670nm Photobiomodulation Modulates Bioenergetics And Oxidative Stress, Decreasing Inflammatory Mediator Production In An In Vitro Model Of Diabetic Retinopathy

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Abstract

Diabetic retinopathy (DR), the most common complication of diabetes mellitus, is associated with oxidative stress, nuclear factor- κ B (NF κ B) activation, and excess production of vascular endothelial growth factor (VEGF) and intracellular adhesion molecule-1 (ICAM-1). Current therapies are invasive, frequently ineffective, and have adverse effects. The application of far-red to near-infrared (NIR) light (630-1000nm) reduces oxidative stress and inflammation *in vitro* and *in vivo*. Thus, we hypothesize that 670nm light treatment will diminish oxidative stress preventing downstream inflammatory mechanisms associated with DR. We used an *in vitro* model system of rat Müller glial cells grown under normal (5 mM) or high (25 mM) glucose conditions and treated with a 670 nm light emitting diode array (LED) (4.5 J/cm²) or no light (sham) daily. We report that a single 670 nm light treatment diminished ROS production and preserved mitochondrial integrity and ATP production in this *in vitro* model of diabetic retinopathy. Furthermore, treatment for 3 days in culture reduced NF κ B activity to levels observed in normal glucose and prevented the subsequent increase in ICAM-1. The ability of 670nm light treatment to prevent early molecular changes in this established DR model system suggests light treatment could become an early therapeutic option for DR.

Introduction

Diabetes affects 10% of the population in the United States¹ and causes complications throughout the body due to sustained high circulating glucose which damages the vasculature resulting in chronic macro- and micro-vasculature dysfunction². In particular, microvasculature damage leads to retinopathy, neuropathy and nephropathy in diabetic patients. Indeed, diabetic retinopathy (DR) has become a leading cause of blindness in the United States¹. The pathophysiology of diabetic retinopathy is complicated, with early pathologic changes observed in diabetic retinopathy that include mitochondrial dysfunction, oxidative stress and inflammation^{3,4}, and later changes including elevated concentrations of vascular endothelial growth factor (VEGF) and a breakdown of the inner blood-retinal barrier, resulting in the extracellular fluid accumulation in macula and disturbed vision^{2,5-7}. This study is focused on the early pathological changes associated with DR and a potential mitigation strategy with treatment with far-red to near-infrared light, or photobiomodulation (PBM). PBM has been shown to act on mitochondria-mediated signaling pathways to preserve mitochondrial function, attenuate oxidative stress, stimulate the production of cytoprotective factors, preventing cell death in *in vitro* and *in vivo* experimental models⁸⁻¹⁰. In retinal cell culture and animal models of DR, 670 nm light therapy resulted in decreased ROS production^{11,12} and slowed disease progression^{9,13,14}. In a rat model of DR, Tang *et al.* found that 670 nm light reduced retinal ganglion cell death and improved retinal function. Importantly, PBM has been shown to attenuate inflammation and oxidative stress and protect retinal function in experimental and clinical studies^{11,12}. The mechanism by which PBM exerts a beneficial effect is not clearly elucidated. Studies have shown that 670 nm light is absorbed by cytochrome-c-oxidase promoting mitochondrial bioenergetic function and increasing ATP synthesis^{8,9,11,13}, however other pathways have been

mechanistically implicated including alteration of transcription^{9,15}, release of nitric oxide^{16,17}, and activation of kinases such as Akt^{18,19}. Regardless of the exact mechanism, studies in both mice and humans have shown improved vision with PBM treatment^{10,12}. The goal of this study is to increase the mechanistic understanding of how 670nm light improves retinal outcomes. We hypothesize that 670nm PBM will mitigate cellular changes caused by high glucose through modulation of reactive oxygen species and mitochondrial function leading to decreased inflammatory marker production. We plan to test this hypothesis in a Müller retinal glial cell model of DR.

The importance of Müller cells in retinal disease is becoming increasingly understood. These cells are the principle glia of the retina, spanning the entire retina with intimate contact of both retinal blood vessels and retinal neurons²⁰. Normally, Müller glial cells function to recycle neurotransmitters, retinoic acid compounds, and ions (including potassium K⁺), to control of metabolism and supply of nutrients or the retina, and to regulate of blood flow and maintenance of the blood retinal barrier^{21,22}. In DR, hyperglycemia-induced retinal injury activates Müller cells leading to a stress response that promotes inflammation and angiogenesis^{5,6,23,24}. Thus, Müller glial cells significantly contribute to diabetic retinopathy^{20,22-28}. In this study, we show improved cellular changes with 670nm treatment in Müller glial cells when cultured in a high glucose environment.

Results

PBM mitigates reactive oxygen species produced by high glucose.

Oxidative stress is a common and significant factor in DR. Increasing intracellular ROS activates NFkB and other pathogenic mechanisms leading to diabetic retinopathy. Therefore, ROS is expected to increase early in disease.

Müller cells acutely exposed to high glucose produced a 50% greater ROS concentration than those cultured under normal glucose sham conditions (n = 3, p < 0.001). Müller cells in high glucose and treated with 670 nm light exhibited significantly decreased ROS concentrations to concentrations similar to those measured under normal glucose conditions (n = 3, p < 0.0001, compared to high glucose sham). It was not anticipated that PBM would modulate Müller cells exposed to normal glucose conditions due to a lack of stress applied. As expected, 670 nm light did not affect the amount of ROS generated intracellularly (n = 3) (Fig. 1).

Mitochondrial Function is Diminished with High Glucose and partially restored with 670nm Light

Mitochondrial function was assessed through various measures to examine enzyme activity, membrane potential, and ATP production. We chose to look at each of these to determine how quickly mitochondrial function is altered. The membrane potential is maintained by the electron transport chain and is altered in response to cellular redox changes. We expect these changes to occur earlier than changes seen in ATP content, which is the end product measured in this study to discover the extend of early mitochondrial changes.

Müller cell NADPH-dependent oxidoreductase activity was reduced by nearly 50% following 24 hours in high glucose medium ($n = 3$, $p < 0.01$), indicative of a reduction in mitochondrial respiration and cellular energy capacity. A single treatment with 670 nm light (dose 4.5 J/cm²) increased NADPH-dependent oxidoreductase activity (Fig. 2A) by 60% from high glucose conditions, to values similar to those measured in cells cultured in normal glucose concentrations. However, this increase was not significant.

Next, we examined mitochondrial membrane potential. Mitochondrial membrane potential (MMP) is the most reliable indicator of mitochondrial integrity and function. The MMP value range of between -136 to -140 mV has been considered optimal for maximum ATP production for all living organisms. Even small decreases from this range result in substantial reductions in ATP production and increases in ROS production²⁹. A decrease in TMRE fluorescence in our assay indicated a reduction in MMP and disrupted mitochondrial energy production, which causes increased ROS production and the activation of apoptotic signaling, leading to severe DR. Müller glial cells in high glucose conditions for 24 hours and treated with 670 nm light showed significantly increased TMRE values compared to the high glucose sham group ($n = 3$, $p < 0.0001$). This increase shows the restoration of mitochondrial membrane potential in addition to improved mitochondrial function, indirectly. At the same time, the high glucose 670 nm light-treated Müller cells showed no significant difference from the normal glucose sham group ($n = 3$) (Fig. 2B).

Lastly, we examined cellular ATP content (Fig. 2C). In high glucose conditions, ATP concentrations trended towards a decrease compared to the normal glucose sham conditions ($n = 3$, $p = 0.10$). This decrease, albeit not significant, shows that mitochondrial function may be altered in as little as 24 hours of high glucose exposure. After 24 hours and treatment with 670 nm, Müller cells in high glucose media showed a trend of increased ATP concentrations similar to cells grown in normal medium.

As expected, ATP concentrations in Müller glial cells cultured in normal glucose concentrations and treated with 670 nm light did not differ from normal glucose controls. Although, cellular ATP concentrations did not reach significance, they fit the overall trend of impaired mitochondrial function.

High Glucose Causes Transcriptional Changes of NFkB And Leads To Increased Production of Pro-DR Markers

With early changes in reactive oxygen species and mitochondrial function, we next looked at downstream transcriptional changes associated with high glucose. The transcription factor, NFkB, is modulated by

reactive oxygen species levels in high glucose conditions³¹ to lead to inflammatory protein transcription. We examined NFκB in our system through a luciferase reporter assay. When cells were cultured in high glucose, there was a significant increase in NFκB activity in comparison to cells cultured in normal glucose (n = 4, p < 0.0001). As shown in Fig. 3, a 70% increase in luminescence in the high glucose cultured groups was observed in comparison to those cultured in normal glucose. NFκB activity was reduced to that observed in normal glucose conditions when treated with 670 nm PBM. This reduction was significantly different than high glucose/sham treated samples. As indicated by a p value of 1 (n = 3), there was no statistical difference between the level of NFκB activation in the normal glucose group receiving sham treatment in comparison to the high glucose group receiving PBM treatment. These results indicate that PBM entirely attenuated hyperglycemia induced activation of NFκB.

Downstream of NFκB, we then examined VEGF via qPCR to determine if hyperglycemia altered VEGF in Müller glial cells. Following 3 days in hyperglycemic culture conditions, VEGF transcripts were 1.5 times larger than VEGF levels under normal glucose conditions (Fig. 4A). We chose this earlier time point since we were looking at mRNA levels of VEGF and we expect those to change in a similar time frame as transcriptional activation of NFκB. Unexpectedly, high glucose, PBM treated Müller glial cells did not show decreased levels of VEGF transcript expression in comparison to high glucose sham treated conditions. This could be due to regulation of VEGF levels by other factors in addition to NFκB, see discussion.

Lastly, we measured ICAM-1 concentrations by western blot in cell lysates from Müller cells cultured in either high or normal glucose medium. We maintained cells in culture for 6 total days. This is longer than the time frame used for NFκB activity analysis to provide time for gene transcription and protein translation to occur. As shown in Fig. 4b, we measured an increase in ICAM-1 concentrations in cell lysates via western blot from cells cultured in high glucose compared to those cultured in normal glucose. ICAM-1 levels were increased 2.5-fold in cells cultured in high glucose compared to cells grown in normal glucose. Likewise, 670nm light treated cells showed reduced ICAM-1 levels, similar to that seen in the normal glucose controls.

Discussion

Novel to the present study, the mechanism of action of PBM utilizing 670 nm light in Müller cells was studied after 24 hours and up to 5 days under high glucose conditions. Our studies showed that as little as 24 hours of high glucose exposure is enough to disrupt mitochondrial function and increase ROS production in Müller glial cells. These early ROS and mitochondrial function changes initiate a cascade of signaling events leading to NFκB activation after 3 days and ICAM-1 and VEGF production. Indeed, treatment with 670nm light attenuated ROS production within an hour and these beneficial effects were also seen in improved mitochondrial function. Daily, short treatment with 670nm light continued to mitigate detrimental signaling effects seen with high glucose alone including reduced NFκB transcriptional activity and decreased production of ICAM-1. Interestingly, we saw no light treatment benefit on the production of VEGF implicating its production by another pathway in addition or in lieu of

NFkB. These findings are consistent with other model systems and pathways in which PBM has been shown to be beneficial^{9,15}.

Oxidative stress has been implicated as an initiating factor in DR pathogenesis^{7,22,23,30,31}. In cell models of DR, oxidative stress is increased in multiple cell types including human Müller glial cells treated with TBHP³², RPE grown in high glucose, and RGC grown in high glucose¹². In our study, ROS increases in 24 hours of high glucose treatment. This increase in ROS is mitigated in one hour by one, short light treatment (670nm, 180s). These findings are consistent with others where PBM has been shown to attenuate oxidative stress in many disease models, including DR^{8,9, 11–13,33}. However, unlike the current study, the majority of these studies have investigated the effects of 670 nm PBM on oxidative stress and ROS production several days after exposure to cytotoxic stressors including high glucose.

Increased oxidative stress can lead to mitochondrial changes. Correspondingly, high glucose causes increased mitochondrial fragmentation in rat Müller glial cells after 7 days in culture. These cells also displayed a change in mitochondrial membrane potential and decreased oxidative phosphorylation and glycolysis³⁴, which is thought to lead to cell death. In our study, we observed loss of mitochondrial permeability transition pore (MPTP) in as little as 24 hours in high glucose suggesting this is an early event in response to elevated glucose levels. However, we did not investigate the order of insult as oxidative stress can arise from mitochondrial functional loss and mitochondrial functional loss can also lead to increased oxidative stress. Consistent with our study, Zhang et al. showed that a mild oxidative stress (100 mM H₂O₂) produced an increase in ROS generation, a 30% reduction in mitochondrial metabolic activity and a 20% reduction in mitochondrial membrane potential within 6 hours in primary cultures of human Müller cells and a human cell line (MIO-M1)³². Thus, acute exposure to either high glucose or to oxidative stress increases ROS generation and disrupts mitochondrial function.

Loss of mitochondrial membrane potential is known to lead to reductions in oxidative phosphorylation³⁴, we measured cellular ATP content in the presence of high glucose at 24 hours. Although we did observe a trend in a diminution of ATP levels in cells exposed to high glucose medium, this diminution was not statistically significant. It is likely that ATP levels may decrease further at a later time point should the high glucose insult continue. In fact, Devi et al. demonstrates an intracellular ATP decrease of 20% in high glucose conditions (25 mM) compared to normal controls (5.5 mM)²³. Furthermore, PBM may be beneficial as ATP levels trended upward similar to normal glucose levels when cells grown in high glucose were treated with 670nm light.

We observed an increase in NFkB activity at 3 days in culture, likely due to the increased ROS and decreased mitochondrial health. In our study, a 70% increase in NFkB transcriptional activity in the high glucose cultured, sham treated groups was observed in comparison to cells cultured in normal glucose and not receiving treatment (Fig. 2). These findings are consistent with previous work^{31,35} where increased NFkB activity was observed in response to increased oxidative stress through high glucose in Müller glial cells³¹, in monocytes (THP-1)³⁶ and pericytes³⁷. Importantly, NFkB concentrations in PBM-

treated high-glucose-exposed cultures was not different from that measured in cells maintained under normal glucose. We believe this attenuation is likely due to the decreased ROS and early prevention of mitochondrial changes.

NFkB is a transcription factor that regulates the production of inflammatory and other molecules. In DR, NFkB regulates the production of ICAM-1 and VEGF^{12,30,31}. Indeed, we observed similar changes in ICAM-1 production as those observed in NFkB activity. In high glucose, Müller glial cells produced 60% more ICAM-1 compared to normal glucose controls, similar to previous studies³¹. This increased production was mitigated with PBM. Our *in vitro* data on the effects of PBM on ICAM-1 concentrations in high glucose-cultured Müller glial cells was comparable to the *in vivo* effects of PBM in diabetic rodents^{11,12}. Since ICAM-1 is highly regulated by NFkB, it is likely that for the observed decrease in NFkB transcriptional activity there will be a corresponding decrease in ICAM-1 level³⁸⁻⁴⁰. We measured ICAM-1 levels by Western blot on samples treated for 5 days, based on a prior study by Chen *et al.* (2012) who observed elevated levels of ICAM-1 when cells were cultured in high glucose for a range of one to seven days³⁰. After 6 days of in high glucose conditions, a two-fold increase in ICAM-1 concentration relative to levels of GAPDH was observed (Fig. 3). Our findings are in accordance with previous studies. Elevated levels of ICAM-1 were, as well, observed in STZ diabetic mice in comparison to non-diabetic control animals^{12,41}. Likewise, in a Müller glial cell culture model of DR, a 4-fold increase in ICAM-1 levels was reported for cells cultured in high (25 mM) glucose in comparison to cells cultured in normal glucose conditions for 5 days. Saliba *et al.* observed an increase in leukostasis, superoxide, and ICAM-1 levels in diabetic mice when compared to non-diabetic control mice. These effects were ameliorated in groups treated with 670 nm LED at a dosage of 6 J/cm². Importantly, these animals had an improvement in retinal function as measured by ERGs¹¹. Two months post-induction of diabetes in the STZ diabetic rats the activation of NFkB was increased by 60% in retinal samples compared to non-diabetic counterparts⁴². Corresponding to this increase in NFkB activity was a reduction in the antioxidant capacity of superoxide dismutase and an increase in oxidative stress, as measured by the presence of 8-isoprostane in the blood and ratio of GSSG/GSH.⁴² The increase in NFkB activity and oxidative stress, as well as the reduction in antioxidant capacity of superoxide dismutase, was partially attenuated in diabetic rats which were treated with the antioxidant resveratrol⁴².

Also, VEGF levels increased with high glucose treatment, consistent with many other reports^{24,25,43,44}. Unexpectedly, we observed no change in VEGF concentration under high glucose conditions compared to high glucose cultured cells treated with 670nm light. This data indicates that in this cell culture model of DR, VEGF and ICAM-1 are not regulated in the same manner. Regulation of VEGF can occur through multiple pathways in addition to NFkB⁴⁴. Indeed, Sun *et al.* reports multiple transcriptional proteins which regulate VEGF production including NFkB, p38, TNFa, CREB, vPA, IL-1B, Era, Stat3, and HCA1. Using a primary rat Müller cell model, Sun *et al.* implicate STAT3 in the regulation of VEGF in response to high glucose stimuli. In primary rat Müller cells, VEGF secretion was shown to be controlled by calcium with its transcriptional regulation via CAMKII and HIF1A⁴³. In neuronal cells, NDMA causes an influx of calcium

leading to increased production and release of VEGF ⁴⁵. Although it is generally accepted that within the injured retina, glial cells are the major source of VEGF ^{25,45}, they are not the only source. In conditional VEGF knockout mice, a Cre/lox system was used to determine the significance of Müller glial cell-derived VEGF. When VEGF synthesis was blocked in Müller glial cells of the conditional mice, a 44.5% decrease in VEGF retinal concentration was observed in comparison to their WT counterparts ²⁵. However, these results also indicate that more than 50% of VEGF is attributed to additional sources, including neuronal cells and RPE cells.

In summary, our findings of the chronic exposure to high glucose show early changes in ROS production and mitochondrial decline, both of which are mitigated with 670nm treatment. Prolonged high glucose culture conditions also lead to NFkB activation and ICAM-1 production. These changes are prevented with 670nm light treatment. Thus, 670nm light can prevent the acute effects of high glucose in a diabetic retinopathy cellular model system and reduce the production of pro-inflammatory mediators, providing a mechanism by which PBM is beneficial.

Methods

Cell Line

Experiments were performed on rat Müller glial cell line generously provided by John Mieyal, Case Western Reserve University. Müller cells were exposed to high glucose (25mM) or normal glucose (5mM) Dulbecco's modified Eagle's medium (Invitrogen 11995 and Invitrogen 11885, respectively) to mimic hyperglycemic and normal conditions. Cell medium was changed daily to maintain constant glucose load. For mitochondrial assays and oxidative stress assays, Müller cells were grown in a phenol free DMEM normal (5mM) or high glucose media (25mM). Phenol free normal glucose media (Invitrogen 11054-020) was supplemented with 5% L-glutamine and high glucose phenol free media (Invitrogen 21063-029) was supplement with 110mg/L sodium pyruvate to sustain Müller cell growth in culture.

Light Treatment

Cell cultures were exposed to 670 nm light from a light emitting diode (LED) array (10 cm x 25 cm) (Quantum Devices Inc. Barneveld, WI) positioned on top of the culture plate at a dose of 25mW/cm² for 180 seconds (total of 4.5J/cm²). Sham-treated cells were handled in a similar manner except that the LED array was not illuminated. PBM-treatment occurred one time per day over 24 hours, 72 hours or 96 hours, depending on the assay. All assays were conducted one hour following the final light treatment.

MTT Assay

Cells were plated in triplicate in 96 well plates at a density of 30,000 cells/well, cultured in the respective high or normal glucose media conditions. At 24 hours, respective media were exchanged and incubated

for one hour. After 1 hour, cells were treated with either 670nm light or sham light for 180 seconds. After one hour, media was removed, and cells were washed with 1x PBS. Then, cells were incubated with 5mg/mL MTT in medium for 3 hours at 37°C. MTT solution was solubilized in 10% Triton x100 and isopropanol with 0.1N HCl (final concentration) and was shaken for 15 mins in the dark. Each well was thoroughly mixed and absorbance was read at 590 nm (Biotek Synergy HT).

TMRE Mitochondrial Membrane Potential:

Cells were plated in triplicate in 96 well plates at a density of 30,000 cells/well in normal or high glucose conditions for 24 hours. At 24 hours, respective media were exchanged and incubated for one hour. After 1 hour, cells were treated with either 670nm light or sham light for 180 seconds. One hour later, media was removed, and cells were washed with 1x PBS. The TMRE assay (Abcam, Cambridge, MA) proceeded per manufacturer's instructions. Briefly, cells were incubated in 200 nM TMRE dissolved in their respective cell mediums for 30 mins. Cells were washed with PBS and fluorescence (EX/EM: 549/575 nm) was read in 100uL PBS/0.2% BSA solution.

ATP Measurement

Cells were plated in triplicate in 96 well opaque-walled plates at a density of 30,000 cells/well in phenol free normal or high glucose conditions for 24 hours. At 24 hours, respective medias were exchanged and incubated for one hour. The cells were treated with 670 nm light (4.5 J/cm^2) or sham light for 180 seconds. After 30 mins, plates were removed from 37 °C and incubated at room temperature for another 30 mins. Then an equal volume of CellTiter-Glo 2.0 Reagent (100uL) was added to Müller cells in their respective media (100uL). Plates were shaken for 10 minutes at room temperature. Luminescence was read (gain 115–135) and compared to an ATP standard curve.

Assessment of ROS

Cells were plated in triplicate in 96 well plates at a density of 30,000 cells/well in normal or high glucose conditions for 24 hours. At 24 hours, respective medias were exchanged and incubated for one hour. The cells were treated with 670 nm light (4.5 J/cm^2) or sham light for 180 seconds. One hour after light treatment, cells were washed with 1x PBS and a 40 mM DCFDA solution (in respective media) was overlaid immediately, per manufacturer's instructions (Cayman Chemical Inc., Ann Arbor, MI). DCFDA solution was allowed to incubate at 37 °C for 1 hour. After 1 hour, DCFDA solution was removed and replaced with 100uL of a 1x buffer and read via fluorescence in a plate reader with EX/EM 484nm/528 nm with a gain of 100–120.

Transfection of NFkB

NFκB activity was measured as previously described by Shelton et al. 2007³¹. Briefly, 20,000 Müller cells/well (6 well dish) were grown for 3 days. At day 3, cells were approximately at a confluency of 60–70%. Cells were washed twice with 1x PBS. They were then co-transfected according to manufacturer instructions with a cocktail containing 1 ml/well Opti-MEM, 5 µl/well lipofectamine 2000, 1 µg/well NFκB plasmid and 0.1 µg/well Renilla plasmid (Shelton et al. 2007). Transfection was allowed to proceed for 8 hours. Post transfection, the transfection cocktail was aspirated and replaced with DMEM, 5 mM glucose, 2% FBS, 1 % penicillin streptomycin, 110 mg/L sodium pyruvate for 3–4 hours. Cells were then switched to appropriate treatment media, high (25 mM) or normal (5 mM) glucose. Cells were maintained in either high or normal glucose conditions for 3 days and were either treated with 670nm light (4.5 J/cm²) or sham treated for 180 sec daily. Following treatment, lysates were collected in 1x passive lysis buffer (PLB) and assessed via a Dual Luciferase Reporter assay (Promega catalog number E1910). Luminescence measured was normalized to Renilla.

Assessment of ICAM-1:

Cells were cultured and treated with light once a day for 5 days. One hour after treatment with light on day 5, cells were harvested and lysed on ice for 15min in 1xRIPA buffer (Alfa Aesar, catalog number J60629) containing 1x protease inhibitor cocktail (Thermo Scientific, catalog number 87786). 100ug of cell lysate boiled at 95°C for 15 min in Laemmli buffer (10% glycerol, 2% SDS, .01% bromophenol blue, 200mM Tris HCl pH 6.8, 20mM DTT). Samples were run on a SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Prior to transfer, the membrane was soaked in methanol for 2 minutes, then washed with Transfer Buffer (0.3%w/v Tris, 1.45% w/v glycine, and 10% v/v methanol). Anti-rabbit ICAM-1 (Cell Signaling, catalog number 49155) and anti-rabbit GAPDH (Cell Signaling, catalog number 21185) were used to probe the membrane at 1:500 and 1:5000, respectively. An anti-goat, anti-rabbit horseradish peroxidase-linked secondary antibody (1:10,000, Jackson Labs, catalog number 111-035-144) followed by detection using SuperSignal™ Chemiluminescent HRP Substrate and exposure to film. ImageJ software was used to compare protein levels normalized to GAPDH.

VEGF qPCR:

Müller glial cells were grown in culture for 3 days, plated at a density of 80,000 cells in 10cm dishes. Each plate was treated with either sham or 670nm light at an intensity of 4.5J/cm². Cells were washed with 1xPBS and harvest in Trizol. RNA was extracted per manufacturer's instructions. Biorad iScript cDNA kit was used to create cDNA. qPCR was performed using Biorad SybrGreen Super Mix on a step1plus machine following manufacturers' instructions. Primers used were for VEGF (FP: CCGCAGACGTGTAAATGTTCC, RP: GACGGTGACGATGGTGGTGT) and actin (FP: TGACGTGGACATCCGCAAAG, RP: CTGGAAGGTGGACAGCGAGG). Samples were run in triplicate and normalized to the sham, normal glucose control. Values were calculated via DDCT.

Statistical Analysis

Samples were measured in duplicate or triplicate. All glucose and light conditions were normalized to the normal glucose sham condition. Averages, standard deviations and standard errors were calculated. Differences between each sample group were analyzed by ANOVA, followed by Bonferroni post-hoc testing. The alpha for statistical analysis was set at a p value of 0.05.

Declarations

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Author contributions:

E.S.L. conceived the paper. H.J.N, G.S., A.E.H. and B.A. conducted the experiments with the support of E.S.L. E.S.L. and J.T.E. interpreted the data; E.S.L. wrote the manuscript with contributions from J.T.E. All authors read and approved the final manuscript.

Competing interests:

J.T.E.'s work has been funded by LumiThera, Inc. She has received compensation as a member of the scientific advisory board of LumiThera, Inc. and owns stock in the company. She has also received compensation as a member of the scientific advisory board of and MultiRadiance Medical, Inc. E.S.L., H.J.N, G.S., A.E.H and B.A. declare no competing interests.

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Figures

Figure 1

670 nm Light Treatment Diminishes Oxidative Stress under High Glucose Conditions in Müller Glial Cells. Bar graph displaying ROS production in Müller glial cells. Cells were cultured in either normal glucose (black bars and white bars) or high glucose (gray bars and hashed bars) medium for 24 hours. 670nm or sham control was applied to the cells at 4.5 J/cm². Oxidative stress was analyzed using DCF-DA. *** p<0.001, **** p<0.0001. n=3, done in triplicate

Figure 2

670nm PBM Restores Mitochondrial Function under High Glucose Conditions. Müller cells were cultured in either normal glucose (black bars and white bars) or high glucose medium (gray bars and hashed bars) for 24 hours. 670nm (white bars and hashed bars) or sham control (black bars and gray bars) was applied to the cells at 4.5 J/cm². All assays were normalized to the normal glucose sham levels. (A) NADPH-dependent oxidoreductase activity was analyzed using MTT, n=3. (B) Mitochondrial membrane potential was analyzed using TMRE. n=3, done in triplicate. (C) ATP levels was analyzed via CellTiter-Glo (Promega) assay, n=3, (p=0.10). * p<0.05, ** p<0.01, **** p<0.0001.

Figure 3

670nm Light Treatment Diminishes NFκB Activity under High Glucose Conditions in Müller Glial Cells. Bar graph showing Müller cells were cultured in either normal glucose (black bars and white bars) or high glucose medium (gray bars and hashed bars) for 72 hours. 670nm (white bars and hashed bars) or sham control (black bars and gray bars) was applied to the cells at 4.5 J/cm² daily. NFκB activity was analyzed

via reporter gene assay, normalized to the transfection control, Renilla. * $p<0.05$ ** $p<0.01$ $n=4$, performed in duplicate.

Figure 4

670nm PBM Suppresses Downstream Mediators of Inflammatory Signaling. Bar graph representing Müller glial cells were cultured in either normal glucose (black bars and white bars) or high glucose (gray bars and hashed bars) medium for 72 hours. 670nm (white bars and hashed bars) or sham control (black bars and gray bars) was applied to the cells at 4.5 J/cm² daily while in culture. (A) VEGF mRNA was analyzed via qPCR and normalized to actin. mRNA levels were calculated by DDCT and normalized to Normal glucose sham, $n=3$, done in duplicate. $p=0.0147$ (B) ICAM-1 was analyzed via western blot and normalized to GAPDH as the loading control via ImageJ. Inset includes a representative western blot image, $n=4$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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