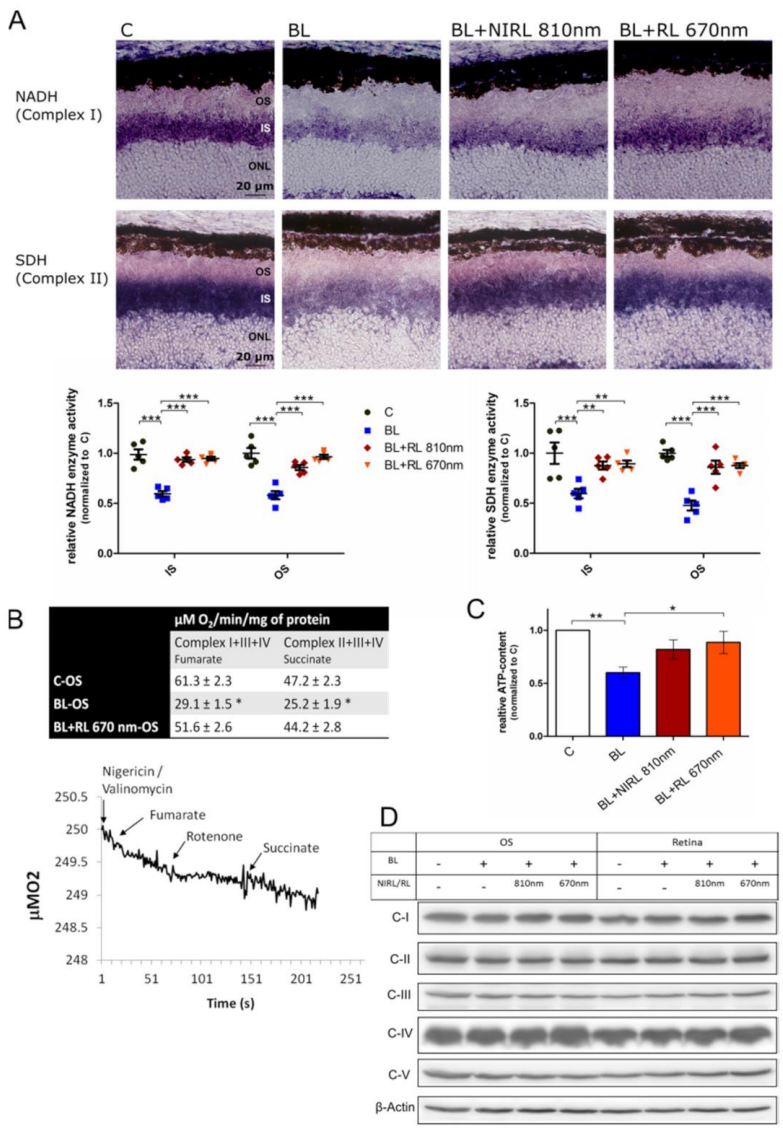

Figure 3. Late event analysis illustrated that oxidative phosphorylation levels in BL applied cells are raised when exposed to NIRL and RL. (A) Histochemical staining quantitative analysis of NADH (Complex I) and SDH (Complex II) to depict enzyme activity. (B) OXPHOS pathways I+III+IV and II+III+IV stimulated by fumarate and succinate respectively. Control-OS, BL-OS, and BL+RL 670 nm-OS were tested. Purified OS oxygen consumption standards recorded in the table shown. (C) ATP bioluminescence assay indicated ATP quantification of retina lysates. (D) Western blot analysis conducted with OS and retina lysates with OXPHOS antibody mixture. β-Actin served as the loading control. Figure 3 is reproduced from Heinig et al. (2020), Figure 3.

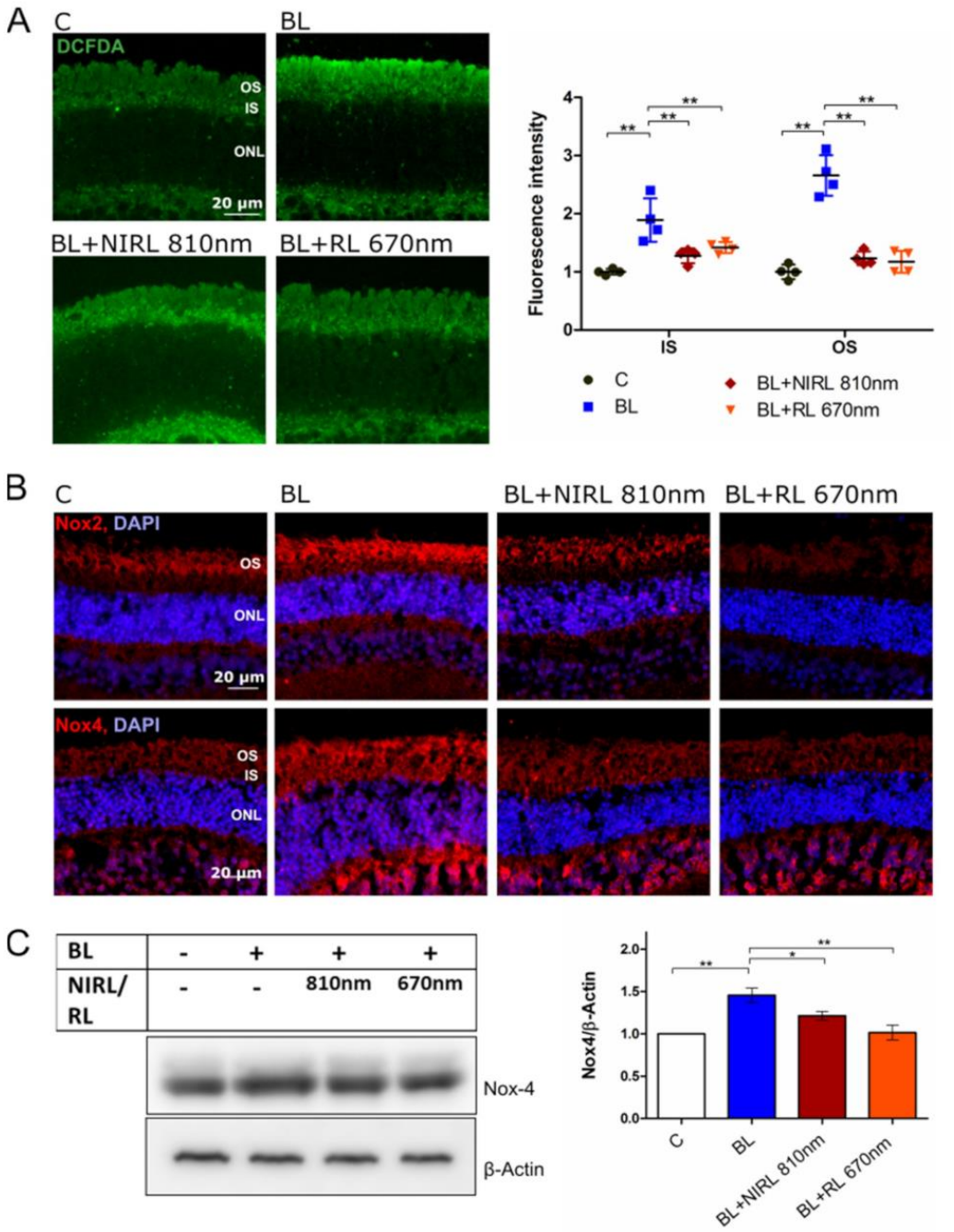

Figure 4. ROS intensity levels decrease upon RL and NIRL exposure. (A) CM-H2DCFDA staining in retinal sections. Fluorescence intensity levels of IS and OS show production of ROS displayed in the graph. (B) Immunohistochemical staining of Nox2 and Nox4 proteins in the retina. (C) OS western blot analysis utilized antibodies against Nox4 and β-Actin. Figure 4 is reproduced from Heinig et al. (2020), Figure 4.

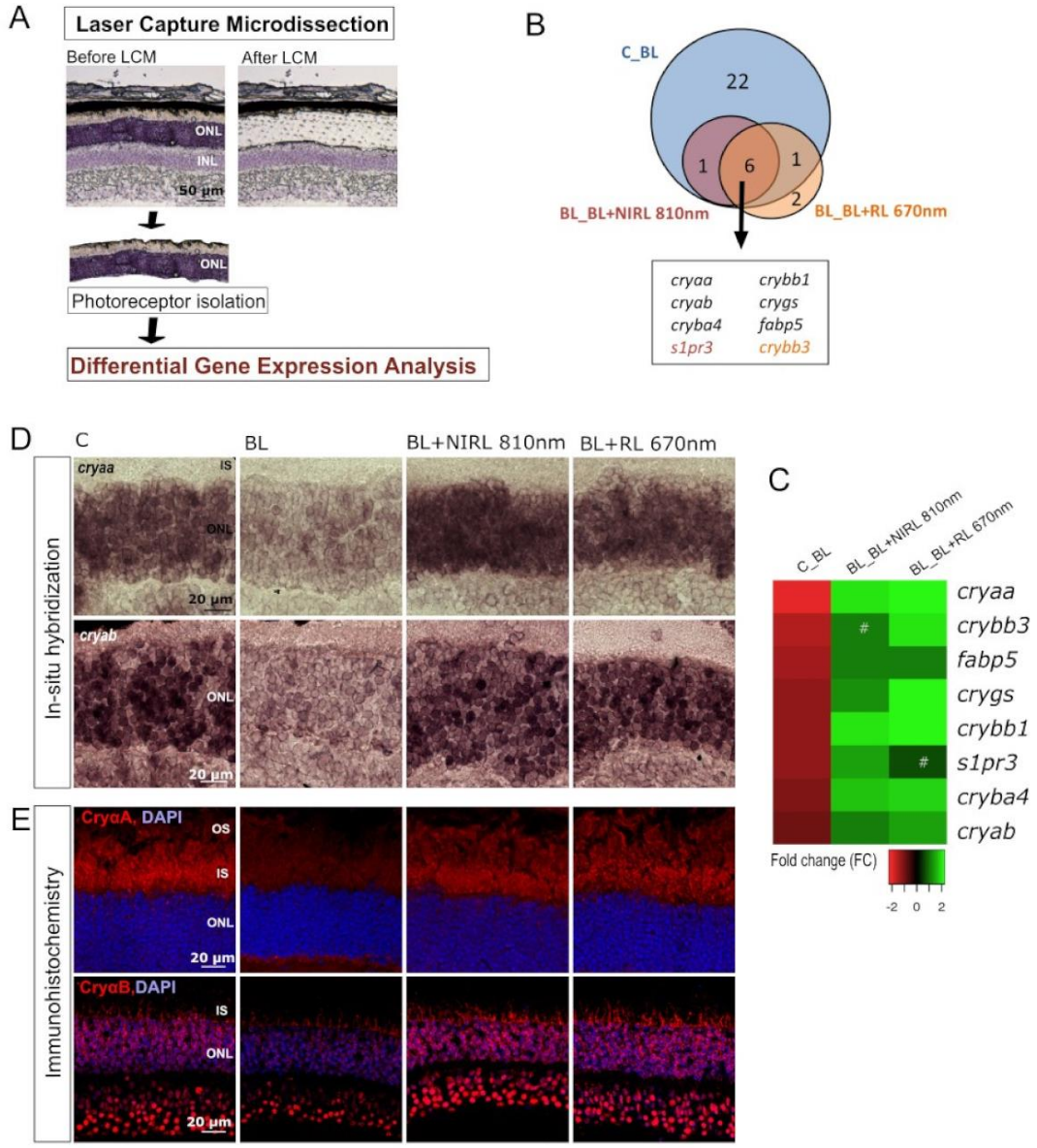

Figure 5. Differential gene expression analysis of photoreceptors were regulated by RL and NIRL. (A) Laser Capture Microdissection (LCM) used for analysis of varied gene expression. (B) Venn diagram visual representation of DEGs overlap between C vs. BL, BL vs. BL+NIRL 810 nm and BL vs. BL+RL 670 nm. (C) Significant gene overlapping presented in the heatmap, distributed by fold change. The # symbol indicates that there is no significance. (D) In-situ hybridization for αA-crystallin and αB-crystallin, presented as cryaa and cryab mRNA, in the retina. Figure 5 is reproduced from Heinig et al. (2020), Figure 5.

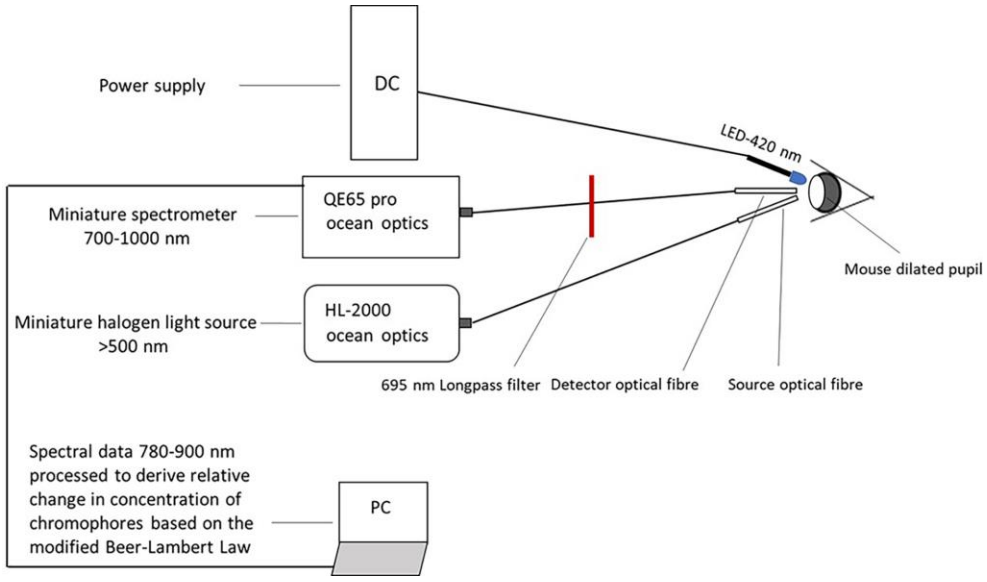

Figure 6. Setup of different light sources. Figure 6 is reproduced from Kaynezhad et al. (2022), Figure 1.

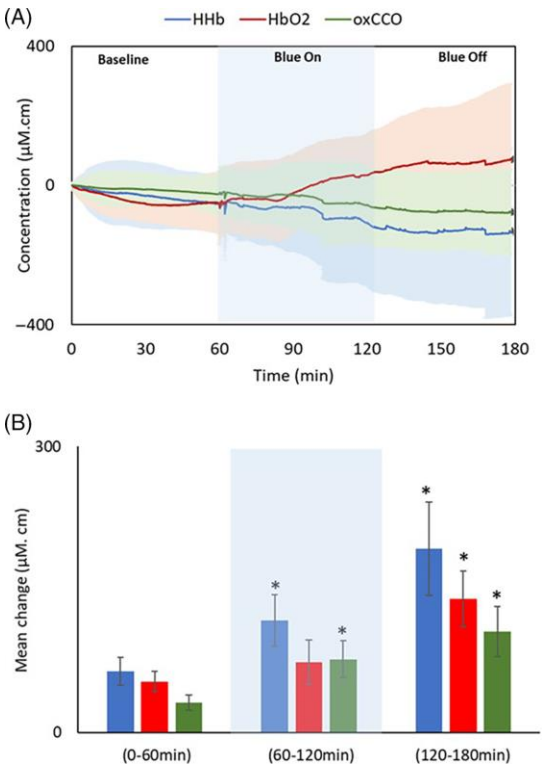

Figure 7. Retinal mitochondrial and blood signals are unstable upon BL 420 nm exposure. Deoxygenated hemoglobin (HHb) changes are presented by the blue line and bars. Oxygenated

hemoglobin (HbO_2) changes are depicted by the red line and bars. Oxidized cytochrome-c-oxidase (oxCCO) changes are shown by the green line and bars. (A) Mean concentrations for each signal for the baseline (0 to 60 min), exposure to blue LED (60 to 120 min), and recovery period following BL exposure (120 to 180 min). (B) Histogram shows mean changes utilizing data from (A). Standard error is represented by error bars. Figure 7 is reproduced from Kaynezhad et al. (2022), Figure 2.

Discussion

The study conducted by Heinig et al. evidently provided efficacy surrounding PBM after BL exposure. Utilizing the wavelengths of 670 nm RL and 810 nm NIRL allowed for ameliorative effects on the retina to take place. Figure 2A presents that when exposed to BL, Bax and Caspase-9 proteins illuminate with a heavily red frequency, supported through further quantitative analysis in Figure 2B. Amplification of protein expression in photoreceptor IS displays its importance in representing transformed mitochondria. Caspase-9 protein is an indication of cell death introduction through lysosomal membrane permeabilization. Here, Caspase-9 is reduced following PBM, which leads to membrane stability.

The Bax protein reduction also indicates lowered cytochrome c production. Cytochrome c is one key signaling apoptotic molecule, which can play a role in mitochondrial metabolism. With the decrease in mitochondrial cytochrome c, mitochondrial permeability transition (MPT) could be stimulated providing an alternate pathway to apoptosis (Cai et al., 1998). Cytochrome c can also lead to increased superoxide radical production, which was inhibited by the increase in Bcl-2 protein expression. Increased Bcl-2 protein expression was presented through thick protein bands displayed in the western blot. Furthermore, Bcl-2 can act as an antioxidant and prevent

ROS production that follows cytochrome c release, inhibiting mitochondrial apoptosis (Cai et al., 1998).

RL and NIRL exposure increased enzyme activity in the IS and OS, with outer nuclear layer (ONL) activity remaining consistent in complex I yet unacknowledged in the study. The ONL contains the photoreceptor nuclei and is supposed to present a loss following BL exposure, but looks similar to the NIRL and RL exposures in complex I. In complex II, there is a brightness and visible difference when the ONL is exposed to BL but its thickness remains consistent. Interestingly, respiratory chain complex protein expression level did not change following BL, RL, or NIRL exposure (Heinig et al., 2020). OS are unable to synthesize proteins, which illustrates the consistent thickness of enzyme activity (Heinig et al., 2020).

Stimulation of fumarate demonstrated more complex activity compared to succinate stimulation of complex II+III+IV. In particular, fumarate reductase can be housed within facultative or anaerobic bacteria, while succinate dehydrogenase is found in the aerobic respiratory chain including the Krebs cycle (Maklashina et al., 2006). Fumarate's ability to live in environments lacking oxygen can suggest its better efficiency in oxygen consumption compared to succinate, as shown in Figure 3B. There was an increased instability through the electron transport chain due to the decrease in oxygen consumption induced by BL, which was able to be recovered by RL and NIRL exposure (Heinig et al., 2020).

Retinal ATP production also significantly increased following RL and NIRL stimulation, which indicates increased metabolic function of retinal cells (Figure 3C). RL proved to be more effective than NIRL, which can be explained by its targeting of photon mitochondrial absorption by wavelength. In a previous study by George et al. (2018), RL at 636 nm and NIRL at 825 nm were utilized to measure oxidative stress generated by human fibroblast radicals. Cells that were

stimulated by NIRL demonstrated double the amount of cellular energy related to oxidative stress than RL (George et al., 2018). The production of ROS leading to oxidative stress therefore can be correlated to light wavelength, with RL's wavelength ranging from 600 to 760 nm to be most effective against apoptosis (George et al., 2018). Therefore, the more oxygen consumed correlates with the more ATP produced (Heinig et al., 2020). Furthermore, increased ATP leads to improved mitochondrial function, which then reduces oxidative stress.

Although protein expression was consistent among complexes I through V, complex IV that tested the MTCO1 antibody appeared to have thicker bands compared to the other complexes, meaning more protein present. Cytochrome c oxidase (CCO) found in complex IV acts as the final electron acceptor for the electron transport chain and main photoacceptor for PBM (Heinig et al., 2020). Since CCO acts as a chromophore, it consists of copper and heme to obtain RL and NIRL (Heinig et al., 2020). Therefore, metabolic energy can improve. Most importantly, these results showing consistent protein expression across all complexes suggest that CCO is not the only primary target of inhibiting oxidative stress by PBM (Heinig et al., 2020). These results display that complexes I and II are also PBM targets besides complex IV, which can lead to improved metabolic conditions (Heinig et al., 2020).

CM-H_2DCFDA dye was used to indicate ROS, which solidified that RL and NIRL reduce the expression of pro-apoptotic Nox4 protein. Notably in Figure 4A, RL most closely resembled the control in comparison to NIRL, portraying better efficacy surrounding RL to ameliorate retinal cell damage. The staining of retinal sections exposed to NIRL illustrated a curvature, while RL illustrated a more linear structure. This curvature could depict the abrupt significant decrease in ROS levels that could allow for the retina to adjust promptly before being exposed to RL to fully ameliorate. Another explanation could be that the NIRL is not evenly distributed or

illuminated, leading to shape differentiation. Interestingly, ROS levels decreased more upon NIRL exposure in IS and more upon RL exposure in OS, which can further support improvement resembling the control in both light stimulation sources. Moreover, it is evident in Figure C that RL is able to better reduce Nox4 protein expression to resemble the control compared to NIRL, but there is still a significant difference shared between RL and NIRL.

Laser capture microdissection (LCM) and RNA sequencing were able to help identify significant gene expression changes through regulatory factor analysis. These altered genes that consisted of cryaa and cryab displayed enhanced mitochondrial activity following RL and NIRL stimulation (Heinig et al., 2020). BL prevented α-crystallin protein expression, while RL and NIRL significantly increased cryaa and cryab expression involved in neuroprotection and anti-apoptotic activity. Specifically, these results of altered genes show that PBM is able to regulate the NF-κB pathway in order to enhance retinal cell activity and survival (Heinig et al., 2020). Through activation of NF-κB, a redox-sensitive transcription factor, RL and NIRL can stimulate a relatively enhanced amount of ROS and aid in protective effects on the retina (Heinig et al., 2020). PBM can suggest that excessive ROS generation causes injury while a moderate level of ROS can prove to be protective (Heinig et al., 2020). They were able to restore more gene expression in cryaa mRNA than cryab, as presented in Figure C. RL was also able to restore more gene expression than NIRL. Lack of cryab led to the rise in ROS production and oxidative stress, which affected MPT and caspase-3 activation (Heinig et al., 2020).

Regulatory gene expression was shown to correlate to efficacy for creating therapy tools, including RL and NIRL. Similarly, a study by Guo et al. analyzed altered gene expression in human bone marrow stem cells upon RL 633 nm LED and infrared light (IR) 830 nm LED exposure (Guo et al., 2015). RL and IR wavelengths both deregulated 292 genes (Guo et al.,

2015). Different wavelengths of light can lead to differentiated genes and deregulation. This study shows that light therapy can be applied to other branches of research, including stem cells.

Concretely stating whether PBM provides cumulative positive effects remains challenging due to a diverse set of studies that can be run with differentiating trials and time periods, wavelengths, model organisms, and sources of light. Where the mechanism for PBM can become unclear pertains to the different wavelengths of light that can play a role in levels of oxidative stress. For instance, a study conducted by Rupel et al. examined the effect different wavelengths of PBM has on in vivo in cancer patients with oral mucositis and in vitro in keratinocytes, oral mucosa, and neutrophil polymorphonuclear (PMN) granulocytes (Rupel et al., 2018). They discovered that the RL laser with a wavelength of 660 nm significantly increased ROS levels in PMNs, and a wavelength of 970 nm NIRL exhibited antioxidant function. ROS levels significantly decreased when exposed to only 800 nm or 660 nm, 800 nm, and 970 nm combined (Rupel et al., 2018). This study raises an interesting point of whether single wavelengths or combined wavelengths are the most effective in amelioration from oxidative stress.

The study by Kaynezhad et al. exhibited the high metabolic rate and mitochondrial density retinal photoreceptors possess (Kaynezhad et al., 2022). BL exposure of 420 nm significantly interrupted retinal mitochondrial metabolism and oxygenation conditions after an hour. These results support mitochondrial density being the greatest in the retina and mainly absorbing light at 420 nm, which porphyrins also take part in (Kaynezhad et al., 2022). This is due to the fact that porphyrins are components of hemoglobin, which correlates to the most significant change in hemoglobin concentration and stability in hemodynamics seen in Figure 7 (Kaynezhad et al., 2022). For longer periods of time, retinal mitochondrial and blood signals

become more unstable upon environmental BL 420 nm exposure. The result that amelioration did not occur until 60 minutes after BL exposure may suggest that BL damage is not severe until 60 minutes or more of exposure.

Considering the translation of these results to human eyes pertaining to how BL affects mice eyes, there is an issue in application. Mice rarely are exposed to continuous levels of BL in comparison to humans. If humans were to continuously stimulate their eyes to 420 nm wavelengths, mitochondrial and electron transport chain activity could be disrupted (Kaynezhad et al., 2022). Wavelengths ranging around 670 nm enhance electron transport chain and mitochondrial activity, ATP generation, mitochondrial membrane potential, and vision (Kaynezhad et al., 2022).

While BL negatively impacts the retina, including mitochondrial oxidative stress, apoptosis, and metabolic variability, BL can also have positive effects of application. A study conducted by Uhl et al. examines how BL induces apoptosis in HeLa cancer cells (Uhl et al., 2021). Utilizing the MG132-Cage, these HeLa cells were stimulated by BL 405 nm for ten minutes or exposed to no light (Uhl et al., 2021). The amount of HeLa cells decreased to 41% following 18 hours and 2% following 24 hours of BL exposure (Uhl et al., 2021). It has also been shown that BL is able to promote and regulate cellular functions, including in human umbilical vein endothelial cells. Kan et al. discovered that BL at a wavelength of 453 nm had a biphasic effect on these cells (Kan et al., 2021). Low intensity BL encouraged cell viability and angiogenic VEGF pathway activation, which can be related to BL wound healing (Kan et al., 2021). However, high intensity BL upregulated apoptosis (Kan et al., 2021).

As studies like these attempt to conclude whether BL is entirely detrimental or not, it is vital to contemplate the future of eye protection and therapy tools such as PBM and BBLs.

According to Dalal et al., blue-turquoise light at 450 to 500 nm is deemed positively influential for health, while blue-violet light at 380 to 450 nm is deemed unhealthy and injurious (Dalal et al., 2020). BL comprises around one-third of the total visible light encountered in irradiation (Dalal et al., 2020). Although there is no evidence that LEDs are permanently detrimental to the human retina under normal use as Touitou and Point noted (Touitou and Point, 2020), what is the time scope for normal? In order for clearer and more universal clarification regarding the effect BL has on a long term basis, complications and uncertainty arise. Future research concerning BL can conduct further trials on humans to weigh the positive and negative outcomes of utilization. There must be consistency in interest to find a consensus regarding the amount of time one can spend exposed to BL before effects such as retinal mitochondrial oxidative damage, apoptosis, and metabolic instability can become irreversible even with neuroprotective, light filtering, and LED or laser therapy tools.

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