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Photobiomodulation Modulates Bioenergetics and Oxidative Stress in an in Vitro Model of Diabetic Retinopathy

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Photobiomodulation Modulates Bioenergetics and Oxidative Stress in an in vitro Model of Diabetic Retinopathy

by

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ABSTRACT

PHOTOBIOMODULATION MODULATES BIOENERGETICS AND OXIDATIVE STRESS IN AN IN VITRO MODEL OF DIABETIC RETINOPATHY

by Alexandria Hall

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Under the Supervision of Elizabeth Liedhegner, PhD and Janis Eells, PhD

Diabetic Retinopathy (DR) is the most common complication of diabetes mellitus and a leading cause of blindness. The pathophysiology of DR is complicated, involving mitochondrial dysfunction, oxidative stress, inflammation and vascular degeneration. The most common therapeutic approach for DR includes the use anti-vascular endothelial growth factor (VEGF) drugs to reduce vascular proliferation. These treatments are invasive, frequently ineffective and have numerous adverse effects. A non-invasive more effective therapy is clearly needed. An alternative, non-invasive therapy using far-red light (photobiomodulation, PBM) may be an improvement over current therapy. PBM has documented efficacy in experimental and clinical studies of retinal disease including DR. Müller glial cells play a critical role in retinal metabolism and are among the first cells to demonstrate metabolic changes in response to retinal stress or disease and play an important role in the pathophysiology of DR. High glucose treatment of Müller cells increases the activity of Nuclear Factor Kappa beta (NFκB) resulting in DR-associated protein synthesis. Previous studies in our laboratory have shown that that mimicking hyperglycemia in cultured Müller cells activates the NFκB signaling pathway resulting in an increase in intracellular adhesion molecule 1 (ICAM-1) and that 670 nm PBM blocks this activation [Fisher, 2015]. However, the upstream mechanisms by which PBM

reduces NFκB activity remain to be elucidated. *We hypothesized that 670 nm photobiomodulation would attenuate hyperglycemia-induced mitochondrial dysfunction and protect against oxidative stress in a rat Müller glial cell model of diabetic retinopathy.* We tested this hypothesis in the following two aims: **Specific Aim 1:** Determine the effects of 670 nm PBM on mitochondrial function in a cultured Müller cell model of DR at 24 hours. **Specific Aim 2:** Determine the effects of 670 nm PBM on oxidative stress and glutathione status in a cultured Müller cell model of DR at 24 hours. We showed for the first time that as little as 24 hour of exposure to high glucose conditions disrupted mitochondrial metabolic activity, and decreased mitochondrial membrane potential, and ATP production. A single treatment with 670 nm light (4.5 J/cm^2) restored metabolic activity, mitochondrial membrane potential and ATP production to values measured under normal glucose conditions within one hour following treatment. We also showed that a 24-hour exposure to high glucose stimulated ROS production. These effects were also reversed by a single treatment of 670 nm light within one hour. We conclude that this *in vitro* model of diabetic retinopathy in a dish has considerable potential for use in the development and testing of therapeutic agents to treat diabetic retinopathy. Our model can assess disruptions in metabolic activity beginning with ROS generation and mitochondrial redox changes culminating in increased production of angiogenic and inflammatory mediators characteristic of diabetic retinopathy.

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ADP: Adenosine 5′-diphosphate

AMP: Adenosine 5′-monophosphate

ARPE: Human retinal epithelial cells

AII: Angiotensin II

BSO: Buthionine sulfoximine

Cu/Zn SOD: Copper/ Zinc superoxide dismutase

DCF: 2’ ,7’ –dichlorofluorescin diacetate

ETC: Electron transport chain

GSH: Reduced glutathione

GPx: Glutathione peroxidase

GSSG: Oxidized glutathione

H2O2: Hydrogen peroxide

ICAM: Intracellular adhesion molecule

IMAC: Mitochondrial inner membrane anion channel

LED: Light emitting diode

MnSOD: Manganese superoxide dismutase

MES: 2-(N-morpholino)ethanesulfonic acid

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Nrf2: NF-E2-related factor 2

NOX: NADPH oxidase

NIR: Near infrared

NADPH: Nicotinamide adenine dinucleotide phosphate

PKC: Protein kinase C

PBM: Photobiomodulation

ROS: Reactive oxygen species

RPE: Retinal pigment epithelium

RGC5: Retinal ganglion cells

Stz: Streptomycin

TBHP: Tertbutyl hydrogen peroxide

TMRE: Tetramethylrhodamine ethyl ester

Trx: Thioredoxin

TXNIP: Thioredoxin inhibitor

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Introduction

Diabetes mellitus

Diabetes mellitus is a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism [8]. In diabetes mellitus, the ability of the body to properly utilize the glucose is impaired resulting in hyperglycemia. Glucose utilization is impaired due to either a lack/reduction in insulin production (type I diabetes) secondary to the β-cell destruction or due to body's difficultly in utilizing insulin (type II diabetes). The resulting hyperglycemia in both forms of diabetes is key in the development of diabetic complications.

Diabetes is emerging as an epidemic of the 21st century. According to International Diabetes Federation, every 7 seconds a person dies from diabetes. The number of patients with this chronic disease world-wide is rising at an alarming rate. Across the world, 382 million people have diabetes, and this number is projected to rise to 592 million in less than 25 years [1]. In this life-long disease, sustained high circulating glucose damages the vasculature resulting in chronic macro- and micro-vascular complications throughout the body. Damage to the macro-vasculature increases the prevalence of heart disease, stroke and peripheral arterial disease, and to the microvasculature, in retinopathy, neuropathy and nephropathy in diabetic patients. As diabetes reaches epidemic proportions, the need for the development and testing of safer and more effective therapies for this disease and its complications is imperative.

Diabetic retinopathy

Diabetic retinopathy is the most common microvascular complication of diabetes and a leading cause of blindness in the United States [9]. The pathophysiology of diabetic retinopathy is complicated, involving oxidative stress, 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 resulting in disturbed vision [8, 18, 42, 47]. Control of diabetes-associated metabolic anomalies including hyperglycemia, hypertension and hyperlipidemia play a major role in the development of microvascular complications associated with diabetic retinopathy. Current interventions for diabetic retinopathy include non-pharmacologic therapies (laser photocoagulation, vitrectomy) and pharmacological therapies involving the intraocular injection of anti-angiogenic agents or corticosteroids that target the excessive vascular growth and inflammation, respectively [30, 47]. These interventions are invasive, expensive and only partially effective.

A Brief Review of Retinal Anatomy and Physiology

The fundamental organization of the retina is conserved across verteb rates. The retina contains five major neuronal cell classes (photoreceptors, bipolar cells, amacrine cells, horizontal cells and retinal ganglion cells) with two prominent support cells, Müller glial cells and retinal pigment epithelial cells, providing metabolic and homeostatic support (Figure 1) [6, 8]. As light enters the eye, rod and cone photoreceptors convert light energy into neural activity. This conversion is made possible by light-sensitive pigments located on the outer segments of the photoreceptor cells. The photoreceptors synapse with bipolar cells. Bipolar cells synapse the retinal ganglion cell which transmit the visual signal along their axons (optic nerve) and ultimately, to the brain (Figure 1) [1]. The retinal pigment epithelium envelopes the photoreceptors facilitating the diffusion of nutrients, key metabolites and oxygen from the choroidal vessels. Horizontal cells, amacrine cells and Müller cells mediate and support the synapses between retinal neurons through the collection and synthesis of glutamate (Figure 1) [1, 48]. The Müller cell has processes that span through the inner- and most of the outer-retina which is unique to this cell type.

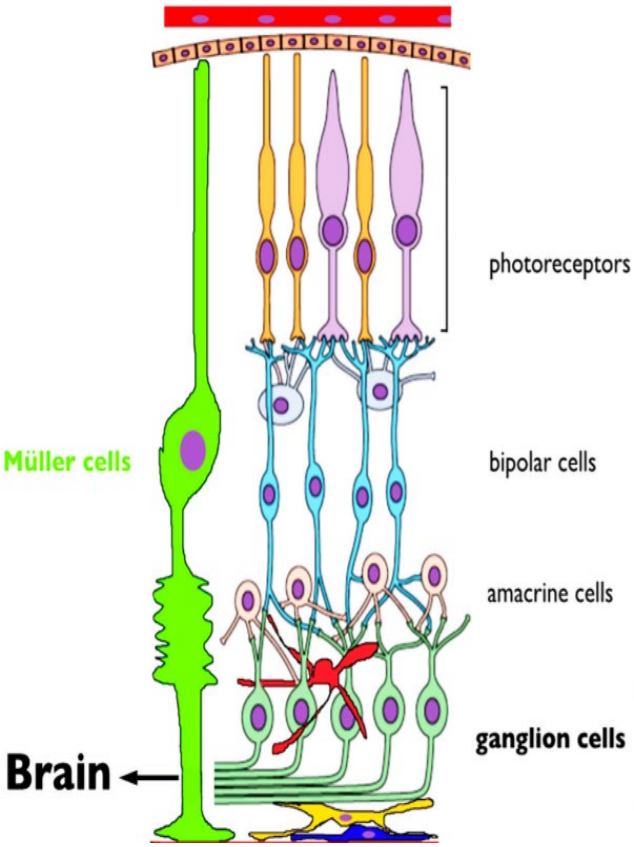

Figure 1: The complexity of the retinal layers from the inner retina (top) to the outer retina (bottom). Retinal cells communicate with each other to transmit a signal to the brain to produce vision. Adapted from Ryskamp *et al.* 2014

These processes allow Müller cell to maintain multiple synapses with different cells throughout the retina. Pericytes surround the vascular endothelial cells in all retinal layers, forming the blood-retinal barrier (Figure 1) [1, 48].

The retina is the highest oxygen-consuming organ in the human body. Inner retinal neurons have the highest metabolic rate of all central nervous tissue, and the oxygen consumption rate of the photoreceptors is, uniquely, several times higher again. The inner segments of the photoreceptor cells are rich in mitochondria providing the ATP required by the ionic pumps which depolarize the cell, driving the 'dark current'. Modulation of the dark current by light is the beginning of vision [48]. As a result, photoreceptors consume more oxygen per gram of tissue weight than any cell in the body [48,49]. This intense oxidative phosphorylation in their inner segments, coupled with high concentrations of polyunsaturated fatty acids in their outer segments naturally renders the retina susceptible to oxidative stress and lipid peroxidation [48]. The retina also contains abundant photosensitizers and is exposed to visible light [49]. Normally, oxidative damage is minimized by endogenous antioxidants and repair systems. With aging and retinal disease, there is an increase in mitochondrial dysfunction and oxidative damage and corresponding decrements in antioxidants and repair systems, resulting in retinal dysfunction and retinal cell loss, leading to visual impairment [48].

In DR, chronic hyperglycemia promotes metabolic dysfunction culminating in excess production of ROS in retinal cells [12, 22, 33, 38, 39, 45]. This surplus of ROS activates pathogenic signaling pathways in the retina leading to inflammation, angiogenesis, and disease. In addition, high concentrations of ROS deplete antioxidant defense systems, subjecting the retina to additional oxidative damage. Although microvascular changes are

integral to diabetic retinopathy, the neural retina is also profoundly affected. Experimental and clinical studies have provided evidence that neural retinal defects are among the earliest detectable changes in diabetes [8]**.** Among the retinal cell types susceptible to hyperglycemia-induced oxidative stress are retinal ganglion cells, photoreceptor cells, retinal pigment epithelial cells, pericytes and Müller glial cells [10, 12, 22, 38, 39, 47]. Furthermore, *in vitro* and *in vivo* studies have shown that Müller glial cells play a very important role in the pathogenesis of diabetic retinopathy [6, 10, 23].

Müller glial cells

Müller cells are the principle glia of the retina. Müller glia are the only cells that span the retina and have intimate contact with both the retinal blood vessels and retinal neurons [6]. Müller cells serve a number of essential functions in the healthy retina including the uptake and recycling of neurotransmitters, retinoic acid compounds, and ions (such as potassium K+), the control of metabolism and supply of nutrients or the retina, and the regulation of blood flow and maintenance of the blood retinal barrier [33, 34]. In DR, hyperglycemia-induced retinal injury activates Müller cells resulting in increased synthesis of survival factors [13, 33]. These factors promote a stress response by activating intracellular pathogenic pathways that promote activation of the inflammatory transcription factor, NFκB leading to the production of ICAM and VEGF, responsible for angiogenesis in DR [8, 10, 13, 18, 58]. Previous *in vitro* studies in other laboratories as well as our own lab have shown that Müller cells upregulate NFκB and VEGF when exposed to high glucose media [10, 3, Fisher, 2015, Hall, 2017]. Müller glial cells significantly contribute to diabetic retinopathy, especially through the secretion of VEGF

[4, 7, 10, 13, 34, 40, 43, 44]. VEGF promotes to growth of blood vessels throughout the retina which obstruct and physically block vision. For this reason, cultured Müller glial cells have been widely utilized to study diabetic retinopathy and have become a well-established model of this disease state.

DR Pathogenesis

The pathogenesis of diabetic retinopathy is complex involving the disruption of multiple intracellular pathways [8, 18]. Early pathologic changes observed in diabetic retinopathy include mitochondrial dysfunction, oxidative stress and inflammation [22, 24]. Oxidative stress results from increased production of reactive oxygen species (ROS), including superoxide and hydrogen peroxide (Figure 2) [3, 19, 24]. ROS oxidize intracellular proteins, lipids and nucleic acids disrupting normal signaling and culminating in disease. Under physiological conditions, small amounts of superoxide leak from the electron transport chain and are converted to hydrogen peroxide by mitochondrial superoxide dismutase (Figure 2) [3, 24]. Hydrogen peroxide diffuses out of the mitochondria into the cytosol and serves an important signaling molecule. Intracellular ROS concentrations are regulated by a complex array of antioxidant systems to maintain low intracellular ROS concentrations [2, 3, 25, 31]. Under hyperglycemic conditions, the excess production of ROS overwhelms the antioxidant systems to a threshold resulting in oxidative stress (Figure 2) [22, 25, 34, 37].

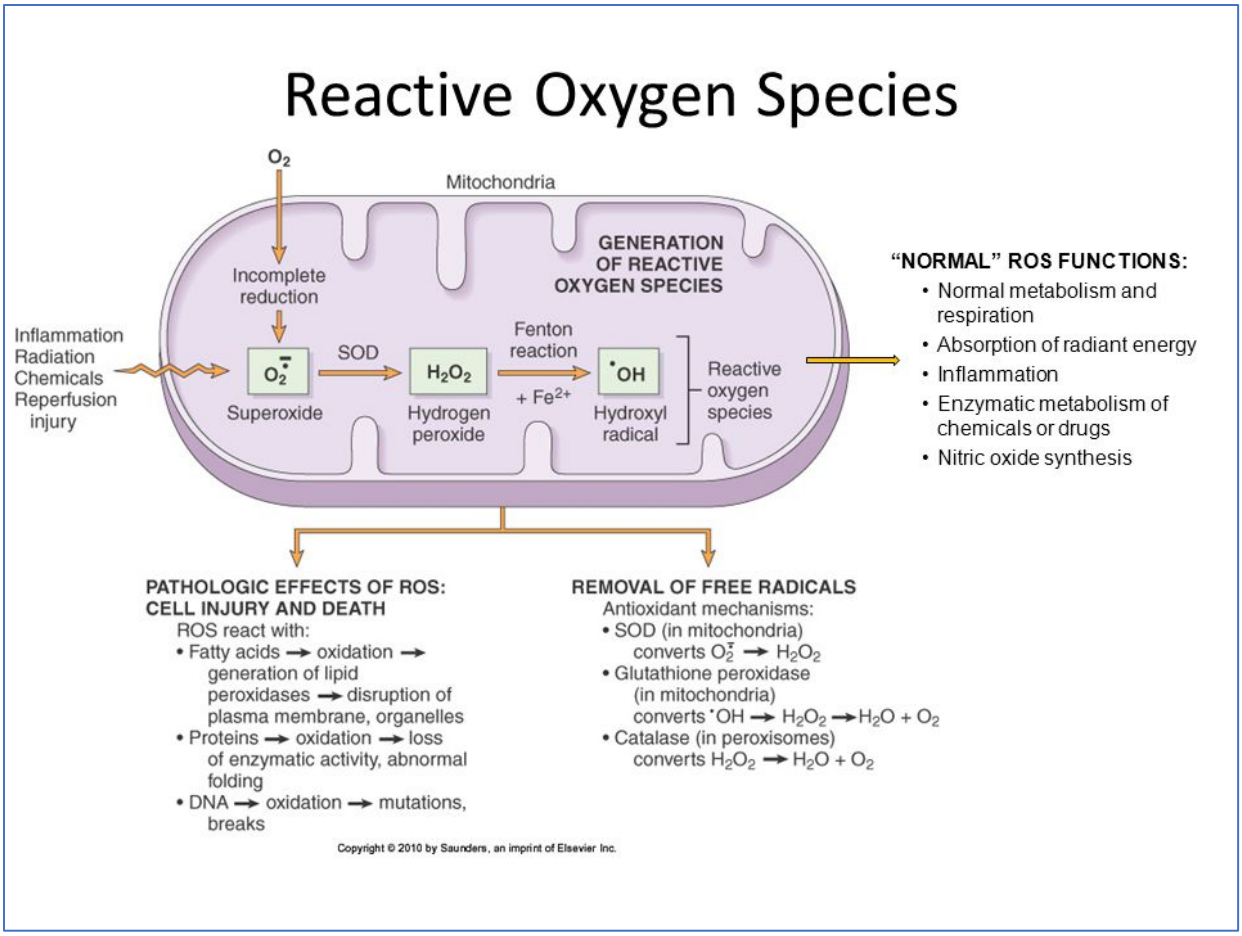

Figure 2: Mechanism of ROS production and ROS action in normal and pathogenic environments. Saunders *et al.* 2010

A major source of the excess ROS production under hyperglycemic conditions is the mitochondrial electron transport chain (ETC) (Figure 3) [3, 29, 31, 42, 47]. Under normal physiological conditions, electrons are donated to complex I or complex II of the ETC and are then passed on to coenzyme Q, complex III, cytochrome c, complex IV and ultimately to molecular oxygen, the final electron acceptor (Figure 3). The transfer of electrons generates a proton gradient across the mitochondrial intermembrane which drives synthesis of ATP by ATP synthase. During this process, small amounts of superoxide

are produced, but are easily cleared by the cell via antioxidant defense mechanisms (Figure 3) [3].

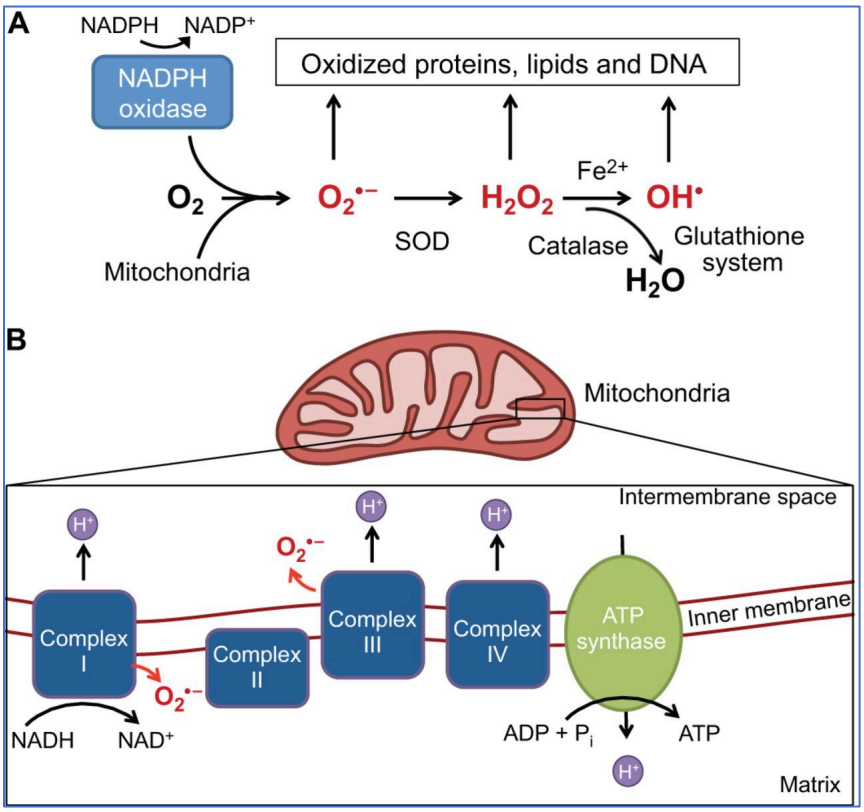

Figure 3: The mitochondrial electron transport chain and production of ROS by electrons escaping coenzyme Q. O_2^-: superoxide; H_2O_2: hydrogen peroxide; SOD: superoxide dismutase. Bigarella *et al.* 2014

In DR, chronic hyperglycemia disrupts the mitochondrial ETC. Increased glucose metabolism results in more electrons passing down the ETC. Excess electrons overwhelm coenzyme Q. Those electrons fail to be transferred to complex III and are instead dispensed prematurely onto molecular oxygen, creating superoxide (Figure 3) [3, 16]. Superoxide is quickly converted by superoxide dismutase into hydrogen peroxide.

Therefore, mitochondria are a major source of ROS production [3, 45, 47] and mitochondrial dysfunction is a major factor in DR pathogenesis.

Cells susceptible to retinal injury have been shown to respond to chronic hyperglycemia, mitochondrial dysfunction, and ROS via five key pathogenic pathways: the polyol pathway, the advanced glycosylated end products (AGEs) pathway, the protein kinase C (PKC) pathway, the hexamine pathway and the angiotensin II (AII) pathway (Figure 4) [8].

Molecular Mechanisms of DR

High glucose alters a number of metabolic pathways; some of the major pathways implicated in the development of retinopathy are PKC activation, accumulation of AGEs and inflammatory mediators, polyol pathway, oxidative stress and activation of the hexosamine pathway (Figure 4) [8, 18]. Experimental models have shown that in the etiology of this progressive disease, activation of NADPH oxidase (*Nox*) increases cytosolic reactive oxygen species (ROS), and sustained accumulation of ROS damages mitochondria further increasing oxidative stress [17, 18]. Superoxide radicals produced by the mitochondrial electron transport chain activate these major pathways, and activation, in turn, can damage the mitochondria propagating the vicious cycle of ROS (Figure 4) [46, 47]. Any connection between a particular metabolic abnormality and the development of diabetic retinopathy, however, is largely speculative, and it is highly likely that no single metabolic abnormality could be the sole cause of the development of diabetic retinopathy. Furthermore, the role of other systemic factors including hyperlipidemia and hypertension in retinal damage and pathology also remains under-investigated.

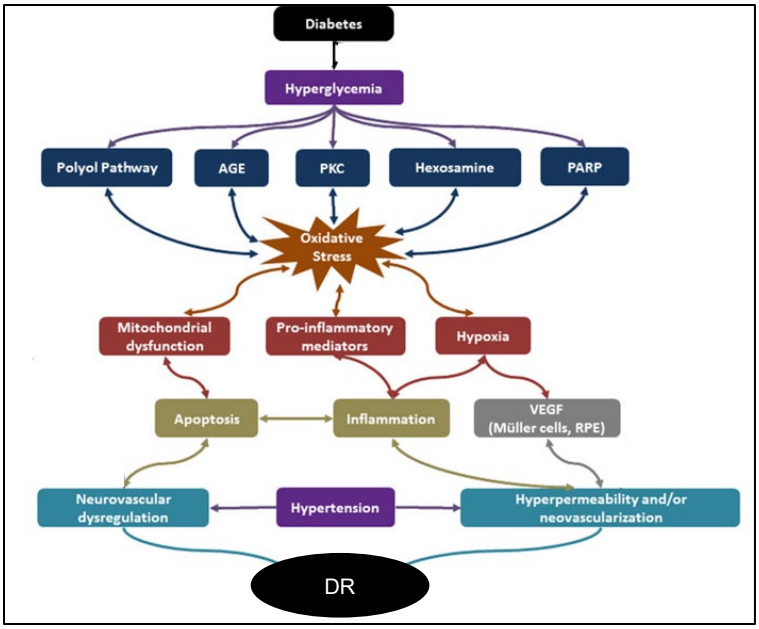

Figure 4: The major pathways involved in DR pathogenesis. Diabetes increases hyperglycemia and activates five major pathogenic pathways. These pathways are caused by and lead to oxidative stress, mitochondrial dysfunction, and inflammation resulting in DR-associated protein synthesis, angiogenesis and DR. AGE: advanced glycosylated end products; PKC: Protein kinase C; AII: Angiotensin II. Adapted from Wang, J. 2017

In the diabetic retina, the polyol and hexamine pathways contribute to oxidative stress by oxidation of proteins and depletion of antioxidants within the cell (Figure 4) [8, 15, 46]. The activation of the AGE, angiotensin II, and PKC pathways result in increased ROS, NFκB activity, and VEGF production [8, 46]. Upregulation of NFκB activity (Figure 5) [Fisher, 2015] increases cytokine production leading to retinal inflammation, angiogenesis, and retinal pathogenesis [8,18, 26, 42]. Angiogenesis in the diabetic retina is attributed to elevated concentrations of VEGF, found late in disease. The development of new blood vessels physically block vision and appear as black spots to DR patients. These blood vessels are weak and prone to rupture as retinal disease progresses.

Ruptured vessels result in a significant breakdown of the blood-retinal barrier and potentiate complete blindness in patients with DR [8, 18, 42].

Previous studies in our laboratory tested the hypothesis that 670 nm PBM would inhibit the activation of NFκB resulting in a reduction in the production two key cytokines involved in vascular proliferation and immune dysregulation, VEGF and ICAM-1**.** These studies employed an *in vitro* model system of cultured retinal Müller glial cells grown in normal (5mM) or high (25mM) glucose conditions to simulate normoglycemia and hyperglycemia, respectively. Cultures were treated with 670 nm LED light (180 sec at 25 mW/cm^2; 4.5 J/cm^2) or no light (sham) for three days (Figure 6). NFκB activity and ICAM-1 concentrations were significantly increased under high glucose conditions (Figure 5, Figure 6). 670 nm PBM restored NFκB activity to values measured under normoglycemic condition levels. 670 PBM also suppressed the production of the downstream inflammatory and angiogenic mediators, ICAM-1 and VEGF, respectively. These data support our central hypothesis that mimicking hyperglycemia in an *in vitro* model of diabetes retinopathy activates the NFκB signaling pathway resulting in an increase in ICAM-1 and VEGF and that 670 nm PBM blocks this activation (Figure 5, Figure 6).

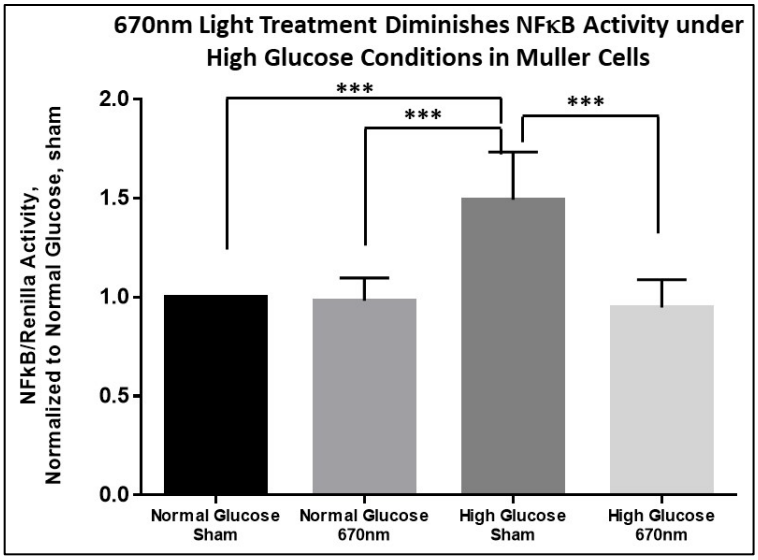

Figure 5: **670 nm Light Treatment Diminishes** NFκB **Activity under High Glucose Conditions In Müller Cells.** Müller cells were cultured in either normal glucose or high glucose medium for 72 hours. 670nm or sham control was applied to the cells at 4.5 J/cm^2 daily. NFκB activity was analyzed via reporter gene assay, normalized to the transfection control, Renilla. *** $p<0.001$ n=4, performed in duplicate. *(Fisher Thesis)*

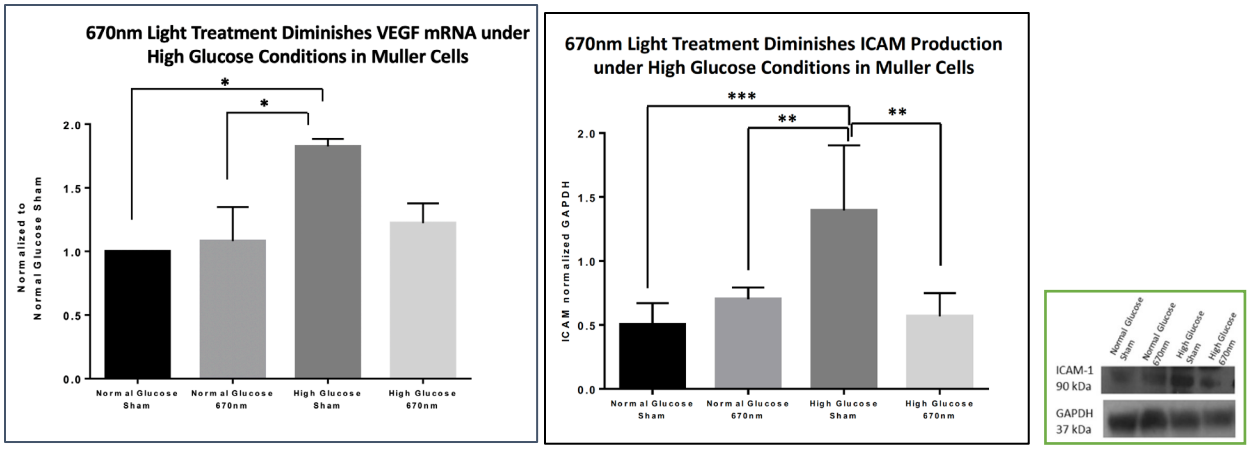

Figure 6: **670 nm Light Treatment Diminishes downstream products of NFκB activity.** Müller cells were cultured in either normal glucose or high glucose medium for 72 hours. 670nm or sham control was applied to the cells at 4.5 J/cm2 daily while in culture. A. VEGF mRNA was analyzed via qPCR and normalized to actin. mRNA levels were calculated by DDCt and normalized to Normal glucose sham, n=2, done in duplicate. B. ICAM was analyzed via western blot and normalized to GAPDH as the loading control via ImageJ. Inset includes a representative western blot image, n=4. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. [Fisher 2015, Hall 2015]

The objective of the present investigation was to use this Müller glial cell *in vitro* model of diabetic retinopathy to elucidate the upstream signaling mechanisms that culminate in the activation of NFκB activity and subsequent increase in the downstream mediators of inflammatory signaling, ICAM-1 and VEGF. Our second objective was to determine the effect of 670 nm PBM on these signaling mechanisms.

Mitochondria and ROS Production

Mitochondria are essential for cell function and cell survival, including energy production and regulation of cell metabolism and apoptosis [42, 52]. Mitochondria are also a primary source of ROS, generated as a byproduct of the electron transport chain (ETC) (Figure 4) [3, 29, 31, 42, 45, 47]. The ETC produces about 85-90% of total ROS within the cell [3,

42]. Mitochondria are also a target of ROS in disease [2, 42]. Hydrogen peroxide causes structural damage to this organelle [31, 42], further inducing mitochondrial dysfunction. However, the cause of mitochondrial dysfunction in a diabetic state is unclear. Some studies suggest that the increased glucose concentration overwhelms the electron transport chain by increasing the rate of oxidative phosphorylation [8], generating ROS. Other studies suggest that mitochondrial DNA is damaged, resulting in dysfunctional proteins that will form essential sections of the ETC complexes, also causing excess ROS and mitochondrial dysfunction [7]. Impaired mitochondrial function is commonly observed in chronic diseases including diabetes mellitus [16, 17, 18, 31, 52]. Mitochondrial dysfunction under pathological conditions is promoted by increased oxidative stress, which can stimulate post-translational modifications of mitochondrial proteins and/or oxidative damage to mitochondrial DNA and lipids [12].

Mitochondrial morphology is essential in function of the mitochondria and the whole cell. In dysfunctional mitochondria, morphology is altered, mitochondrial membrane potential is reduced, and the integrity of the mitochondrial membrane is disrupted [17, 25]. When the integrity of the mitochondrial membrane fails, mitochondria send apoptotic signals that lead to pore formation in the mitochondrial membrane to induce apoptosis. This mitochondrial permeability transition pore forms a pore in the inner mitochondrial membrane promoting the release of cytochrome c from the inner membrane space into the cytosol [38, 39, 45]. This significantly disrupts cell metabolic activity and initiates apoptosis via the mitochondrial apoptotic pathway; contributing to DR pathogenesis.

Oxygen consumption is another measure of mitochondrial function. As oxygen is the final electron acceptor of the ETC; the reduction of oxygen to water can be measured. Maximal

and steady state oxygen consumption was measured in high and normal glucose conditions at three- and six-days post exposure [39]. At day-three, rat retinal endothelial cells were not affected by glucose concentration. However, after six days post high glucose exposure, retinal endothelial cells significantly decreased both steady state and maximal oxygen consumption [39]. These results were independent of the number of mitochondria present in the cell [39], indicating a decrease in mitochondrial function in retinal endothelial cells exposed to high glucose conditions.

Role of Mitochondria in Müller Glial Cells

Retinal Müller cells play a crucial role in maintaining retinal homeostasis [6]. Moreover, Müller cell function requires considerable energy production. Although previous literature has primarily emphasized the importance of glycolysis as the main energy provider, recent studies highlight the need of mitochondrial ATP production to maintain Müller cell function especially under conditions of chronic oxidative stress [10, 19, 34, 40]. Therefore, the mitochondria in Müller cells play an important role in disease pathogenesis. Under high glucose conditions, we anticipated metabolic activity to decrease due to stress. Devi *et al.* (2012) found that cytochrome c oxidase remains active after prolonged high glucose exposure in retinal Müller cells [10]. This indicates that the cells and mitochondria remain functional under chronic hyperglycemic conditions and may activate mechanisms to survive.

Interestingly, Müller cells in high glucose conditions decreased intracellular ROS levels at four hours, however, was increased at day-three [10]. ATP generation increased at four

hours, and then decreased after 24 hours [10] and remained low post high glucose exposure [10]. These results suggest that initial exposure to high glucose levels promotes the modulation of ATP and ROS levels around 24 hours. This may lead to an increase in antioxidant defense activity to compensate and decrease ROS levels. Eventually the antioxidant systems may become overburdened resulting in increased and persistent levels of intracellular ROS and decreased ATP generation.

In Müller glial cells exposed to high glucose conditions for seven days, the mitochondrial morphology was significantly disrupted compared to cells grown in normal medium [37]. Further, these cells exhibited a decrease in oxygen consumption rate and extracellular acidification (glycolysis) and an increase in mitochondrial membrane potential and cytochrome c release [37]. With chronic exposure, Müller cells produce DR-associated proteins leading to disease. Therefore, in a Müller cell model of DR, compromised mitochondrial respiration leads to DR.

In the Müller cell model system, it is currently unknown whether mitochondrial dysfunction or ROS production initiates the disease pathway. However, it is apparent that oxidative stress affects mitochondrial function in Müller cells in high glucose conditions. ROS accumulation correlates with a decrease in antioxidant defense systems [10]. Therefore, prolonged exposure to high glucose conditions leads to mitochondrial dysfunction and increased ROS. Excess ROS depletes the available antioxidants, leading to further increased ROS within Müller cells, and disease.

Antioxidant Defense Systems

Under normal physiological conditions, low levels of ROS act as signaling molecules that are kept in balance by intracellular antioxidant defense systems. In disease, oxidative stress increases and this balance is disrupted. The stability between ROS as signaling molecules and a surplus of ROS contributing to oxidative stress depends on the reduction potential of complexes of the mitochondrial ETC and the ability of antioxidant systems to remove ROS [2][34].

It is estimated that under physiological conditions 2% of the oxygen consumed in aerobic respiration is converted into ROS, specifically superoxide anion [3]. The primary enzymes that function to convert harmful ROS to less harmful species are superoxide dismutase (MnSOD and Cu/Zn SOD), glutathione peroxidase (GPx) and catalase (Figure 7) [2, 3, 25, 31].

In diabetic retinopathy, altered redox cycling has been correlated with excess ROS and depleted antioxidant defense mechanisms [21]. Therefore, absence of antioxidants directly contributes to ROS and oxidative stress in disease. Furthermore, during chronic hyperglycemia excess generation of ROS overwhelms the antioxidant pathways resulting in increased ROS and RNS formation and activation of oxidative stress pathways [21, 59].

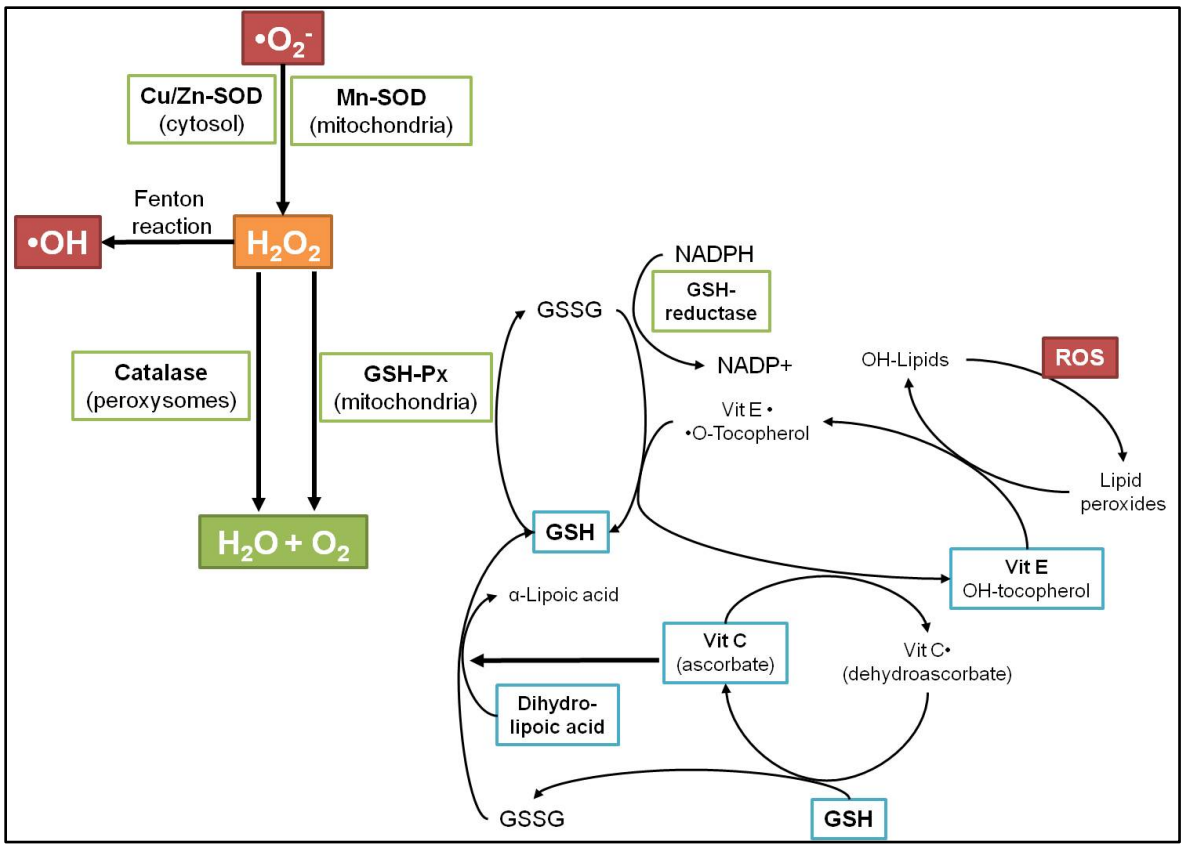

Figure 7: **Intracellular antioxidant mechanisms.** Mn-SOD and Cu/Zn-SOD convert superoxide to hydrogen peroxide. Glutathione peroxidase and catalase further reduce hydrogen peroxide to water and oxygen. Glutathione peroxidase uses a system to replenish reduced glutathione as a substrate. O_2^{-}: superoxide; SOD: superoxide dismutase; H_2O_2: hydrogen peroxide. Lazo-de-la-Vega-Monroy et al. 2013.

SOD is the first line of defense against superoxide. SOD is present in nearly all cells and converts $^{\bullet}O_2^{-}$ into H_2O_2 (Figure 7). As the hydrogen peroxide may still react with other ROS, degradation is necessary by either one of the other two antioxidant enzymes, GPx or catalase [10, 12].

GPx catalyzes the degradation of H_2O_2 by reduction, where two glutathione (GSH) molecules are oxidized to glutathione disulfide (GSSG). Regeneration of GSH by GSH-reductase, requires NADPH, which is oxidized to NADP+. Catalase, on the other hand, is localized primarily in peroxisomes, and so it detoxifies the hydrogen peroxide that diffuses from the mitochondria to the cytosol, converting it into water and molecular oxygen [10, 12].

Additionally, some nonenzymatic antioxidant mechanisms help regenerate GSSG back into GSH. Antioxidant vitamins including A, C, E and alpha-lipoic acid are among these mechanisms. Although all these antioxidant defenses work together to eliminate H_2O_2 (and thus superoxide) from the cell, in the presence of reduced transition metals (Cu, Fe), H_2O_2 can be transformed into $^{\bullet}OH$, which is a highly reactive ROS, by the Fenton reaction (Figure 7) [10, 23].

Glutathione

Reduced glutathione (GSH) is key antioxidant in Müller glial cells. The antioxidant capacity of GSH is the greatest within the cell [2]. GSH is thought to be the main contributor of redox regulation and has been suggested to be the primary ROS scavenger [3]. In the presence of ROS, GSH is oxidized to oxidized glutathione (GSSG) via glutathione peroxidase (GPx).

The redox pair, GSH/GSSG, is found in the cytoplasm and in the mitochondrial intermembrane space and matrix [3]. Mitochondria contain about 15% of total cellular GSH [2]. The ratio of GSH to GSSG within the cell is indicative of degree of oxidative stress [2] where low ratios are indicative of oxidative stress. GSSG influences ROS

accumulation within the mitochondria and promote the disruption of the mitochondrial membrane potential [2]. In addition, ROS influence the formation of the mitochondrial permeability transition pore by oxidizing GSH, causing its opening and the initiation of apoptosis [2]. Thus, during DR, a decrease in the GSH/GSSG ratio contributes to excess ROS and leads to oxidative stress, mitochondrial dysfunction, and disease.

There are multiple hypotheses as to why glutathione becomes depleted. One includes increase in neurotransmitter production with the purpose of detoxifying the rest of the retina [6]. This may outcompete for the precursors required for glutathione synthesis, therefore decreasing the amount of GSH in the cell and may contribute abnormal redox signaling and oxidative stress. Another hypothesis includes the modulation of GSH through the effects of high glucose on intracellular transcription factors responsible for the generation of antioxidants [50, 51]. One study found that high glucose affects the binding capabilities of NF-E2-related factor 2 (Nrf2), a transcription factor responsible for transcribing GSH [51]. When Nrf2 is bound, it cannot translocate into the nucleus to transcribe GSH. Therefore, DR conditions may affect the transcription of GSH, resulting in decreased antioxidant capacity, progression disease pathogenesis through increased oxidative stress. Lastly, glutathione may be depleted due to the burden of excess intracellular ROS. Reduced GSH may attempt to clear ROS but may be depleted more rapidly than it is being produced; resulting in more ROS production.

In a Müller cell model of DR, antioxidant levels are expected to decrease. However, levels of GSH within the cell have been variable in many models involving retinal degeneration, injury and disease [23]. Some studies report a decrease in GSH [19] as expected, while others report an increase in GSH [3, 23] which could be associated with upregulation of antioxidants to decrease oxidative stress.

As previously discussed, Müller cells exposed to hypoxic conditions for three days had increased levels of ROS [34]. However, with the addition of the antioxidant, ebselen, ROS levels decreased [34]. This suggests that the presence antioxidants may be enough to decrease disease prevalence.

In high glucose conditions, Müller cells exposed to tert-butylhydroquinone, a chemical that promotes oxidative stress, were found to increase GSH levels by 77% compared to control cells after 12 hours [19], which is unexpected. However, in the same study, Müller cells exposed to high glucose media for one week had decreased levels of GSH [19]. These observations suggest that under conditions of oxidative stress, GSH may be initially upregulated to clear excess ROS. Yet after chronic oxidative stress, GSH may be depleted resulting in more oxidative stress and disease progression.

Due to the variability of GSH concentrations in Müller cells, other components of the glutathione mechanism maybe altered in disease as well. Müller cells exposed to hypoxic conditions for three days had similar levels of GPx to those exposed to normal conditions [34]. In the same hypoxic conditions, the addition of ebselen unexpectedly resulted in

increased GPx levels. Additionally, GPx activity is upregulated when GSSG is increased [2]. However, a deficiency in GPx was found to be accompanied by oxidative stress, enhanced cell death, and increased angiogenic factors in the hypoxic retina [35]. This study suggests that GPx may play a role in the variability of GSH levels in Müller cells in high glucose conditions [34] and may be a compensatory mechanism for GSH redox cycling. Therefore, the variability of GSH levels seen in Müller cell may closely related to the regulation of the glutathione mechanism and the duration of hyperglycemic exposure. Thus, GSH levels are particularly important in the Müller cells of DR patients.

Treatment of Diabetic Retinopathy (Diabetic Macular Edema)

The primary cause of vision loss in diabetic patients with diabetic retinopathy is diabetic macular edema (DME). Alteration of the blood–retinal barrier (BRB) and inflammation are hallmark features in the pathogenesis of DR and DME [8, 18, 41, 42, 47]. Inflammation plays a crucial role with involvement of chemokines and cytokines including vascular endothelial growth factor (VEGF). VEGF is a potent cytokine and vaso-permeability factor that has been targeted in multiple, large clinical trials. Several anti-VEGF drugs are used as first line therapy in the treatment of diabetic macular edema (DME). These drugs are administered by injection in the vitreous humor on a monthly basis and have been shown to be effective in vision improvement and prevention of vision loss, However, many DME patients do not show complete response to anti-VEGF drugs despite multiple intravitreal injections with these drugs. Also, the effect seems to be transient in those responders, and many patients do not show complete resolution of fluid [30, 47, 61]. Other interventions for DR include non-pharmacologic therapies (laser photocoagulation,

viterectomy) and pharmacological therapies involving the intraocular injection of anti-angiogenic agents or corticosteroids that target the excessive vascular growth and inflammation, respectively [30, 41, 47]. All of the currently used intraocular interventions are invasive, expensive and only partially effective. A novel non-invasive therapeutic approach that targets retinal cell metabolism and inflammation directly would provide a considerable improvement over current therapeutic approaches.

Photobiomodulation

Photobiomodulation (PBM) by light in the far-red to near-infrared (NIR) range of the electromagnetic spectrum applied directly to damaged tissue, 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 models (Figure 8) [5, 16, 32]. PBM is a non-invasive, inexpensive and effective treatment modality for diseases resulting in retinal injury [5, 11, 16]. Recent studies by Tang et al. have demonstrated the efficacy of 670 nm PBM in four patients with macular edema. The long-term objective of our studies is to develop PBM as a stand-alone or adjunct therapy for the treatment of DME and other retinal diseases.

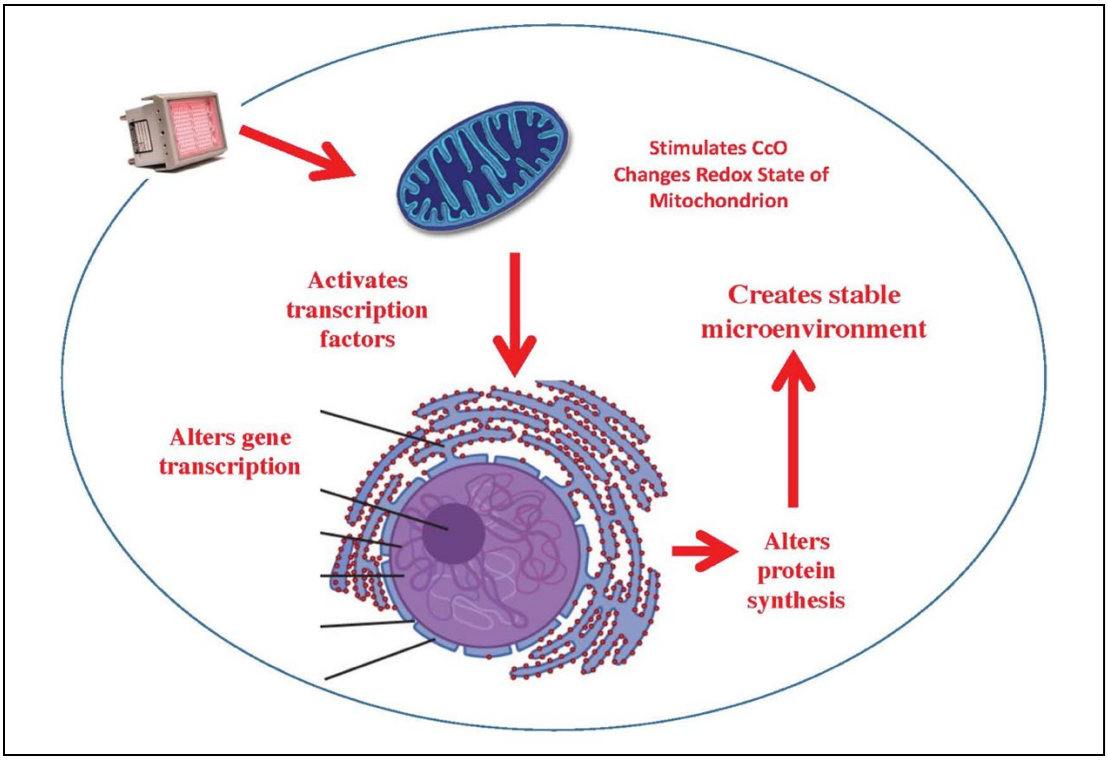

Figure 8: Hypothesized Mechanism of Acton of Photobiomodulation. Far-red and Near-infrared light target the mitochondria and activate the production cytoprotective molecules to create a stable intracellular microenvironment. Cco: cytochrome c oxidase

Far-red and NIR PBM have been investigated in several models of retinal disease. PBM has been shown to attenuate inflammation and oxidative stress and protect retinal function in experimental and clinical studies [28, 36]. Studies have shown that 670 nm light is absorbed by cytochrome-c-oxidase resulting in stimulation of mitochondrial bioenergetic function and increased ATP synthesis (Figure 8) [5, 11, 16, 28].

Sivapathasuntharam *et al.* (2017) demonstrated the effects of 670 nm light therapy on mitochondrial function in aging mice and found that increased retinal mitochondrial function correlated with improved vision. Cytochrome c oxidase levels were increased after 670 nm light exposure [32] compare to no light treatment. These findings suggest

that 670 nm light improved mitochondrial and retinal function and may be advantageous therapy for retinal disease.

Similarly, in cell culture and animal models of DR, 670 nm light therapy was beneficial resulting in decreased ROS production [28, 36] and slowed disease progression [11, 14, 16]. In a rat model of DR, Tang *et al.* (2013) found that 670 nm light reduced retinal ganglion cell death and improved retinal function. However, little is known about upstream mechanisms in the Müller cell model of DR. Therefore, our objectives are to evaluate how 670 nm light modulates early mechanisms in DR.

SUMMARY AND GAP IN KNOWLEDGE

Mitochondrial dysfunction and oxidative stress are primary contributors to the pathogenesis of diabetic retinopathy. Many studies have shown that that ROS increases in response to high glucose conditions [8, 46]. Similarly, the mitochondria become dysfunctional and antioxidants are depleted, furthering ROS production. ROS activates NFκB leading to increased VEGF and DR pathogenesis [8, 15,18, 26, 42, 46]. Dysfunctional mitochondria have been shown to activate pathologic and apoptotic signaling leading to decreased energy production and cell demise, potentiating DR symptoms. Previous studies indicate that PBM therapy may be beneficial to treat DR symptoms, however, the intracellular mechanisms that PBM modulates is not well understood.

Previous studies show that PBM is beneficial in improving mitochondrial function and decreasing oxidative stress, both by decreasing ROS and promoting antioxidant mechanism. Further, NFκB has been found to be decreased after treatment with 670 nm

light in Müller glial. These studies suggest that PBM may be beneficial in this pathogenic pathway induced by diabetes.

The importance of Müller cells in retinal disease is becoming increasingly understood. As retinal support cells, Müller cells are responsible for cell homeostasis and retinal maintenance. Currently, few studies are studying the PBM mechanisms on mitochondrial dysfunction, oxidative stress and glutathione status mitochondrial dysfunction on a DR Müller cell model. Therefore, more studies are necessary to understand the importance of Müller cell function and how light treatment modulate pathogenic signaling within the cell. The following studies were performed to assess PBM mechanistic modifications early in DR pathogenesis. We have chosen to examine various aspects of mitochondrial dysfunction and simultaneous redox balance via ROS and glutathione status in Müller glia.

HYPOTHESIS AND SPECIFIC AIMS

Diabetic retinopathy (DR) is the most common cause of visual impairment and blindness in the United States [9]. As a result of diabetes mellitus, excess glucose in the blood disrupts normal cellular mechanisms in the retina, leading to oxidative stress, inflammation and neovascularization [8, 15, 46]. In DR, abnormal cellular signaling has been suggested to be initiated by mitochondrial dysfunction and excess production of reactive oxygen species (ROS) [8, 10]. Excess ROS results in the activation of unregulated transcription of growth factors responsible for inflammation and angiogenesis [4, 8, 10]. Consequently, current therapies aim to prevent blood vessel

growth to avert the progression of DR by targeting the downstream molecule, vascular endothelial growth factor, responsible for angiogenesis. While these treatments slow disease progression, they are invasive, costly and can cause serious adverse effects [30]. Photobiomodulation (PBM) is a noninvasive and unique therapeutic treatment, which uses far-red to near-infrared light in the 630-1000 nm range of the light spectrum [5]. PBM has been found to act by multiple mechanisms and has been studied in a variety of animal and cellular models of retinal disease, including models of DR [5, 10, 11, 14, 16, 28]. In animal models of DR, PBM has been shown to attenuate inflammation and enhance retinal function [5]. However, upstream mechanisms by which PBM is able to decrease inflammation remain elusive. We *hypothesize that 670nm PBM will ameliorate mitochondrial dysfunction and improve redox status in a rat Müller cell model of diabetic retinopathy.*

Specific Aim 1) Determine the effects of 670 nm PBM on mitochondrial function in a cultured Müller cell model of DR. Our *working hypothesis* is that treatment with 670nm PBM will improve bioenergetics and increase ATP concentrations in Müller glial cells exposed to increased glucose concentrations.

Specific Aim 2) Determine the effects of photobiomodulation on antioxidant status in a cultured Müller cell model of DR. Our *working hypothesis* is that treatment with 670nm PBM will attenuate ROS production and increase intracellular concentrations of the reduced form of the antioxidant, glutathione, in Müller glial cells exposed to increased glucose concentrations.

Our goal is to better understand how PBM modulates disease and characterize this treatment paradigm as a potential early intervention to prevent DR progression.

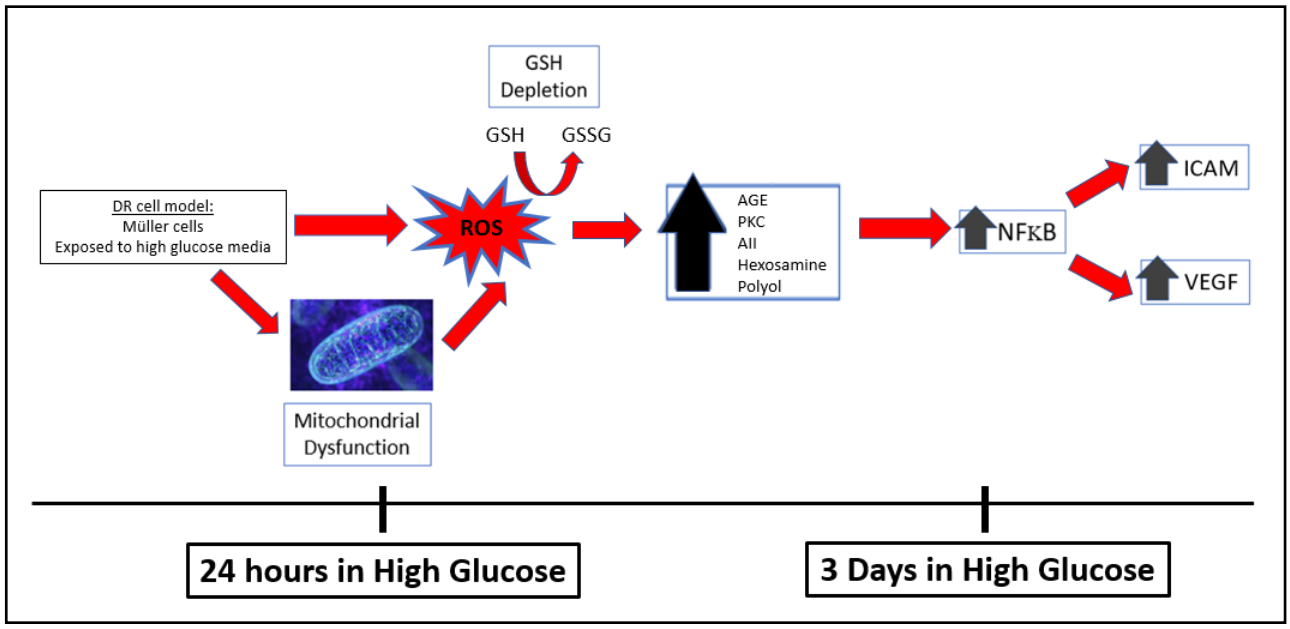

Figure 9**: Proposed timeline for Müller glial cells in high glucose conditions.** Previous studies in our lab show that NFκB, ICAM and VEGF are modulated after 3 days or 72 hours in high glucose conditions. However, upstream mechanisms have yet to be elucidated. ROS: reactive oxygen species; GSH: reduced glutathione; GSSG: oxidized glutathione; NFκB: Nuclear Factor κ beta; VEGF: vascular endothelial growth factor; ICAM: Intracellular adhesion molecule; AGEs: advanced glycosylated end products; PKC: protein kinase c; AII: angiotensin II

MATERIALS AND METHODS

Cell Line: Experiments were performed on rat Müller glial cells. Müller cells span 70% of the retina providing structural and metabolic support [4]. Müller cells have recently been shown to be a target for DR therapy due to their secretion of high levels of VEGF in DR conditions [8, 10, 46]. Previous studies have shown that Müller cells upregulate NFκB activity in 3 days under diabetic conditions [Fisher, 2015], thus making them an appropriate target for PBM. Müller cells were obtained from 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. 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. Light treatment consisted of 670 nm irradiation at an irradiance of 25 mW/cm^2 for 180 seconds resulting in a radiant exposure or dose of 4.5 J/cm^2 *[dose calculation: irradiance (W) x exposure time (sec) = radiant exposure J/cm^2 0.025 W/cm^2 x 180 sec = 4.5 J/cm^2].* Cells grown in normal glucose (5 mM) or high glucose (25 mM) medium were treated with 670nm light one time at 24 hours. Sham-treated cells were handled in a similar manner except that the LED array was not illuminated. All assays were conducted one hour following PBM-treatment. Studies in the Eells laboratory have demonstrated NFκB activity and transcriptional changes after three days in hyperglycemic conditions [Fischer, 2015]. We reasoned that high-glucose mediated mitochondrial dysfunction and oxidative stress were early events in this *in vitro* model of diabetic retinopathy. Therefore we examined the effects of high-glucose exposure and PBM after 24 hours in culture.

Cell Type	Conditions	Treatment
Rat Muller glial cells	Low (5mM)	No light (sham)
Rat Muller glial cells	Low (5mM)	670nm
Rat Muller glial cells	High (25mM)	No light (sham)
Rat Muller glial cells	High (25mM)	670nm

Table 1: Summary of treatment groups

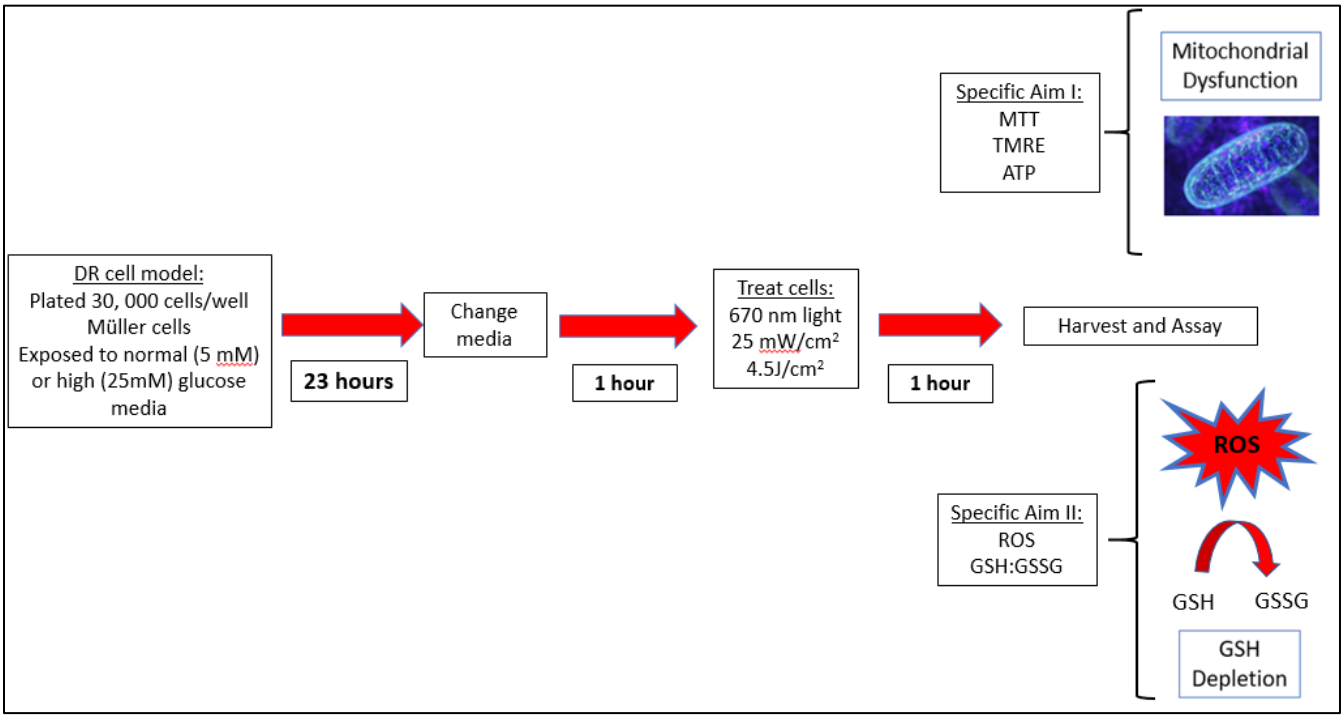

Figure 10: **Summary of Müller cell protocol.** Müller cells were plated at 30,000 cells per well in a 96 well plate, exposed to either 5mM or 25mM glucose conditions. After 23 hours, media was changed, and cells incubated for another hour. Müller cells were treated with 670 nm light for 180 seconds and allowed to incubate for one hour. Cells were harvested and assayed per individual protocol.

Assessment of metabolic activity as a measure of mitochondrial function

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay assesses metabolic activity indirectly by measuring the reduction of yellow tetrazolium

salt. MTT contains lipophilic side groups and is thus able to pass the cell membrane where it is reduced in viable cells by mitochondrial or cell plasma enzymes including oxidoreductases, dehydrogenases, oxidases, and peroxidases using NADH, NADPH, succinate, or pyruvate as electron donor. This results in a conversion of MTT to the water-insoluble formazan which has a purple color.

Retinal Müller glial cells were plated in triplicate in two 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. One plate was treated with 670 nm for 180 seconds. After one hour, media was removed, and cells were washed with 1x PBS. Then 100 µL of MTT and medium solution (5mg/mL MTT) was incubated for 3 hours at 37 ºC. The MTT solution was exchanged with an MTT solvent (10%Triton x100 and isopropanol with 0.1N HCl (final concentration) and was shaken for 15 mins in the dark. The MTT solvent was pipetted several times through the shaking process to allow proper cell lysis in sample wells. Absorbance was read at 590 nm.

Determination of mitochondrial membrane potential as a measure of mitochondrial function

Mitochondrial membrane potential (ΔΨm) was measured as a parameter of mitochondrial function in DR conditions using the TMRE Mitochondrial Membrane Potential Assay Kit (Abcam, Cambridge, MA) Tetramethylrhodamine ethyl ester (TMRE) is a positively charged molecule that permeates cells and accumulates in metabolically active mitochondria due to the highly negative membrane potential across the outer

mitochondrial membrane. Mitochondrial dysfunction is characterized by a reduction in the ΔΨm and is measured as a reduction in TMRE staining.

Müller glial 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. One plate was treated with 670 nm for 180 seconds at an intensity of 4.5 J/cm^2. After one hour, media was removed, and cells were washed with 1x PBS. A 200 nM TMRE solution was in respective glucose medium prepared from 1mM TMRE stock; 100 uL solution was added per well and incubated for 30 mins. The TMRE solution was removed and cells were washed with then read in 100uL PBS/0.2% BSA solution. Fluorescence was read via plate reader with EX/EM: 549/575 nm. FCCP (carbonyl cyaninde 4-(trifluoromethoxy) phenylhydrazone) at a concentration of 20uM was prepared from 50mM FCCP stock solution as a positive control. FCCP is an ionophore uncoupler of oxidative phosphorylation and destroys mitochondrial membrane integrity, disrupting membrane potential.

Assessment of intracellular ATP concentrations as a measure of mitochondrial function

We utilized ATP concentrations were measured as an additional index of mitochondrial bioenergetic function. ATP concentrations were determined using Promega CellTiter-Glo 2.0 Assay (Promega Inc., Madison, WI). Concentrations of phosphorylated adenosine nucleotides, including the universal energy carrier adenosine 5′-triphosphate (ATP) and its metabolites adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate

(AMP), define the energy state in living cells and are primarily dependent on mitochondrial function. ATP is the energy exchange factor that connects anabolism and catabolism. It is required for major reactions and processes that occur in living cells, including muscle contraction, phosphorylation and active transport. Luminescence provides a simple and effective tool for measuring ATP concentrations. This Promega ATP detection assay uses firefly luciferase to convert ATP and luciferin to oxyluciferin and light. The light emitted in this reaction is directly proportional to the concentration of ATP present. Using the ATP detection standard, quantitative measurement of cellular ATP content can be achieved with a dynamic range of 12 fmol to 10 pmole of ATP.

Müller glial 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. One plate was treated with 670 nm light for 180 seconds at an intensity of 4.5 J/cm^2. After 30 mins, plates were removed from 37 ^{o}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 2 mins to induce cell lysis and incubated at room temperature for 10 mins to establish the correct temperature gradient. Luminescence was recorded at a gain from 100-120. Sample values were compared to an ATP standard curve.

Assessment of ROS concentration in Müller cells

Intracellular production of ROS in the form of hydrogen peroxide (H_2O_2) was measured using the dichlorodihydrofluorescein diacetate (DCF-DA) method (Cayman Chemical Inc., Ann Arbor, MI) [44]. The DCF assay is a fluorescence assay that measures the concentration of hydrogen peroxide in the cell. This is done with the addition of a DCF-DA dye that fluoresces when oxidized into DCF by ROS (H_2O_2). Fluorescence was measured using a BioTek plate reader (BioTek, Inc., Winooski, VT) at an excitation wavelength of 475 nm and emission wavelength of 529nm.

Müller glial 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. One plate was treated with 670 nm for 180 seconds at an intensity of 4.5J/cm^2. Cells were washed with 1x PBS and a 40 uM DCFDA solution (in respective media) was overlaid immediately. DCFDA solution was allowed to incubate 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. A positive control of TBHP (tertbutyl hydrogen peroxide) (inducer of ROS production) was utilized to confirm assay results.

Measure of glutathione status in Müller cells

Glutathione (GSH) is an essential intracellular antioxidant. GSH and is thought to be the main contributor of intracellular redox regulation and a key ROS scavenger [3]. Intracellular concentrations of this critical antioxidant, glutathione were assessed using an enzyme recycling assay (Cayman Chemical Inc., Ann Arbor, MI). The GSH assay

employs the enzymatic recycling of the glutathione to quantify levels of GSH via glutathione reductase. DTNB (5,5'-dithio-*bis*-2-nitrobenzoic acid) reacts with the sulfhydryl group of GSH and produces the molecule TNB (5-thio-2-nitrobenzoic acid), producing a yellow color. The new molecule GSTNB is then reduced by glutathione reductase to again produce GSH and TNB. In this process, the rate of TNB production is proportional to the rate of the glutathione mechanism and concentration of GSH in our sample. The TNB molecule was measured at the absorbance of 405 nm. Utilizing the glutathione mechanism in this assay allows the measurement of both reduced and oxidized glutathione to reflect total glutathione in a ratio. The GSH assay was used to assess antioxidant status to quantify the ratio of reduced to oxidized glutathione in Müller cells after treatment with glucose and light, as described above. This colorimetric assay was performed on the four groups of cells and compared to standard curve. Cellular proteins will be precipitated out prior to analysis to ensure measurement of only glutathione.

Müller glial cells were plated in 6-well plates at a density of 1,000,000 cells/well in normal or high glucose conditions for 24 hours. At 24 hours, respective media were exchanged and incubated for one hour. One plate was treated with 670 nm for 180 seconds at an intensity of 4.5J/cm^2. After one hour, media was removed, and cells were washed with 1x PBS. Müller cells were scraped, collected and centrifuged for 10 min at 1000xg at 4 ^{o}C. Cell pellet was homogenized in 400uL of cold buffer (50mM phosphate buffer pH6-7 containing 1mM EDTA). Samples were sonicated on ice with an amplitude setting of 40% for 20 seconds, twice to lyse cells. Samples were then centrifuged at 10,000xg for 15 mins at 4 ^{o}C. The supernatant was deproteinated by adding 40uL of 10% W/V MPA

(metaphosphoric acid) to the sample and vortexed. Samples were incubated at room temperature for 5 mins and centrifuged at 2000xg for another 5 mins. After the supernatant was removed, 50uL of 4M TEAM (triethanolamine) reagent was added to each sample and vortexed. Half of each sample was removed and added to a new tube to assess both total glutathione and oxidized glutathione separately. A 1M 2-vinylpyridine (10.8uL 2-VP, 89.2uL of 100% ethanol) reagent was prepared and 10uL was added per 1 mL of sample. Samples with 2-VP measure oxidized glutathione. Incubated 2-VP samples at room temperature for 60 mins, while samples that do not contain 2-VP will incubate on ice for 60 mins. The assay cocktail (MES buffer, co-factor mixture, enzyme mixture, DI water and DTNB) was added to a 96 well plate with 150 uL/well. Then 50uL of sample was added to each well. The plate was covered and was shaken in the dark for 25 mins and read at an absorbance of 405 nm. A reduced glutathione (GSH) and oxidized glutathione (GSSG) standard curve was prepared. Buthionine sulfoximine (BSO) was used as a positive control where 0.1 mM was placed in cellular media for 20 hours to deplete glutathione levels to show our assay can measure changes in cellular GSH.

Determination of GPx activity

Glutathione peroxidase (GPx) activity will also be measured (Cayman Chemical Glutathione Peroxidase Assay). Previous studies indicate that a defect in GPx activity could result in oxidative stress and progression of DR [35]. Therefore, this assay was used to measure GPx activity in Müller cells after treatment with glucose and light. This assay measures GPx activity indirectly via the glutathione redox coupled reaction. After GPx catalyzes GSH to GSSG, it is recycled back to GSH by glutathione reductase and

oxidation of the cofactor, NADPH to $NADP^+$. The oxidation of NADPH to $NADP^+$ is measured at an absorbance of 340 nm and is related to GPx activities in the cell. Determining GPx activity describes the rate of GSH oxidization to GSSG in the presence of ROS where an increased rate is suggestive of oxidative stress. This colorimetric assay will be performed on the four treatment groups.

Müller glial cells were plated in 6 well plates at a density of 1,000,000 cells/well in normal or high glucose conditions for 24 hours. At 24 hours, respective medias were exchanged and incubated for one hour. One plate was treated with 670 nm for 180 seconds at an intensity of $4.5J/cm^2$. After one hour, media was removed, and cells were washed with 1x PBS. Cells were scraped and centrifuged for 10 mins at 2000xg at 4 ^oC. Sample pellets were homogenized in 400uL of cold buffer (50mM phosphate buffer pH6-7 containing 1mM EDTA) and centrifuged at 4 ^oC for 15 mins at 10,000xg. After the supernatant was collected 20uL of samples were added to a 96 well plate. An assay cocktail (Assay buffer, Co-substrate, NADPH) was added at a volume of 170uL total per well. Quickly before reading, 20uL of cumene hydroperoxide was added and shaken for several seconds. Absorbance was read kinetically every minute for 5 mins. A positive control of glutathione peroxidase was utilized. Data was collected as a function of absorbance per minute. GPx activity was determined by using the NADPH extinction coefficient of $0.00373\ uM^{-1}$. One unit is defined as the amount of enzyme that will oxidize of 1.0 nmol of NADPH to $NADP^+$ per minute at 25 C. GPx activity = (absorbance per min/$0.00373\ uM^{-1}$) x (0.19 mL/0.02 mL) = nmol/min/mL.

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.

Results

Specific Aim 1: Determine the effects of 670 nm PBM on mitochondrial function in a cultured Müller cell model of DR. Our working hypothesis is that treatment with 670nm PBM will improve mitochondrial function in Müller glial cells exposed to increased glucose concentrations.

Rationale: Retinal Müller glial cells are susceptible to hyperglycemia-induced injury leading to ROS production and NFκB modulation via multiple pathways [8, 18]**.** An early event in the pathology of diabetic retinopathy is the disruption of mitochondrial function leading to downstream signaling culminating in disease. Previous studies in the Eells' laboratory have shown that NFκB activity increases in Müller cells after exposure to high glucose media for 3 days [Fisher, 2015]. Treatment with 670 nm light decreases NFκB activity after 3 days of high glucose exposure, suggesting prevention of DR progression [Fisher, 2015]. Current treatments aim to block VEGF. However, these changes are seen late in disease pathogenesis and, early mechanisms of disease remain to be elucidated.

Investigations into the mechanisms of PBM have shown that FR/NIR photons are absorbed by the mitochondrial photoacceptor molecule, cytochrome c oxidase, triggering

intracellular signaling pathways that culminate in improved mitochondrial energy metabolism, increased cytoprotective factor production, and cell survival [5, 11, 16, 28, 32]. Studies in cultured retinal ganglion cells (RGC5), RPE (ARPE 19) and photoreceptors (661W) have shown that 4 days of exposure to elevated glucose concentrations increased superoxide production, inflammatory biomarker expression, and cell death. 670 nm light (5 J/cm2) administered 2 times per day for 4 days inhibited all of these abnormalities. The studies in our lab have focused specifically on Müller glial cells and investigated the effect of 24 hours of elevated glucose exposure and a single treatment with 670 nm light. We assessed mitochondrial function by measuring metabolic activity, mitochondrial membrane potential and the intracellular concentration of ATP. Because NFκB activity is modulated after 3 days of high glucose exposure, we propose that mitochondrial dysfunction will occur earlier in the disease pathway. Müller cells were exposed to high glucose for 24 hours before treating with 670 nm light and assessment for metabolic bioenergetics. Mitochondrial function was indirectly measured utilizing the MTT assay, TMRE assay, and intracellular concentration of ATP to determine metabolic activity, mitochondrial membrane potential and oxidative phosphorylation as key components of mitochondrial function.

Effects of photobiomodulation on mitochondrial function in a Müller retinal glial cells

A. 670 nm PBM Restores NAPDH-Dependent Oxidoreductase Activity in Müller Glial Cells Under High Glucose Conditions.

The MTT assay is a colorimetric assay for measuring cell metabolic activity. It is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent

cellular oxidoreductase enzymes to reduce the tetrazolium dye, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to its insoluble formazan, which has a purple color. The MTT assay is dependent on mitochondrial respiration and indirectly serves to assess the cellular energy capacity of a cell.

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 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.

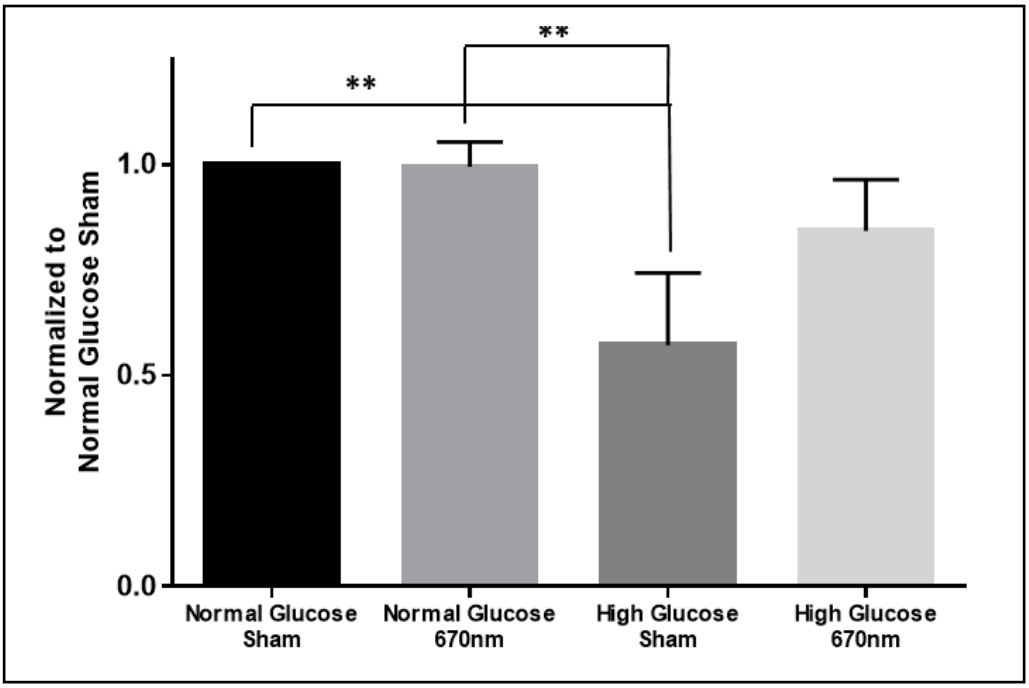

Figure 11: **670 nm PBM Restores NAPDH-Dependent Oxidoreductase Activity in Müller Glial Cells under High Glucose Conditions**. Müller Glial cells were cultured in either normal glucose or high glucose medium for 24 hours. 670nm or sham control was applied to the cells at 4.5 J/cm^2. All assays were normalized to the normal glucose sham levels. NAPDH-dependent oxidoreductase activity was analyzed using MTT, n=3. ** $p<0.01$.

B. 670 nm PBM Restores Mitochondrial Membrane Potential in Müller Glial Cells Under High Glucose Conditions.

Mitochondrial membrane potential is the driving force for electron transport essential for the maintenance of mitochondrial membrane integrity [17, 25]. As the membrane potential drops, the ETC slows, resulting in decreased ATP production and increased ROS generation. A loss of integrity causes disrupted morphology and function and the activation of apoptotic signaling leading to severe DR. Mitochondrial membrane potential was assessed indirectly via TMRE assay to determine one aspect of mitochondrial

function. As mitochondrial membrane potential is lost, TMRE values will decrease. In our study, Müller cells were cultured in either normal or high glucose conditions for 24 hours and treated with or without 670 nm light. Müller cells in high glucose conditions showed significantly disrupted mitochondrial membrane potential, shown by a 50% decrease in TMRE values, compared to the normal glucose sham ($n=3$, $p<0.0001$), suggesting decreased mitochondrial function in diabetic conditions after 24 hours.

Müller 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 ($n=3$).

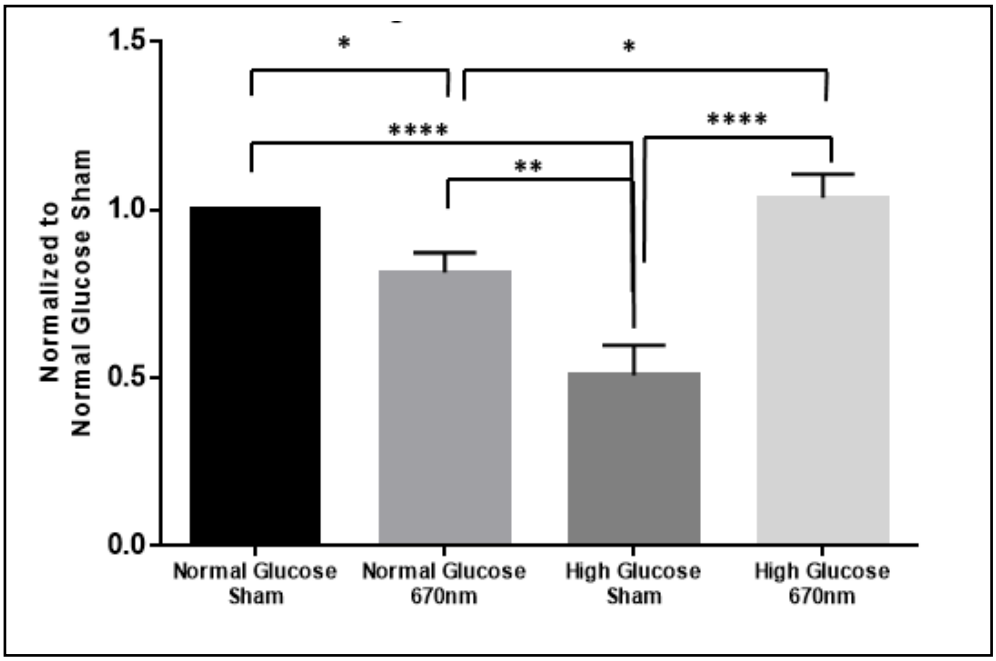

Figure 12: **670 nm PBM Restores Mitochondrial Membrane Potential in Müller Glial Cells Under High Glucose Conditions**. Müller Glial cells were cultured in either normal glucose or high glucose medium for 24 hours. 670nm or sham control was applied to the cells at 4.5 J/cm^2. All assays were normalized to the normal glucose sham levels. Mitochondrial membrane potential was analyzed using TMRE. n=3, done in triplicate. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

C. 670 nm PBM Restores ATP concentrations in Müller Glial Cells Under High Glucose Conditions.

Another key biomarker of mitochondrial bioenergetics is ATP production. Müller cells require an abundant amount of energy to support other cells in the retina and to maintain homeostasis. Over time, hyperglycemic conditions induce mitochondrial dysfunction resulting in increased ROS production and decreased ATP synthesis. Müller cells were exposed to normal or high glucose conditions for 24 hours and intracellular ATP concentrations were determined using a sensitive luminescence-based assay. In high glucose conditions, ATP concentrations were decreased compared to the normal glucose

sham conditions, as expected (n=2). After 24 hours and treatment with 670 nm, Müller cells in high glucose media showed increased ATP concentrations similar to cells grown in normal medium (n=2, p=0.089).

As expected, ATP concentrations in Müller cells cultured in normal glucose concentrations and treated with 670 nm light did were not different from normal glucose controls (n=2).

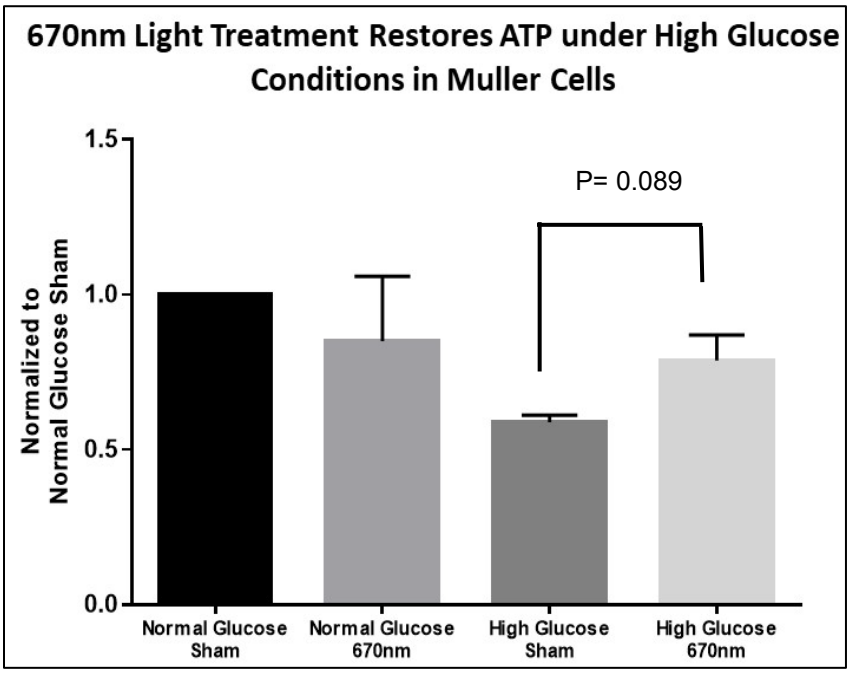

Figure 13: **670 nm PBM Restores ATP Concentrations in Müller Glial Cells Under High Glucose Conditions**. Müller Glial cells were cultured in either normal glucose or high glucose medium for 24 hours. 670nm or sham control was applied to the cells at 4.5 J/cm^2. All assays were normalized to the normal glucose sham levels. ATP concentrations were analyzed using CellTiter-Glo (Promega) assay, n=2), performed in duplicate.

Effects of Photobiomodulation on Antioxidant Status in a Müller cell model

Specific Aim 2) Determine the effects of photobiomodulation on antioxidant status in a cultured Müller cell model of DR. Our *working hypothesis* is that treatment with 670nm PBM will attenuate ROS production and increase intracellular concentrations of the reduced form of the antioxidant, glutathione, in Müller glial cells exposed to increased glucose concentrations.

Rationale: The retina has a high need for antioxidant protection due to constant light exposure, high oxygen consumption by photoreceptor cells and high concentrations of polyunsaturated fatty acids in the outer segments [48] In addition, the outer retina experiences circadian periods of hypoxia (dark) and hyperoxia (light) that add to retinal oxidative stress [48]. Müller glial cells produce the key antioxidant, glutathione. Glutathione is primarily responsible for maintenance of redox balance in the retina [2,3]. Glutathione status is generally expressed as the ratio of reduced GSH to oxidized glutathione (GSSG). An increased GSH:GSSG ratio reflects greater concentrations of reduced GSH than oxidized GSSG indicative of an abundance of reducing power. Under hyperglycemic conditions, mitochondrial stress and DR-related signaling produce significant amounts of ROS, leading to oxidative stress in Müller cells. Under physiological conditions, Müller cells have abundant concentrations of reduced glutathione (GSH) to detoxify excess ROS that is produced. GSH is utilized as a substrate in a redox mechanism that reduces hydrogen peroxide into water via glutathione peroxidase (GPx). In this process oxidized glutathione (GSSG) is formed. This reaction is coupled when GSSG is further reduced by glutathione reductase (GR) back to GSH. The ratio of intracellular GSH/GSSG is indicative of levels of oxidative stress present within the cell

[2]. Antioxidant concentrations of GSH are significantly depleted in the diabetic retina due the overburden of ROS surplus. Thus, during DR, a decrease in the GSH/GSSG ratio contributes to excess ROS and leads to oxidative stress and disease progression. Previous studies have indicated that GPx activity is modulated in hyperglycemic conditions leading to dysregulated redox signaling and oxidative stress [35]. Similarly, studies indicated that retinal cells exposed to high glucose conditions had depleted intracellular antioxidant levels. However, 670 nm light treatment was found to increase antioxidant levels within these cells [10]. Because the glutathione mechanism is the main contributor to redox regulation, we measured ROS and glutathione levels in Müller cells. To measure ROS, we used the Abcam DCFDA assay. To measure components of the glutathione redox mechanism, used the Cayman Glutathione Assay to measure the GSH/GSSG ratio and the Cayman Glutathione Peroxidase activity (GPx) assay to measure redox enzyme activity.

D. 670 nm PBM decreases ROS production in Müller glial cells under high glucose conditions

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

PBM has been shown to attenuate oxidative stress in many disease models, including DR [5, 11, 16, 28, 36, 38]. 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. Ours is the first study to test the effects of a single treatment with 670 nm PBM in light for 180 seconds 24 hours after exposure in

their respective glucose conditions. Müller glial cells acutely exposed to high glucose conditions.

Müller cells exposed to high glucose produced a 50% greater concentrations of ROS (measured as H_2O_2 by this assay) 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 thos measured under normal glucose conditions.) ($n=3$, $p<0.0001$). Müller cells exposed to normal glucose conditions were not anticipated to be modulated by PBM due to lack of stress applied. As expected, 670 nm light did not affect the amount of ROS generated intracellularly ($n=3$).

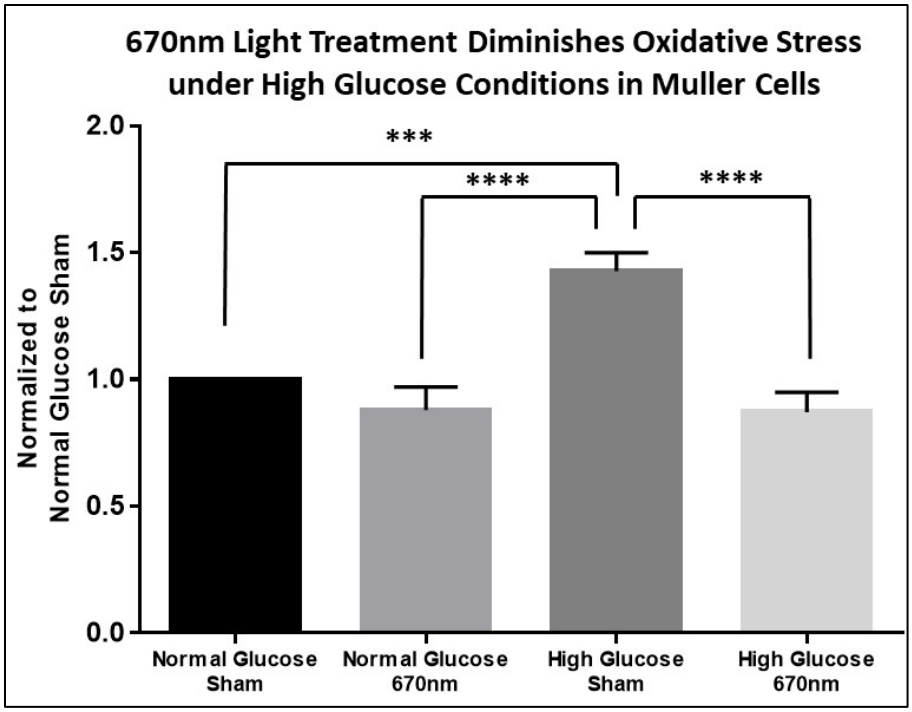

Figure 14: 670 nm PBM Attenuates ROS Production in Müller Glial Cells Under High Glucose Conditions. Müller cells were cultured in either normal glucose or high glucose medium for 24 hours. 670nm or sham control was applied to the cells at 4.5 J/cm^2. Oxidative stress was analyzed using DCF-DA. *** $p<0.001$, **** $p<0.0001$. n=3, done in triplicate

E. Glutathione concentrations in Müller glial cells

Total glutathione, reduced glutathione (GSH) and oxidized glutathione (GSSG) concentrations were determined using a sensitive enzyme recycling assay (Cayman Chemical, Ann Arbor, MI) first reported by Tietze [60]. In this assay, GSH is oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) resulting in the formation of GSSG and 5-thio-2-nitrobenzoic acid (TNB). GSSG is then reduced to GSH by glutathione reductase (GR) using reducing equivalent provided by NADPH. The rate of TNB formation is proportional

to the sum of GSH and GSSG present in the sample and is determined by measuring the formation of TNB at 414 nm.

We experienced difficulty in obtaining reproducible results using this assay. Table 2 shows the results from 3 experiments. The extreme range of total GSH from 3-13 μM between experiments made it impossible to reliably determine the GSH/GSSG ratio.

Muller Glial Cell Glutathione Analysis						
Assay #1						
Treatment	***Total GSH***	***GSSG***	***GSH***	***GSH/GSSG***	***GSH/Total***	***GSH % Control***
	mM	*mM*	*mM*	*mM*		
NG Sham	9.64	1.92	7.72	4.01	80%	100%
NG PBM	6.24	1.24	4.99	4.03		64%
HG Sham	7.19	0.91	6.28	6.85		81%
HG PBM	8.57	1.79	6.78	3.78		87%
Assay #2						
Treatment	***Total GSH***	***GSSG***	***GSH***	***GSH/GSSG***		
	mM	*mM*	*mM*	*mM*		
NG Sham	2.89	0.75	2.14	2.85	74%	100%
NG PBM	3.59	1.23	2.35	1.9		109%
HG Sham	9.77	1.66	8.11	4.87		370%
HG PBM	11.22	3.87	7.34	1.89		342%
Assay #3						
Treatment	***Total GSH***	***GSSG***	***GSH***	***GSH/GSSG***		
	mM	*mM*	*mM*	*mM*		
NG Sham	11.43	1.21	10.22	8.43	89%	100%
NG PBM	10.01	0.77	9.23	11.82		90%
HG Sham	7.43	0.38	7.05	18.66		68%
HG PBM	13.4	0.85	12.54	14.58		122%

Table 2: Analysis of Glutathione Concentrations in retinal Müller glial cells

F. **Glutathione Peroxidase Activity in retinal *Müller* cells**

The conversion of GSH to GSSG is dependent on the activity of GPx. The activity of this enzyme determines how quickly this reaction will occur. GSSG is recycled to GSH by glutathione reductase and NADPH as a cofactor. This is a kinetic assay, where GPx is the rate limiting step and NADPH to $NADP^+$ is absorbed at 340nm, directly measuring GPx activity.

We observed difficulties in the analysis of GPx activity. In our samples, GPx activity did not differ from the background controls (Background:0.0158, Normal Glucose Sham: 0.0131, Normal Glucose PBM:0.0131, High Glucose Sham:0.0139, High Glucose PBM:0.0135). We plated various densities of cells per plate ending with the same result. A positive control using glutathione peroxidase was used to assess the validity of the assay.

Glutathione Peroxidase Analysis					
	Time	NG S	NG P	HG S	HG P
A	1	0.80933	0.805	0.81233	0.814
p19	2	0.79433	0.78933	0.79633	0.797
	3	0.78133	0.77633	0.783	0.78433
	4	0.76933	0.764	0.76967	0.77167
	5	0.75633	0.752	0.75633	0.759
B		NG S	NG P	HG S	HG P
p21	1	0.815	0.81367	0.812	0.807
	2	0.79933	0.798	0.796	0.791
	3	0.78667	0.78367	0.78233	0.77667
	4	0.77367	0.76967	0.76867	0.764
	5	0.761	0.75667	0.75567	0.75133
C		NG S	NG P	HG S	HG P
p19	1	0.80333	0.812	0.80833	0.80967
	2	0.78733	0.79533	0.792	0.79367
	3	0.775	0.78167	0.77833	0.78033
	4	0.76233	0.76767	0.76567	0.76767
	5	0.74967	0.75433	0.75267	0.75967
	Background (mins)			Pos cont	
	1	0.871		1	0.523
	2	0.85367		2	0.39833
	3	0.838		3	0.27533
	4	0.82267		4	0.16133
	5	0.80767		5	0.12633

Table 3: Analysis of Glutathione Peroxidase Activity in retinal Müller cells

DISCUSSION

The objective of the present study was to test the hypothesis that treatment with 670 nm PBM will improve mitochondrial function and attenuate oxidative stress in an established *in vitro* model of diabetic retinopathy. Our studies showed for first time that as little as 24 hours of exposure to high glucose is enough to disrupt mitochondrial function and increase ROS production in Müller glial cells. We also showed for the first time that a single treatment with 670 nm light attenuates the cytotoxic actions of high glucose exposure within one hour. We conclude that this *in vitro* model of diabetic retinopathy has considerable potential for use in the development and testing of therapeutic agents to treat diabetic retinopathy. Our model can assess disruptions in metabolic activity beginning with ROS generation and mitochondrial redox changes and culminating in increased production of angiogenic and inflammatory mediators that are characteristics of diabetic retinopathy.

A. Comparison of our investigations in the retinal Müller glial cell model of diabetic retinopathy with those in the literature.

A number of studies have examined the effects of high glucose and other cell stressors including hydrogen peroxide and hypoxia on mitochondrial function and redox balance in cultured cells. Table 3 compares our findings to those of other studies in retinal Müller glial cells and in other cell types.

REFERENCE	MODEL	INSULT	TREATMENT	RESULTS	PBM EFFECTS
Alex Hall Thesis	Rat Müller glial cell line (rMC1)	5mM glucose 25mM glucose 24 hours	670 nm PBM (4.5 J/cm^2) 180 sec treatment Assays 1-hour post PBM	Decreased mitochondrial metabolic activity Decreased ΔΨm Decreased ATP Increased H_2O_2 Increased GSH/GSSG	Increased mitochondrial metabolic activity Restored ΔΨm Increased ATP Decreased H_2O_2 Decreased GSH/GSSG
[36] Tang *et al.* 2013	Rat Müller glial cell line (rMC1) RGC(RGC5) PR (661W) RPE(ARPE19)	5mM glucose 30mM glucose 7 days	670 nm PBM (10 J/cm^2) 2x/day-4days	Increased superoxide Increased oxidative stress Increased cell death	Decreased superoxide Decreased oxidative stress Decreased cell death
[53] Zungu *et al*. 2019	*Fibroblasts*	Hypoxia and acidosis 4 and 5 days	633 nm PBM (5 J/cm^2) 1 and 4 days Assays 1 & 24 hours post PBM	Decreased ΔΨm Decreased ATP	Increased ΔΨm Increased ATP
[10] Devi *et al.* 2017	Rat Müller glial cell line (rMC1)	5.5 mM glucose 25 mM glucose 2 hours to 5 days	None	Decreased ΔΨm Increased Superoxide Increased H_2O_2 Decreased ATP Increased mitophagy	NA
[37] Tien *et al.*	Rat Müller glial cell line (rMC1)	5mM Glucose 30mM Glucose 4 days	None	Decreased ΔΨm Decreased oxphos & glycolysis Increased mito fragmentation Increased apoptosis	NA
[19] Lu *et al.* 1999	Rat Müller glial cell line (rMC1)	TBH 12 hours	None	Increased GSH 77%	NA
[19] Lu *et al.* 1999	Rat Müller glial cell line (rMC1)	5.5 mM glucose 28mM glucose 1 week	None	Decreased GSH 30%	NA
[57] Zhang *et al.* 2018	Human Müller cell line (MIO-M1) Primary Human Müller cells (HuPMC)	100μM hydrogen peroxide 4 hours	None	Decreased ΔΨm Increased H_2O_2 Decreased NADPH/NADP Increased HSP60 Decreased GSH	NA

Table 4. *Effect of high-glucose and other stressors on mitochondrial function and redox status in cultured cells with or without PBM.*

Tien et al (2017) conducted a comprehensive investigation of the effects of hyperglycemia on retinal Müller cell function. The purpose of their study was to determine if Müller glial

cell loss reported in diabetic retinopathy [48] is attributable to mitochondrial dysfunction by investigating the effects of high glucose on mitochondrial morphology; mitochondrial membrane potential, cellular bioenergetics and cytochrome c release. For these studies rat Müller glial cells (rMC-1) were grown in normal or high glucose medium for 7 days. Their studies showed that cells grown in high glucose for 7 days exhibited significantly increased mitochondrial fragmentation compared to those grown in normal glucose medium. Mitochondrial membrane potential was altered and both oxidative phosphorylation and glycolysis were inhibited under 7 days of high glucose conditions. Cells exposed to high glucose also exhibited a significant increase in cytochrome c release and apoptosis. The authors concluded that high glucose-induced mitochondrial morphology changes and subsequent mitochondrial dysfunction may contribute to retinal Müller cell loss associated with diabetic retinopathy [37].

Our data are among the first to show that an acute exposure (24 hours or less) to high glucose conditions induces oxidative stress and mitochondrial dysfunction. We measured a 50% increase in hydrogen peroxide generation in high glucose conditions. Our data further show that mitochondrial metabolic activity (mitochondrial oxidoreductase activity) was decreased by 40% and mitochondrial membrane potential was decreased by 50% in high glucose conditions after 24 hours compared to normal glucose conditions, indicating that oxidative stress and disrupted mitochondrial function are early responses to hyperglycemic conditions. Similarly, Zhang *et al.* (2018) showed that a mild oxidative stress (100 μM hydrogen peroxide) 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) [57]. Thus, acute exposure to either high glucose or to oxidative stress increases ROS generation and disrupts mitochondrial function.

We further showed that ATP concentrations were reduced nearly 40% by exposure to high glucose. These findings are not statistically significant and preliminary at this time. It will be necessary to repeat the ATP assay in future studies to confirm these observations.

Under normal conditions the proton pumping activity of complexes I, III and IV of the electron transport chain generate the mitochondrial membrane potential. Together with the proton gradient, mitochondrial membrane potential forms the transmembrane potential of hydrogen ions which is harnessed to make ATP [3, 42, 47]. Disruption of the mitochondrial membrane results in a dysregulated proton gradient affecting electron transport and increasing ROS production via ETC complexes. Few studies demonstrate a change in ATP production in high glucose after a short incubation period. Our preliminary data are indictive of a reduction in ATP concentrations following 24 hours of high glucose. Similarly, Devi *et al.* (2010) demonstrates an intracellular ATP decrease of 20% in high glucose conditions (25 mM) compared to normal controls (5.5 mM) [10].

These combined data suggest that after stress insult, mitochondrial function decreases from 6 to 24 hours. After 1 week, mitochondrial further decreases. With exposure to high glucose conditions for 24 hours, cellular stress is further reflected via decreased mitochondrial function, and after 1 week of exposure to high glucose, mitochondrial function is severely disrupted functioning at a quarter of its capacity.

Our studies evaluated the acute effects of high glucose exposure in retinal Müller cells whereas the majority of studies examining the cytotoxic actions of high glucose in retinal Müller cells have measured chronic effects manifested 4-7 days after exposure to high glucose. The findings of the chronic exposure studies have provided evidence for sustained increases in ROS production, disruption of mitochondrial bioenergetics, mitochondrial fragmentation and cell death.

We measured ROS concentrations after 24 hours in high glucose conditions. We found that in as little as 24 hours, intracellular ROS increases in Müller cells when exposed to glucose ($n=3$, $p<0.001$). We measured a 50% increase in ROS concentrations post-high glucose exposure; indicating that ROS generation and mitochondrial dysfunction are prominent and early components DR pathology. Studies in human Müller cells, Zhang *et al.* (2018) showed that ROS increases by 40% after 6 hours of exposure to 100uM TBHP [10]. TBHP exposure simulates mild oxidative stress conditions; mimicking DR-like characteristics. Further, Tang *et al.* (2013) shows that after 4 days in high glucose (30 mM) conditions, superoxide increases by 101% and 119% in RGC and RPE cells, respectively, compared to normal glucose controls (5 mM) ($p<0.05$) [36]. These combined results suggest that from 6 hours of stress to 4 days, ROS is continually increasing. However, to our knowledge, this is the first study to show that ROS increases significantly in Müller cells after 24 hours of exposure to 25 mM glucose conditions.

The retina has a high need for antioxidant protection due to constant light exposure, high oxygen consumption by photoreceptor cells and high concentrations of polyunsaturated fatty acids in the outer segments [48]. In addition, the outer retina experiences circadian

periods of hypoxia (dark) and hyperoxia (light) that add to retinal oxidative stress [48]. Müller glial cells produce the key antioxidant, glutathione. Glutathione is primarily responsible for maintenance of redox balance in the retina [2, 3]. Glutathione status is generally expressed as the ratio of reduced GSH to oxidized glutathione (GSSG). An increased GSH:GSSG ratio reflects greater concentrations of reduced GSH than oxidized GSSG indicative of an abundance of reducing power. Total glutathione, GSH and GSSG concentrations were determined using a sensitive enzyme recycling assay (Cayman Chemical, Ann Arbor, MI) first reported by Tietze [60].

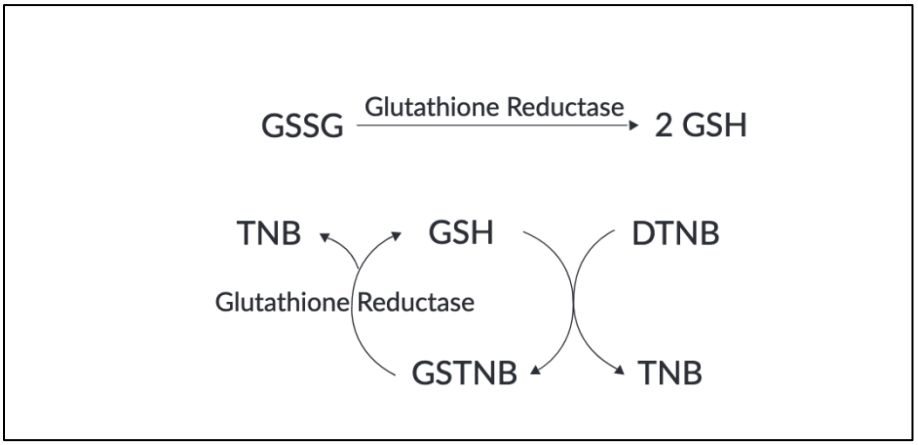

Figure 15: Mechanism of Cayman Chemical Glutathione Recycling Assay. Tietze, 1969

We hypothesized that reduced GSH would be oxidized the GSSG under high glucose conditions and that 670 nm light treatment would attenuate this oxidation. However, we experienced difficulty with this assay and our results showed too much irregularity to be interpreted. Because GSH is such a sensitive molecule, it is easily oxidized to the GSSG form. During experiment, there is much manipulation and time required to measure GSH and may therefore be oxidized or degraded. Additionally, some samples were frozen mid-

experiment. The thawing process during experiment may have also contributed to the degradation of GSH.

Other studies have demonstrated that the GSH:GSSG ratio in DR pathogenesis may be variable [3, 10, 19, 23, 55, 56, 57]. Lu *et al.* (1999) exhibits similar findings to our data where GSH was increased in cultured Müller cells under the effects of mild oxidative stress induced by butylhydroquinone [19]. After 12 hours, GSH was increased by 77% compared to normal conditions. In the same study, Müller cells were exposed to 28 mM high glucose conditions for 1 week. Müller cells in these high glucose conditions showed a decrease in GSH by 30% compared to cells grown in 5.5 mM glucose conditions [19]. These results together are in agreement with data shown in the present study. GSH may increase initially as a normal survival mechanism with the introduction of early oxidative stress after 24 hours. However, after continued stress, GSH becomes depleted due to the burden of ROS [6, 19, 51].

B. Effect of 670 nm Photobiomodulation in the retinal Müller cell Model of DR.

Novel to the present study, the mechanism of action of PBM utilizing 670 nm light in Müller cells was studied after 24 hours under high glucose conditions. One-hour post-PBM treatment, cells were assessed for mitochondrial function and ROS production. We found that after treatment with 670 nm light, mitochondrial metabolic activity post exposure to high glucose increased by 20%, however, this increase was not found to be significantly different from Müller cells grown in high glucose conditions alone (n=3). With a p value of 0.09, it may be possible that if additional sample groups were added, this difference may become significant. Additionally, one of the N of 3 shows that in high glucose conditions did not decrease as much as our other samples, providing the large error bar found in our

results. Similarly, the increase after treatment with light did not show as large of an increase. Therefore, sample size may contribute to these results. However, with our current data, it is important to recognize that our findings show that just one treatment with 670 nm light has a beneficial effect on DR-affected mitochondria in Müller cells with only a 15% decrease from normal conditions.

Further studies suggest that multiple light treatments may be necessary to reach the full potential of PBM and therefore, significance.

In the current study, when Müller cells in high glucose were treated one time with 670 nm light at 24 hours, mitochondrial membrane potential was completely restored to values seen in normal glucose sham conditions ($n=3$, $p<0.0001$). These results show that high glucose damages Müller cells via disrupted mitochondrial membrane potential after a very short incubation period. However, one treatment with light was able to significantly improve the mitochondrial membrane potential.

Interestingly, Müller cells in normal glucose conditions and treated with 670 nm light showed a 20% decrease in mitochondrial membrane potential values from cells grown in the normal glucose sham conditions; suggesting that PBM disrupts mitochondria in normal conditions ($n=3$, $p<0.05$). Although unexpected, this could possibly be a result of one assay among the three that showed a larger decrease than the others. Similarly, in the context of all mitochondrial assays, mitochondrial function does not seem to be different in normal glucose with PBM. Because Müller cells have a high degree of metabolic activity, these cells may be particularly responsive to light. However, it is important to note that Müller cells grown in this normal glucose, light treated conditions still had significantly more functional mitochondria than cells in the high glucose disease

conditions, as determined by the difference in mitochondrial membrane potential (n=3, $p<0.01$).

Few studies have demonstrated changes in mitochondrial membrane potential in a DR-model after 24 hours of glucose exposure. However, Zungu *et al.* (2008) shows that fibroblasts suffer a disrupted mitochondrial membrane potential after exposure to hypoxic and acidic conditions (95% N_2 and 5% O_2, pH 6.7) by 15% after 4 days. However, with treatments on day 1 and 4 (2 total treatments), 632.8 nm helium-neon laser (3mW/cm^2, 5J/cm^2), mitochondrial membrane potential was significantly restored by an 10% and 18% change from stressed conditions.

In our study, one light treatment with 670 nm light intervened in this DR pathway and manipulated ATP levels, increasing trends to concentrations to those seen in the normal glucose sham conditions. Increased levels of ATP due to light treatment is also suggestive of improved ETC function. As a result of light, electron transfer is restored promoting mitochondrial membrane potential and therefore, metabolic activity. Similarly, Tang *et al.* showed that retinal superoxide increased by 50% in RGC cells after 4-day exposure to 30 mM glucose conditions [36]. After 4 days of high glucose exposure and 8 total light treatments (670 nm, 25mw/cm^2, 5J/cm^2), ROS levels decreased by 50% with a p value <0.05, showing concentrations similar to the normal glucose sham. Our combined results suggest that ROS increases at 24 hours and stays elevated through day 4 [36]. Therefore, treatment with light is shown to decrease ROS to normal levels early in disease pathogenesis as well as 4 days after high glucose insult.

Novel to the study, our results suggest that just one treatment using 670 nm light modulates our DR-signaling pathway through the improvement of mitochondrial function and decreasing oxidative stress at the short time period of 24 hours post high glucose treatment ($n=3$, $p<0.001$).

PBM treatment reversed these effects in our study and other DR models [36]. Therefore, we conclude that PBM is a beneficial treatment to prevent these early factors in DR pathogenesis, and novel to our study, we show that PBM may have the potential to be utilized as an early intervention in DR pathogenesis.

We experienced practical difficulties in the analysis of GSH and GPx activity in this study. Both of these assays may require a higher density of cells to distinguish between our different groups. Future studies will need to be conducted to resolve this issue.

Summary, Conclusions and Clinical Significance.

In summary we provide evidence that a single-treatment with 670 nm light rapidly attenuates high glucose-induced mitochondrial dysfunction and oxidative stress at 24 hours; culminating in inhibition of NFκB activation and a reduction in the production of angiogenic and pro-inflammatory mediators in a cellular model of diabetic retinopathy.

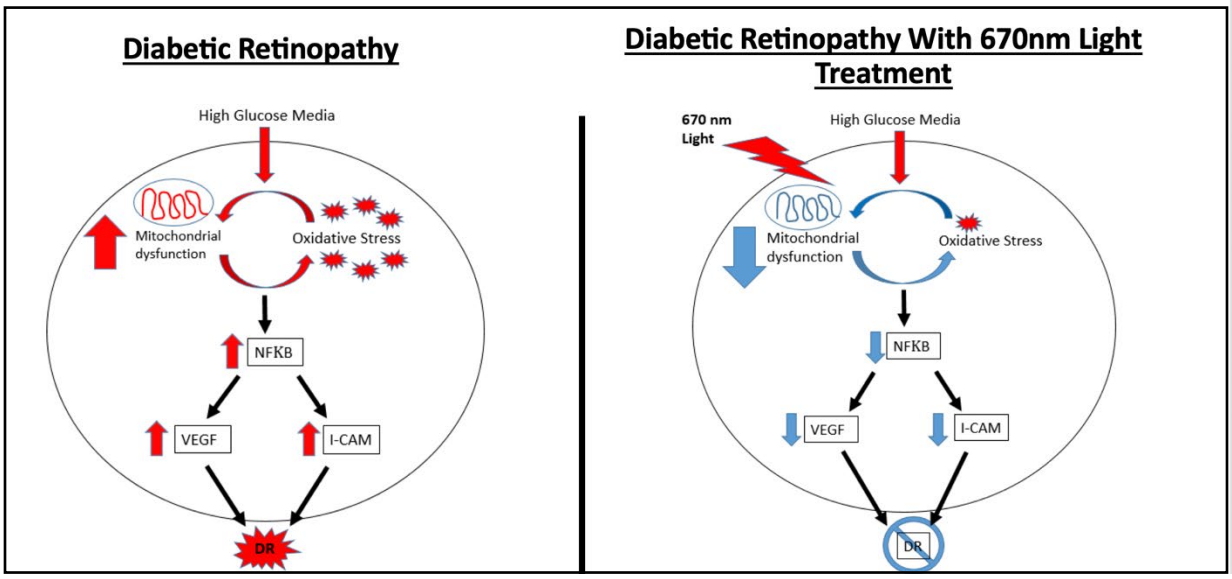

Figure 16: 670 nm Light Intervention in a DR pathway in Müller Cells Exposed to High Glucose Conditions

Our data strongly supports our hypothesis *that 670 nm photobiomodulation attenuates hyperglycemia-induced mitochondrial dysfunction and protects against oxidative stress in a rat Müller glial cell model of diabetic retinopathy.* Our findings also improve our understanding of the acute effects of hyperglycemia in the retina and the potential of 670 nm PBM to ameliorate these effects.

This early interplay between mitochondrial dysfunction, ROS production and depleted glutathione induced by high glucose conditions determine the heath and homeostasis of the cell in DR. If left untreated, this pathogenic cell signaling continues resulting in

blindness. Our data indicate that early intervention in this process using light therapy has the potential to slow the course of diabetic retinopathy or possibly even prevent it.

Our *in vitro* model of DR has considerable potential for use in development and testing of therapeutic agents to treat DR. The DR pathway in our Müller cell model suggested that after one treatment with PBM, high glucose-induced mitochondrial dysfunction and ROS generation was reversed after early exposure to stress. Therefore, the early interventions of PBM on the pathogenesis of DR presented in this study demonstrate that light treatment may be a potential beneficial tool in the early amelioration of diabetic retinopathy.

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