December 2016

The Transcriptional Effects of Photobiomodulation in an In Vitro Model of Diabetic Retinopathy

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THE TRANSCRIPTIONAL EFFECTS OF PHOTOBIOMODULATION IN AN *IN VITRO* MODEL OF DIABETIC RETINOPATHY

by

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A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in Biomedical Sciences

at

The University of Wisconsin-Milwaukee

December 2016

ABSTRACT

THE TRANSCRIPTIONAL EFFECTS OF PHOTOBIOMODULATION IN AN *IN VITRO* MODEL OF DIABETIC RETINOPATHY

by

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The University of Wisconsin-Milwaukee, 2016
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Diabetic retinopathy (DR) is the most common complication of diabetes mellitus and a leading cause of blindness. The pathophysiology of DR is complicated, involving inflammation, oxidative stress, retinal vascular proliferation, and vascular degeneration. Symptomatically, the growth and subsequent rupture of vessels within the frame of view leads to the development of vision loss and eventual blindness. Prior to the development of symptoms, oxidative stress involved in DR leads to the activation of the transcription factor, nuclear factor-κB (NF-κB), resulting in the excess production of vascular endothelial growth factor (VEGF) and intracellular adhesion molecule-1 (ICAM-1), proteins involved in vascular development and immune dysregulation, respectively. The most common therapeutic approach for DR utilizes anti-VEGF agents to reduce vascular proliferation. These treatments are expensive, invasive, frequently ineffective, and have numerous adverse effects, such as retinal detachment, infection, and inflammation inside the eye. A non-invasive alternative therapy is clearly needed. Photobiomodulation (PBM) using far-red to near infrared (NIR) light has been shown to reduce oxidative stress and inflammation *in vitro* and *in vivo* and is an ideal candidate for an alternative therapy. Indeed, PBM slows the progression of DR in animal models

via attenuation of oxidative stress and by reducing the relative level of ICAM-1. We hypothesize that PBM will reduce the activity of NF-κB and reduce the production of VEGF and ICAM-1 in an *in vitro* model of DR**.** To test this hypothesis, we used an *in vitro* model system of cultured retinal Müller glial cells grown in normal (5 mM) or high (25 mM) glucose conditions for either 3 or 6 days to simulate normoglycemia and hyperglycemia. Cultures were treated with 670 nm light emitting diode (LED) (180 seconds at 25 mW/cm^2; 4.5 J/cm^2) or no light (sham) for 3 or 5 days. NF-κB activity and ICAM-1 concentrations were significantly increased under high glucose conditions, as measured by a dual luciferase assay or western blot, respectively. Treatment with 670 nm LED significantly reduced NF-κB activity of high glucose culture cells to values comparable to transcriptional activity measured under normoglycemic condition and decreased the level of ICAM-1. VEGF concentrations were not affected by high glucose or PBM. These data are in partial support of our central hypothesis that in an *in vitro* model of DR, 670 nm light will reduce activation of NF-κB, and reduce the synthesis of ICAM-1 and VEGF. The lack of an observable effect of hyperglycemia or PBM on VEGF concentrations indicates that the stimulation of VEGF secretion requires the activation of additional signaling pathways not induced by high glucose alone.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AGE: Advanced Glycation End Products

AMD: Age-related macular degeneration

ANOVA: Analysis of Variance

ATP: Adenosine Triphosphate

BSA: Bovine Serum Albumin

CcO: Cytochrome c Oxidase

CDC: Center for Disease Control

DMEM: Dulbecco's Modified Eagle's Medium

DNA: Deoxyribonucleic Acid

DR: Diabetic Retinopathy

ELISA: Enzyme-Linked Immunosorbent Assay

FVM: Fibrovascular membrane

GAPDH: Glyceraldyhyde-3-phosphate dehydrogenase

HI-FBS: Heat Inactivated-Fetal Bovine Serum

ICAM-1: Intracellular Adhesion Molecule-1

LB: Lysogeny Broth

LED: Light-Emitting Diode

NIR: Near-Infrared

NO: Nitric Oxide

PBS: Phosphate Buffered Saline

PBM: Photobiomodulation

PDR: Proliferative Diabetic Retinopathy

PKC: Protein Kinase C

PVDF: Polyvinyl difluoride

NF-κB: Nuclear factor-κB

rMC: Rat Müller glial cells

ROS: Reactive Oxygen Species

RAGE: Receptor of Advanced Glycation End Products

STZ: Streptozotocin

TBI: Traumatic Brain Injury

TBS: Tris-Buffered Saline

TBST: Tris-Buffered Saline + Tween 20

VEGF: Vascular Endothelial Growth Factor

ACKNOWLEDGEMENTS

I cannot express enough thanks to my advisors, Dr. Janis Eells and Dr. Elizabeth Liedhegner, for their continued feedback and guidance. I would also like to extend my sincerest gratitude to my committee members, Dr. Jennifer Doll and Dr. Dean Nardelli, for their support and instruction throughout my time at UW-Milwaukee, and I would also like to thank my fellow laboratory members, Betsy Abroe, Nader Mahdi, and Shikan Zheng, for their assistance throughout this project.

My completion of this project could not have been accomplished without the continued support of my parents, Bonnie and Martin. Additionally, I would like to thank my fiancé for his unwavering encouragement. Lastly, I would like to dedicate this work to my grandmother, Ida, who taught me that the only things in life that matter are family and the continued pursuit of knowledge.

I. INTRODUCTION

Diabetes

Over 29 million people in the United States are currently diagnosed with diabetes mellitus (CDC 2014). Diabetes mellitus is a metabolic disease in which the body is unable to regulate glucose utilization, resulting in an asymptomatic increase in blood glucose concentrations referred to as hyperglycemia. Two pancreatic hormones, insulin and glucagon, are important in the regulation of blood glucose concentrations. Glucagon signals for the release of glucose into circulation, whereas insulin promotes the uptake of glucose. In diabetes, though, glucose utilization is impaired in one of two ways: by a lack of insulin production or by a defect in the body's response to insulin. Under normal conditions, insulin is released by the pancreas in response to a rise in concentrations of glucose, free fatty acids, amino acids, and incretins. Within the pancreas, insulin is produced by pancreatic β-cells and secreted into the blood stream in response to elevated glucose concentrations. Once in the blood stream, insulin signals skeletal muscle and adipose tissue to take up glucose, restoring homeostasis. Glucose is then oxidized *via* the tricarboxylic acid cycle and mitochondrial electron transport chain to produce adenosine triphosphate (ATP), the primary cellular energy source. Inadequate insulin production or insulin signaling inhibits these processes, resulting in hyperglycemia.

Although diabetes mellitus is incurable, alterations to lifestyle and drug therapies can assist in the maintenance of stable blood glucose concentrations. Treatment options are dependent on whether an individual is diagnosed with Type 1 or Type 2 diabetes. Type

1 diabetes results from an autoimmune induced destruction of the pancreatic β-cells, which eliminates insulin production. Therefore, treatment of type 1 diabetes requires insulin therapy throughout a patient's life. Type 2 diabetes, however, is associated with insulin resistance and/or abnormal insulin secretion (Gullausseau *et al*. 2008). Unlike type 1 diabetes, type 2 diabetes can be managed, to some degree, by lifestyle changes, such as dietary modifications. Most type 2 diabetics, though, will also require insulin or oral hyperglycemic drug therapy to regulate blood glucose levels.

Despite lifestyle changes and/or drug interventions to regulate blood glucose concentrations, long-term metabolic complications commonly develop in patients with either type 1 or type 2 diabetes. Diabetic complications occur with sustained tissue exposure to high circulating glucose and lead to micro- and macro-vascular complications throughout the body (Yamaoto *et al.* 2001, Aubert *et al.* 2014, Wu and Juurlink 2002, Wendt *et al.* 2003). Macrovascular damage increases the prevalence of heart disease, stroke, and peripheral arterial disease. Microvascular damage contributes to diabetic retinopathy (DR), neuropathy and nephropathy in diabetic patients. Cardiovascular disease, nephropathy, neuropathy, and DR are all considered major complications of diabetes.

The most common diabetic complication, DR, is associated with vision loss as a result of vasculature development and rupture within the retina of the eye. A pathological increase in oxidative stress, promoted by mitochondrial dysfunction, increased signaling through the advanced glycation endproducts (AGE) and receptor of AGE (RAGE)

pathway, as well as increased activation of protein kinase C (PKC) are important for the development of DR (Kanwar *et al.* 2009, Wu and Juurlink 2002, Xia *et al.* 1994, Geralds *et al.* 2009). The symptomatic changes, which occur as a result of these pathways, take place in the retina.

Anatomy of the Eye

Anatomy of the Retina

A healthy retina is essential for good vision. This light-sensitive tissue is located at the back of the eye and is the site of vision transduction. The neural retina is arranged in three cell layers. The rod and cone photoreceptor cells, which are responsible for visual transduction, are arranged in a layer against the back of the eyeball. The second layer of cells, known as the inner nuclear layer, contains bipolar cells, horizontal cells, and amacrine cells. The surface layer of the retina is comprised of ganglion cells. The ganglion cell axons form the optic nerve which relays information to the brain. There are two synaptic regions in the retina: (1) the outer plexiform layer which contains the photoreceptor cell synapses which communicate with bipolar and horizontal cell dendrites and (2) the inner plexiform layer containing bipolar cell and amacrine cell synapses to ganglion cells. Neurotransmitters, including glutamate, γ-aminobutyrate, glycine, acetylcholine, dopamine, and serotonin, are responsible for the signaling across the synapses. The Müller glial cells and retinal vasculature maintain retinal homeostasis by ensuring an adequate nutrient supply and eliminating waste.

Retinal Vasculature and Pericytes

The blood supply to the retina is carried by the central retinal artery, the choroidal blood vessels, and their associated capillaries. These vessels provide oxygen while removing waste throughout the retina. Blood vessels throughout the body are lined with endothelial cells coated with pericyte cells, which forms the blood-retina barrier. The pericyte cells wrap around the endothelial cells of capillaries to provide stability, by maintaining the blood-retinal barrier, and by regulating blood flow (Jo *et al.* 2013, Peppiatt *et al.* 2006).

The Müller Glial Cells

The Müller glial cells are the principal glia in the retina. Müller glial cells support surrounding cells through the release of growth factors and antioxidants, and uptake of extracellular debris. Thus, functional changes in Müller glial cells can impact both neuronal and vascular cells in the retina. The anatomical localization of these cells throughout the retina allows for the maintenance of homeostasis, as well as providing structural and metabolic support to all cell types. The somata of Müller cells reside between either plexiform layer. Trunks extend from the somata in each direction, allowing Müller cells to span the width of the retina. Müller cells assist in the uptake and metabolism of retinal neurotransmitters. Regulation of neurotransmitters by Müller glial cells within the synapse prevents the inappropriate activation of neurons. Furthermore, the Müller cells support and maintain the blood-retinal barrier (Shen *et al*. 2012). Transgenic mice with conditional Müller glial cell dysfunction exhibited accelerated photoreceptor apoptosis, and blood-retinal barrier breakdown, resulting from weakened

tight junctions between endothelial cells (Shen *et al*. 2012). These observations substantiate the neural and vascular protective functions of the Müller cells.

Examination of the Retina

Imaging of the fundus (back of the eye) allows for visual inspection of the retina and macula, as well as the choroid and blood vessels. The majority of fundus examinations are conducted using a fundus camera, ophthalmoscope. These fundus images are principle for the determination of DR. In a healthy retina the macula, is devoid of blood vessels while in a diseased retina, the vasculature show signs of venular bulging and/or retinal thinning (Jonsson *et al*. 2016).

Diabetic Retinopathy

DR is the most common complication associated with diabetes, and it is the leading cause of blindness in the United States (CDC 2008). The early stages of DR are typically asymptomatic, despite the underlying cellular and metabolic changes because there are no physically perceivable changes to vision (Wilkenson *et al.* 2003). These early stages are collectively referred to as the non-proliferative stage, which is characterized by microaneurysms, hemorrhages, exudates, and cotton-wool spots; which are indicative of nerve fiber damage. Notably, angiogenic changes do not develop during this early stage (Nayak *et al.* 2007). The development of retinal abnormalities is monitored by fundus imaging during yearly eye exams. The asymptomatic phase of DR is accompanied by retinal cell death, vascular swelling, and vascular leakage.

Diabetic patients with DR become aware of their disease when they progress to the proliferative diabetic retinopathy (PDR) stage, and visual changes are apparent. The progression of PDR is associated with pathological retinal angiogenesis. The symptoms in this final stage initially begin with spotty vision and ultimately lead to blindness. These symptoms are caused by the inappropriate growth of weak blood vessels that can easily rupture. The spots in the field of vision occur with the leakage of blood from these weak vessels, as well as by excessive inflammation. The angiogenesis and inflammation in DR is associated with a number of different factors. Intracellular adhesion molecule-1 (ICAM-1) and vascular endothelial growth factor (VEGF) are two factors that have been implicated as playing a role in detrimental cellular changes within the retina.

Cellular Changes in Diabetic Retinopathy

Many cell types undergo functional alterations in the progression of DR. These changes denote the progression and the severity of the disease. Pericyte cell loss, due to an increase in oxidative stress, is the first histopathological change in the development of DR (Amano *et al.* 2005). Pericyte loss promotes the hyperdilation of endothelial cells, and early vascular abnormalities. The degeneration of the retinal neuronal cells has also been purported to coincide with the inability of the vasculature to remove waste, and a change in function of the Müller glial cells (Frydkjaer-Olsen *et al*. 2015). The Müller cells have, as well, been shown to shift from a primarily anti-inflammatory state to a pro-inflammatory state in the progression of DR (Gerhardinger

et al. 2005, Harada *et al.* 2006, Zong *et al.* 2010). Analysis of gene expression in Müller cells isolated from retinae of streptozotocin (STZ)-diabetic rats revealed an up-regulation of inflammatory-associated proteins (Gerhardinger *et al*. 2005). Thus, the biochemical and molecular changes that occur in Müller glial cells disrupt their function and result in vascular and neuronal cell dysfunction and death.

Molecular Mechanisms of Diabetic Retinopathy

Oxidative Stress

At the molecular level, multiple pathways are involved in the pathological changes that occur during DR. However, many of these are not fully understood. Figure 1 illustrates pathways that have been associated with the overproduction of reactive oxygen species (ROS) leading to DR. These include mitochondrial dysfunction, the activation of PKC, and the production of AGEs (Kanwar *et al.* 2009, Wu and Juurlink 2002, Xia *et al.* 1994). Additionally, there is decreased antioxidant function further promoting the development of oxidative stress (Kanwar *et al.* 2009). The increased level of oxidative stress further activates intracellular signaling pathways. In STZ diabetic rats, treatment with antioxidants, including, astaxanthin and curcumin, have been shown to alleviate the increased leukostasis, which may result from elevated levels of ICAM-1, and the induction of increased VEGF synthesis (Yeh *et al.* 2016, Obrosova *et al.* 2001). The combination of excessive ROS generation and antioxidant depletion are key factors in the development of DR.

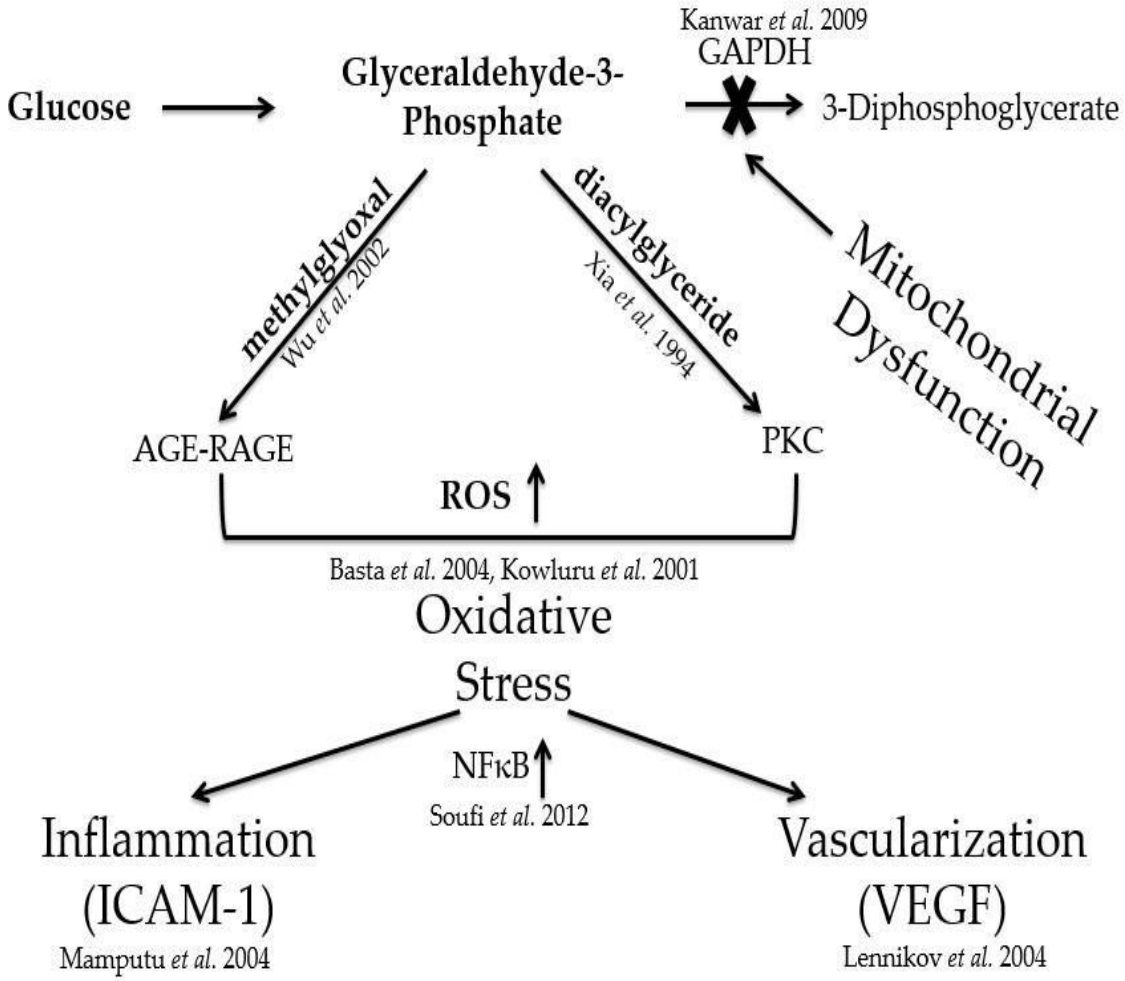


Figure 1: Proposed Pathways Involved in the Development of DR
The depiction of pathways involved in the progression of DR. Early metabolic and antioxidant mitochondrial dysfunction induced by high glucose inhibits the metabolism of glucose as a result of reduced enzymatic activity of GAPDH. The build up of metabolites promotes the development of ROS through both the PKC and AGE-RAGE pathway. The development of oxidative stress has been shown to promote the inappropriate activation of NF-κB leading to the excess synthesis of ICAM-1 and VEGF.

Mitochondrial Dysfunction

Mitochondria are the primary source of ROS in the cell, and mitochondrial dysfunction is thought to be the initial source of oxidative changes that occur in DR (Trudeau *et al.* 2006). Trudeau *et al*. observed early structural and functional changes of mitochondria in rat retinal endothelial cells grown in high glucose medium (30 mM), in comparison to those cultured in low glucose medium (Trudeau *et al.* 2006). After 30 minutes of exposure to high glucose conditions, the mitochondria of the rat retinal endothelial cells exhibited fragmented membranes. Mitochondrial membranes structure recovered to normal within two hours of high glucose treatment (Trudeau *et al.* 2006). However, when rat retinal endothelial cells were cultured in high glucose for a minimum of 48 hours, mitochondrial fragmentation continued for the remainder of the 6-day analysis. Additionally, when rat retinal endothelial cells were cultured in high glucose for three or six days, there was a significant reduction in oxygen consumption and an increase in the release of cytochrome c indicating a change in mitochondrial metabolism (Trudeau *et al.* 2006). These changes in mitochondrial function caused deregulation of many cellular systems due to a reduction in ATP synthesis and increased production of ROS.

In addition to impaired mitochondrial function, the increase production of ROS by the mitochondria impairs glucose metabolism, resulting from a reduction in the enzymatic function of glyceraldehyde-3-phosphate (GAPDH). The impaired enzymatic function of GAPDH has been linked to dysfunction in antioxidant mechanisms (Kanwar *et al*. 2009). In retinal endothelial cells maintained under hyperglycemic conditions, mitochondrial superoxide dismutase, an enzyme known to scavenge ROS, is diminished (Kowluru *et*

al. 2006). Furthermore, under hyperglycemic conditions, overexpression of mitochondrial superoxide dismutase restored GAPDH function (Xueliang *et al*. 2003). The inhibition of GADPH, as observed in diabetes, leads to the build-up of glucose metabolites, including glyceraldehyde 3-phosphate. The accumulation of high concentrations of these glucose metabolites promotes further downstream activation of pathways including the PKC and AGE-RAGE pathway (Figure 1). Increased activation of these signaling cascades further promotes an increase in oxidative stress.

ROS Activation of Intracellular Signaling

Collectively, the development of oxidative stress results in the activation of pathways involved in inflammation and angiogenesis primarily through activation of nuclear factor-κB (NF-κB) (Soufi *et al.* 2012). Indeed, the activation of NF-κB is considered an important step in the progression of DR (Nagai *et al.* 2007, Zhao *et al.* 2012, Romeo *et al.* 2002, Zheng *et al.* 2004), specifically due to its regulation of ICAM-1 and VEGF (Mamputu *et al.* 2004, Shelton *et al*. 2007 Cervellati *et al*. 2014). ICAM-1 is an important adhesion molecule involved in the inflammatory response associated with DR (Mamputu *et al*. 2004, Joussen *et al*. 2001). During normal function, ICAM-1 functions to facilitate leukocyte transmigration by allowing attachment and migration of leukocytes through blood vessels. However, when produced in excess, ICAM-1 leads to an inordinate degree of leukostasis, which was observed in a STZ-induced diabetic rat model of DR (Tang *et al.* 2013). This increase in leukocyte adhesion correlated with increased endothelial cell apoptosis (Joussen *et al.* 2001). The accelerated loss of endothelial cells alters the structure of the vasculature, resulting in leakage and the

rupture of blood vessels and, thus, impairing the adequate blood flow within the vasculature (Joussen *et al.* 2001).

With the impaired vascular function, the reduced blood flow to the retina promotes an ischemic environment. To enable restoration of appropriate tissue oxygen concentrations, angiogenesis is stimulated. Normally, angiogenesis is important for improved blood flow in areas where the vasculature is damaged. However, in DR, overproduction of VEGF leads to excess growth of these blood vessels, which impedes vision. Vision loss occurs as a result of vessel growth and leakage of blood near the macula. Also, irreversible vision loss takes place after extensive blood vessel growth promotes the formation of a fibrovascular membrane (FVM). The surgical removal of FVMs is frequently required to prevent retinal detachment and permanent loss of vision (Kim *et al.* 2015).

Treatment of Diabetic Retinopathy

Therapeutic options for diabetic patients with DR depends on how pronounced their retinal abnormalities are. During the early stages of DR, the treatment includes continued control of diet and blood pressure (Liu *et al.* 2013). As DR progresses, therapies are directed at inhibiting the inappropriate growth of blood vessel. These treatments are monoclonal antibodies designed to neutralize VEGF and thus are referred to as anti-VEGF therapy (Yang *et al.* 2013). Individuals with PDR that abstain from treatment will progress to severe vision loss and eventually become blind.

Currently, anti-VEGF therapies are the gold standard of treatment for DR. Although, photocoagulation therapy is often used in conjunction with the anti-VEGF therapies. This treatment regimen employs a high-intensity laser to seal or destroy damaged and leaking vessels in the retina (Yang *et al*. 2013). If these steps are performed before retinal damage, the reversal of vision loss is likely (Yang *et al.* 2013).

Current treatment strategies for patients with DR reduce vision loss. However, significant side effects are associated with their use. Some common side effects of therapies for DR include scotoma (blind spot), retinal scarring, macular edema, and/or vitreous hemorrhage (Sher *et al*. 2013, Shin *et al.* 2009). In addition to these side effects, the therapeutic effects of these treatments diminish in a short period of time. One study showed improvement of visual acuity in 17 patients treated with intravitreal bevacizumab, an antibody against VEGF. However, visual acuity declined to baseline by three months following treatment, indicating a need for repeated treatments (Preti *et al.* 2012). Targeting the VEGF signaling cascade is just one of many plausible therapeutic options for control of DR. Another possible therapeutic would include targeting upstream molecules which may have equal or greater beneficial effects. One such target that has gained interest is modification of the transcriptional activity of NF-κB, a transcription factor that has been shown to play a role in the progression of DR (Nagei *et al.* 2007, Soufi *et al.* 2012).

NF-κB's Role in Diabetic Retinopathy

NF-κB activation is an important factor in the progression of DR, because of its regulatory effects on proteins involved in inflammation and retinal angiogenesis (Figure 1). NF-κB also plays a beneficial role in many other cellular processes, including inflammation, immunity, cell proliferation, differentiation, and survival. This diverse functionality of NF-κB is a result of extensive various subunits and methods of activation. The need for diverse NF-κB function is due to variability amongst cell types, differences in phosphorylation, and other post-translational modifications (Romeo *et al*. 2002, Werner *et al*. 2005, Brown *et al*. 1994).

Generally, NF-κB is maintained in the cytoplasm when dimerized by IκB. Once NF-κB is activated, at least 150 genes have been reported to be under the transcriptional regulation of NF-κB (Ghose and Bhattacharyya 2015). Three pathways are involved in the activation of NF-κB, including the canonical, non-canonical, and ROS-activated. In DR, the overproduction of ROS promotes the inappropriate and over activation of NF-κB. Regulation of NF-κB activity may provide a therapeutic target for DR. Previous studies show that inhibition or reduced activation of NF-κB slows the progression of DR. Nagei *et al.* (2007) inhibited NF-κB in a STZ diabetic mouse model of DR with dehydroxymethylepoxyquinomicin, reducing inflammation and slowing progression of DR compared to vehicle treatment only groups (Nagai *et al*. 2007). However, due to the vast role of NF-κB in regulating cellular function, long-term inhibition may not be beneficial.

In a similar study, Soufi *et al.* treated STZ diabetic mice with resveratrol, a plant-based antioxidant compound (Soufi *et al.* 2012). Resveratrol treatment resulted in decreased NF-κB activation, as measured by antibody binding of the p65 subunit in nuclear extracts (Soufi *et al.* 2012). Furthermore, the treatment reduced apoptosis and inflammation (Soufi *et al.* 2012). These changes likely occur as a result of attenuating oxidative stress. Thus, attenuation of NF-κB activation by ameliorating hyperglycemia induction of oxidative stress may present an alternative therapeutic target for the treatment of DR.

Photobiomodulation

Photobiomodulation

A potential alternative therapy for DR includes treatment with far-red to near-infrared (NIR) light (630 nm-1000 nm). This light treatment has been shown to cause a biological change including mitigation of oxidative stress, repair of mitochondrial function, and prevent cell death, and is, thus, commonly referred to as photobiomodulation (PBM) (Ball *et al*. 2011). Treatment with low-energy lasers or light-emitting diode (LED) arrays has been reported to be beneficial in models of tissue repair and wound healing (Gaida *et al*. 2004, Gupta *et al*. 2015, Pinto *et al.* 2009). In a model of wound healing, Sprague-Dawley rats treated with 904 nm light had a higher healing efficacy on the induced burn wounds compared to treatment with sulfadiazine cream, a common burn therapy (Gupta *et al.* 2015). Furthermore, PBM has shown promise in clinical trials for the treatment of mucositis and stroke. 670 nm light reduced the incidence of mucositis in the treatment patients undergoing a bone marrow transplant

(Hodgson *et al.* 2012, Whelan *et al.* 2002). In clinical trials of PBM in stroke patients, light therapy has shown promise in both safety and improved outcomes (Zivin *et al*. 2009, Lampl *et al.* 2007). Taken together, studies involving PBM have shown beneficial effects in the disease models chosen. Understanding treatment with far-red to NIR light, though, remains challenging due to varying effects observed between wavelengths and dosage. For example, rats with burn wounds received treatment with 670 nm light at a range of dosages (1, 5, 9, and 19 J/cm^2) or received sham treatment for either three or five days a week. Animals which received treatment with 670 nm light at a dosage of 1 J/cm^2 had a significantly higher rate of healing than rats treated with the various other dosages, as well as rats which received sham treatment (Al-Watban and Delgado 2005). Additionally, the wavelength selected for treatment has differential effects. Comparative analysis of treatment with 665, 730, 810, and 980 nm light (36 J/cm^2) was performed in a mouse model of traumatic brain injury (TBI) (Wu *et al.* 2012). TBI was induced in adult male BALB/c mice by the controlled drop of a weight onto their skull. Animals received a single dose of their respective treatment fours-hours post-TBI (Wu *et al.* 2012). Mice performed various tasks for measurement of neurological injury from post-injury day 0 to postinjury day 30 (Wu *et al.* 2012). Animals treated with 665 and 810 nm light post-injury had a significant improvement in neurological function in comparison to TBI control animals (Wu *et al.* 2012). However, there was no significant difference in neurological function between animals treated with 730 nm or 980 nm light, compared to animals which were sham treated (Wu *et al.* 2012). Therefore, understanding treatment with light in the far-red to NIR range remains challenging due to varying effects observed between wavelengths and dosage. The beneficial effects of

treatment with light in the far-red to NIR range reported thus far, support further investigation into optimal dosages and wavelengths.

Photobiomodulation in Models of Oxidative Stress

Beyond the alleviation of symptoms, another therapeutic effect observed after treatment with PBM is the diminution of oxidative stress. In a number of models, of which oxidative stress has pathological significance, treatment with far-red to NIR light has been shown to reduce oxidative stress and alleviate symptoms and or pathological markers of the disorder (Tang *et al.* 2013, Saliba *et al.* 2015, Silva Macedo *et al.* 2015, Purushothuham *et al.* 2014). In cell culture models of oxidative stress, PBM was able to diminish ROS. ARPE-19 cells, immortalized retinal pigment epithelial cells, and human primary retinal pigment epithelial cells treated with hydrogen peroxide showed diminished ROS when treated with 670 nm light. The authors report that although ROS is diminished, it was not to a sufficient level to preserve mitochondrial membrane potential and reduce cell death (Fuma et al. 2015). Similarly, a reduction in both NO and ROS was observed in dental pulp cells treated with LPS and 855 nm light (Montoro et al. 2014).

Similar results were observed *in vivo*. Male 2-month-old Wistar rats were exposed to formaldehyde (1%, 90 in), as a model of acute lung inflammation, and treatment with 660 nm light decreased oxidative stress while simultaneously increasing the presence of antioxidants in comparison to non-treated rodents exposed to formaldehyde (Silva Macedo *et al*. 2016). Furthermore, in two different mouse models of Alzheimer's disease five month K3 mice and seven month APP/PSI mice were treated for 90

seconds with 670 nm light for five days per week over a four-week period, which resulted in reduced lesion size in comparison to non-treated Alzheimer's mice (Purushothuham *et al*. 2014). Corresponding to these reductions in pathological markers was a decrease in the presence of 8-hydroxy-2'-deoxyguanosine, a marker of deoxyribonucleic acid (DNA) oxidative damage (Purushothuham *et al*. 2014). These studies are just a few examples demonstrating the effects of PBM on oxidative stress and disease manifestations.

Photobiomodulation in Retinal Disorders

Several studies using 670 nm light on retinopathies have demonstrated therapeutic effects for this treatment. For example, in Long Evans rats treatment with 670 nm LED has been used to protect against the toxic effects of methanol (Eells *et al.* 2003). Methanol exposure leads to formic acid production and metabolic acidosis resulting in injury to the retina and optics nerve as observed through abnormal ERGs. Additionally, methanol treatment leads to histological abnormalities including retinal swelling and disruption of the mitochondria in photoreceptors (Eells *et al.* 2003). These symptomatic and histological changes observed in methanol-intoxicated rodents were significantly reduced in animals treated with 670 nm light for 144 seconds at a dosage of 4.5 J/cm^2, at 5, 24, and 50 hours after intoxication (Eells *et al.* 2003). In a rodent model of retinitis pigmentosa, treatment with 670 nm light also regulates cytoprotection and reduces the loss of photoreceptors (Machida *et al.* 2013). Furthermore, retinal function was improved in animals treated with 670 nm light compared to control (Kirk *et al.* 2013). Treatment with 670 nm light also promotes improvements in visual acuity in age-related

macular degeneration (AMD) (Merry *et al.* 2013). In a small patient sample, nine individuals with AMD received treatment with 670 nm light at 50-80 W/cm^2, 4-7.68 J/cm^2, and a duration of 88 +/- 8 seconds three times per week for six weeks. Patients treated with 670 nm light had a significant improvement in visual acuity, when compared to measures of visual acuity collected prior to treatment (Merry *et al.* 2013).

Photobiomodulation in Models of Diabetic Retinopathy

Collectively, previous studies show that PBM attenuates oxidative stress and ameliorates disease manifestations in retinal disorders. It is, therefore, likely that PBM may be beneficial for the treatment of DR. 670 nm light treatment of retinal ganglion cells, Muller glial cells and RPE cells cultured in high glucose concentrations to simulate diabetes reduced superoxide production and cell injury (Tang *et al.* 2013). In the STZ rat model of DR, 670 nm light treatment at the onset of diabetes, significantly reduced the development of lesions, leukostasis, and reduced the increase in ICAM -1 synthesis associated with disease (Tang *et al.* 2013). Similar observations were made in the STZ-mouse model of DR (Saliba *et al.* 2015). Additionally, Saliba *et al*. reported that 670 nm light treatment of advanced stage diabetes in rodents also slowed disease progression (Saliba *et al*. 2015). A recent series of case reports also provides clinical evidence that 670 nm light reduces retinal edema associated with diabetes (Tang *et al.* 2015). Beyond these findings, little is known about the intracellular effects of PBM in models of DR; however, work in other areas has helped provide insight behind the mechanism of action for PBM.

Mechanism of Action of PBM

Therapeutically beneficial effects of PBM have been observed in a number of disorders such as Alzheimer's disease, wound healing, and various retinal diseases (Purushothuham *et al*. 2014, Gupta *et al*. 2015, Eells *et al.* 2003, Tang *et al.* 2013). The improved functional outcomes and diminution of disease causing lesions have been found to coincide with an improvement in mitochondrial function (Cummins *et al.* 2013). Mitochondrial dysfunction is an early step in the development of an assortment of disorders, particularly in the retina, a mitochondrial rich environment (Trudeau *et al.* 2006, Marshall *et al.* 2015, Maleki *et al.* 2013). Photobiomodulation has been shown in several studies to modulate mitochondrial function (Agrawal *et al*. 2014, Cummins *et al.* 2013, Wong-Riley *et al*. 2005). In a rodent model of optic nerve injury, mice were either sham treated or treated with 670 nm LED (88 seconds, 60 mW/cm^2, 5 J/cm^2), for one or seven days after injury. Sham-treated mice with optic nerve injury presented with a change in mitochondrial structure and activity, as determined by transmission electron microscopy. The changes to the mitochondrial structure in the model of optic nerve injury were attenuated in the injured mice receiving 670 nm light (Cummins *et al*. 2013). The effects of NIR light on mitochondrial function has been hypothesized to induce functional changes in the mitochondrial photoacceptor molecule, cytochrome c oxidase (CcO). In cultured primary neurons, treatment with 670 nm light significantly increased the activation of CcO leading to improved mitochondrial function as indicated by an increase in ATP synthesis (Wong-Riley *et al*. 2005, Agrawal *et al*. 2014). Furthermore, treatment of primary neurons with 670 nm light protected from induced cell death by potassium cyanide, an inhibitor of CcO (Wong-Riley *et al*. 2005). Recent studies have

shown that NIR irradiation results in redox alterations in the CcO complex activating intracellular signaling cascades which culminates in improved mitochondrial function and increased synthesis of cytoprotective factors (Ball *et al.* 2011, Demontis *et al.* 2002). Beyond these findings, due to the prolonged effect of brief treatment with light in the far-red to NIR range, it is likely that PBM induces a cascade of events leading to the stimulation of gene expression, protein synthesis, and oxidative metabolism. Although the mechanism has not been fully elucidated, it has been postulated that the light photons modify the structure of CcO, improving electron transport and increasing ATP production (Lohr *et al*. 2009). Also, it has been observed that light treatment culminates in the release of a small burst of ROS or nitric oxide (NO) and activation of transcription factors which modulate gene transcription (Lohr *et al*. 2009, Zhang *et al*. 2009). Naturally, CcO can bind either oxygen or NO on the heme of iron or copper center. As suggested by Lohr *et al*. a NIR photon photodissociates NO from CcO of mitochondria with abnormal function. The release of NO promotes oxygen to bind CcO and re-establish mitochondrial function. This release of NO is referred to as the burst, and may promote transcription (Figure 2). In regards to mitochondrial associated genes, gene transcriptional changes after light treatment result in the upregulation of cytoprotective cytokines, as previously mentioned, and antioxidants such as mitochondrial superoxide dismutase (Agrawal *et al*. 2014, Huang *et al*. 2013, Zhang *et al*. 2003, *Tang et al*. 2013).

Additionally, treatment with light in the NIR spectrum has been found to increase the expression of CcO in an animal model of AMD (Begum *et al.* 2013). In the complement factor H knockout (CHF -/-) mouse model of AMD, animals were exposed to 670 nm

LED for 6 minutes twice a day (Begum *et al.* 2013). This NIR treatment was continued for 14 days. The CHF -/- mice exposed to light treatment had a significant increase in CcO as measured by immunostaining in sectioned eyes in comparison to those collected from sham-treated CHF-/- animals (Begum *et al.* 2013). Collectively, these studies further provide evidence that treatment with PBM promotes improvement to mitochondrial activity through changes in mitochondrial-associated gene regulation (Begum *et al.* 2013).

Microarray analysis was used to elucidate additional pathways and cellular changes that occur after treatment with PBM. Gene expression profiles performed on treated human fibroblasts indicate that PBM regulates 111 genes. The genes regulated by PBM include those involved in cell proliferation, suppression of apoptosis, cell growth, redox regulation, and mitochondrial energy metabolism (Song *et al.* 2003). While CcO is a likely photoacceptor molecule, other downstream transcriptional changes occur which may or may not result from CcO activation. Therefore, additional research is needed to determine PBM mechanism.

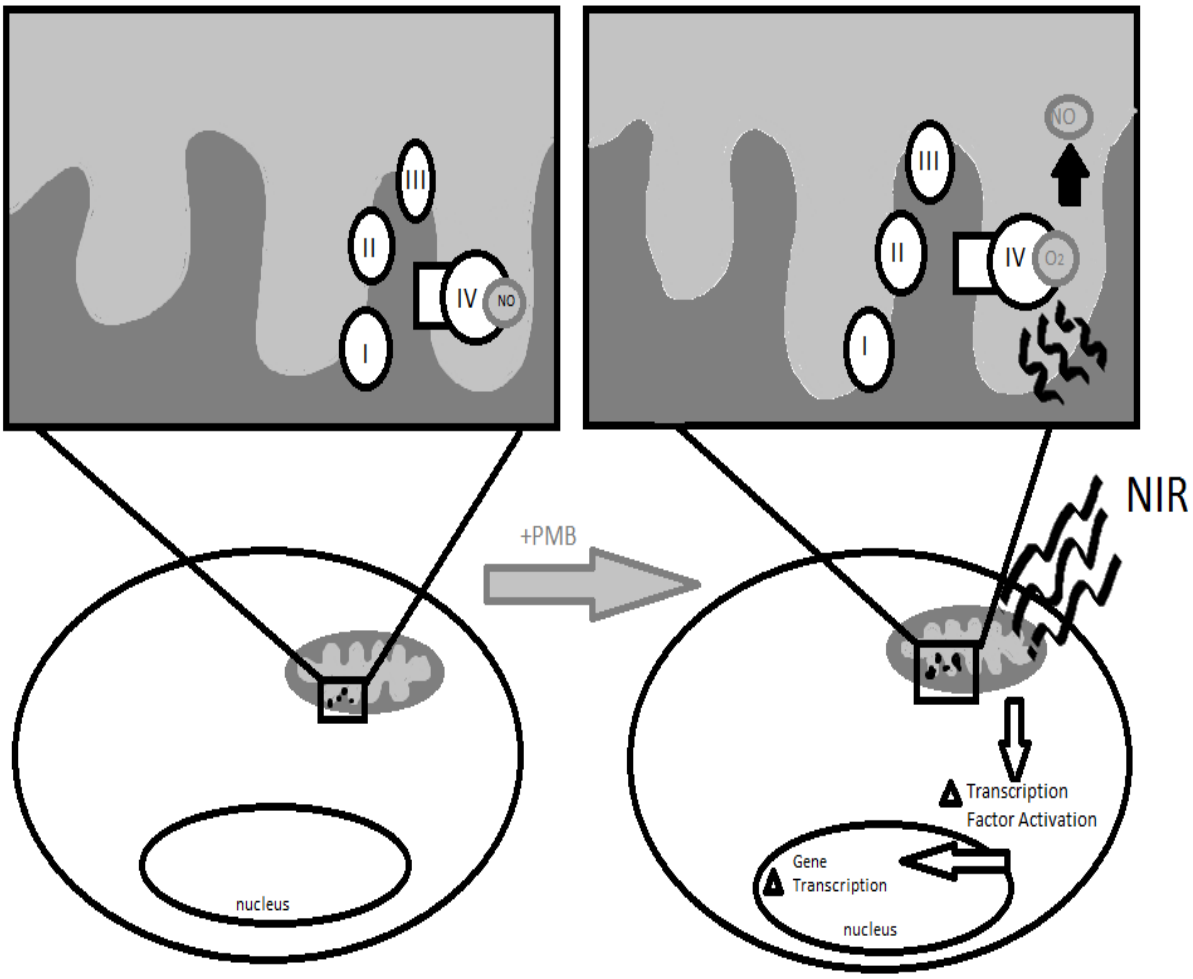


Figure 2: Image Depicting the Theorized Effects of PBM on CcO
Work by Lohr *et al.* (2009) and Zhang *et al.* (2009) suggests that in a state of mitochondrial dysfunction CcO preferentially binds NO over oxygen. Treatment with NIR light photodissociates NO, therefore allowing oxygen to bind. This binding of oxygen promotes improvement to mitochondrial function and sub sequentially induces changes in activation of transcription factors and gene transcription. (△ represents a change)

Effects of Photobiomodulation on NF-κB

In a small assortment of models, PBM has been found to modulate the activation of the transcription factor NF-κB. In a rodent model of burn healing, animals treated with 904 nm (0.2 J/cm^2) light for either 4 or 7 days post injury exhibited a decrease in NF-κB translocation to the nucleus (Gupta *et al*. 2015). Although this is not a direct measure of transcriptional activity, it is indicative of a reduction in the activation of NF-κB. Interestingly, differential observations were reported by Mafra de Lima *et al.* (2009) in regards to the effects of far-red to NIR light treatment on the activity of NF-κB. Bronchi from Wistar rats were dissected and treated with 655 nm at a dose of 2.6 J/cm^2 (Mafra de Lima *et al.* 2009). After treatment, relaxation of bronchi smooth muscles was restored. When an NF-κB antagonist was introduced, these effects were blocked. Therefore, in this model, NF-κB activation by 655 nm is preferential (Mafra de Lima *et al.* 2009). Outside of these studies, little is known about the effects PBM has on NF-κB activity in additional models or with regard to other treatment protocols. The varying effects of PBM on NF-κB activity could be due to the dual role of NF-κB, for eliciting either a pro-inflammatory or anti-inflammatory response, or differences between cell type studies and the manner in which NF-κB was activated (Liu *et al.* 2007, Fong *et al*. 2008). These conflicting results highlight a need for additional research to understand the effect of PBM on NF-κB transcriptional activity. Analyzing the effects of PBM on NF-κB in a model of DR may help clarify inconsistencies.

Summary and Gap in Knowledge

The progression of DR has been linked to increased activity of NF-κB, as well as increased levels of ICAM-1 and VEGF (Mamputu *et al.* 2004, Lennikov *et al.* 2004). It is well established that NF-κB activation occurs in response to elevated ROS (Kowluru *et al*. 2003, Kowluru *et al*. 2007, Wautier *et al*. 1994). Furthermore, 670 nm light has been shown to inhibit oxidative stress associated with pathological changes in the development of DR. Tang *et al.* confirmed that the inhibition of oxidative stress was correlated with a decrease in ICAM-1 levels and leukostasis (Tang *et al.* 2013). Beyond this work, little is known about changes in intracellular signaling and transcriptional activity. Research by Gupta *et al*. and Mafra *et al*. investigated the effects of PBM on NF-κB activity, however, the observed changes in NF-κB activity were dependent on the models analyzed (Gupta *et al.* 2015, Mafra de Lima *et al.* 2009). PBM modulation of signaling in the retina has yet to be elucidated. We have conducted studies to characterize the mechanisms by which 670 nm light affects retinal signaling pathways in a cultured cell model of DR. These studies examine the effects of 670 nm light on activation of NF-κB and cellular level of ICAM-1 and VEGF in a Muller glial cell model of DR.

To perform these studies, Müller glial cells were cultured in either normal or high glucose to replicate normoglycemic and hyperglycemic conditions. Groups from each culture condition received either 670 nm LED treatment at a dosage of 4.5 J/cm^2 or sham treatment. These conditions allowed for analysis of hyperglycemic conditions in the selected model, as well as determine the therapeutic effects of 670 nm LED

treatment on attenuation of hyperglycemic-induced differences. The Müller glial cell model was selected because of their principle role of maintaining homeostasis for both the vasculature and for neuronal cells. Thus, the ability to modulate hyperglycemic-induced changes in Müller glial cells may be biologically important for ameliorating changes in additional cell types.

II. HYPOTHESIS AND SPECIFIC AIMS

DR is the leading cause of blindness in the United States and accounts for 10,000 new cases annually. The early stages of disease are asymptomatic despite the induction of mitochondrial dysfunction, increased AGE-RAGE interaction, and excess activation of PKC pathways, resulting in an increase in oxidative stress. This, in turn, leads to increased inflammation and retinal neovascularization, due in part to the activation of NF-κB (Mamputu *et al*. 2004, Shelton *et al*. 2007). Two of the proteins regulated by NF-κB and involved in inflammation and retinal neovascularization, ICAM-1 and VEGF, are implicated in the progression of DR and loss of vision.

Intraocular injections of anti-VEGF agents is the current therapy for DR; however, this approach is invasive and subject to undesirable side effects (Yang *et al.* 2003). PBM using 670 nm LED arrays is currently under investigation as a potential therapeutic option. Tang and colleagues have shown that 670 nm light attenuates DR in an experimental rat model of DR and in patients with diabetic non-center edema (Tang *et al.* 2013, Tang *et al.* 2015, Saliba *et al.* 2015). In the STZ-diabetic rodent model of DR, an observable reduction in leukostasis, which correlated with the reduction in ICAM-1

was also observed when animals were treated with 670 nm LED (Tang *et al.* 2013). Additionally, these observations corresponded to a reduction in oxidative stress. Based on the relationship between the induction of oxidative stress and the increase activation of NF-κB, as well as the relationship between increased activity of NF-κB and levels of ICAM-1 and VEGF in DR, we predict that PBM will reduce NF-κB activity and reduce the relative level of ICAM-1 and concentration of VEGF. Therefore, we *hypothesize that in an in vitro model of DR, PBM will reduce the activity of NF-κB, and reduce the level of ICAM-1 and concentration of VEGF.* Characterization of the effects of PBM on NF-κB activity and changes in the level of ICAM-1 or VEGF will advance our understanding of the mechanism of action of PBM. Mechanistic studies are essential to move this treatment modality into the clinic for treatment of DR. We plan to test this hypothesis with the following specific aims:

Specific Aim 1: Determine the effects of PBM on the activity of NF-κB.

Working Hypothesis: *PBM will reduce activation of NF-κB in a model of DR*.

Specific Aim 2: Determine the effects of PBM on the levels of VEGF and ICAM-1.

Working Hypothesis: *PBM will reduce the concentration of VEGF and the level of ICAM-1 in a model of DR.*

III. MATERIALS AND METHODS

Cell Line

Müller glial cells are in direct contact with multiple cell types in the retina and are important for the maintenance of homeostasis. Prior research analyzed changes in Müller glial cells cultured under hyperglycemic conditions. High glucose culture conditions resulted in an increase in transcriptional activity of NF-κB (Shelton *et al.* 2009). It has also been observed that under hyperglycemic conditions Müller glial cell production ICAM-1 and VEGF and ICAM-1 is increased (Bai *et al.* 2009, Shelton *et al.* 2007). Thus, Müller glial cells develop abnormalities consistent with those observed in DR, and this model will facilitate the analysis of the actions of PBM on intracellular signaling systems. Because we are using a cell culture model, we can examine signaling within one cell type. However, because we are using an isolated cell culture system, signaling which naturally occurs between cell types cannot be examined and will require further work in an animal model. For the scope of this study, an isolated cell culture system is ideal to better understand intracellular signaling activated by 670 nm light.

Culture Conditions

Immortalized rat Müller glial cells (rMC) were obtained from John Mieyal at Case Western Reserve University. Stock cells were maintained in a solution of 10% DMSO in 10% FBS with Dulbecco's Modified Eagle's Medium (DMEM) and stored in liquid nitrogen. For tissue culture experiments, cells were suspended in DMEM with 1% penicillin-streptomycin, 10% heat-inactivated fetal bovine serum (HI-FBS), and 110

mg/L sodium pyruvate. For light treatment experiments, cells were cultured for up to six days in either high (25mM) or normal (5 mM) glucose with 1% penicillin-streptomycin, 2% HI-FBS and 110 mg/mL sodium pyruvate. Media was replaced daily, or twice daily for transfected cells to maintain stable glucose concentrations. As shown in Table 1 samples collected on the last day of treatment indicate that levels of glucose were maintained for cells cultured in 100 mm dishes. However, for cells cultured in a six-well dish culture glucose levels were not maintained for the full 24 hours. Therefore media was changed every 12 hours to retain stable glucose concentrations (Table 2). Cultures were stored in a 37°C incubator with 5% CO_2. For six well or 100 mm plates, 20,000 cells/well or 80,000 cells/100 mm dish were plated, respectively.

Table 1: Glucose Levels After 24 Hours of Incubation

Sample	Calculated Glucose Concentration
Normal Glucose (stock media)	1.86 mg/mL
High Glucose (stock medial)	5.78 mg/mL
Normal Glucose (100mm dish)	1.53 mg/mL
High Glucose (100 mm dish)	6.12 mg/mL
Normal Glucose (6 well plate)	0.54 mg/mL
High Glucose (6 well plate)	5.93 mg/mL

Table 2: Low Glucose Levels After 12 Hours of Incubation

Sample	Calculated Glucose Concentration
Normal Glucose (Control)	1.75 mg/mL
Normal Glucose (6 well dish)	1.43 mg/mL

670 nm light Treatment

Cultures were treated once daily between 11:00 – 12:00 pm. Cells cultured in high or low glucose were placed on the surface of the 670 nm 75 cm^2 LED array (Spectralife, QDI, Barneveld, WI) (Table 3). PBM treatment consisted of 670 nm irradiation for 180 seconds at a power intensity of 25mW/cm^2, resulting in an energy density of 4.5 Joules/cm^2 (0.025 W/cm^2 x 180 sec = 4.5 J/cm^2). Sham-treated cells were handled in a similar manner, except the LED device was not turned on. Cells were either light- or sham-treated for three days (NF-κB assessment) or 5 days (ICAM-1 and VEGF assessment) depending on assay protocol. Following treatment, conditioned media and/or cell lysates were collected and stored at -80°C for future processing.

Table 3: Summary of Treatment Regimen for Experimental Groups

Group	Media	Treatment
1	High Glucose (FBS+25mM Glucose)	Light
2	High Glucose (FBS+25mM Glucose)	Sham
3	Normal Glucose (FBS+5mM Glucose)	Light
4	Normal Glucose (FBS+5mM Glucose)	Sham

Plasmid Growth and Purification

DH5 α bacterial cells, containing NF-κB or Renilla plasmids, were obtained from John Mieyal at Case Western Reserve University. To initiate a starter culture, stabs from frozen stock cultures (maintained in 20% glycerol) were incubated in lysogeny broth (LB) containing 100 µg/mL ampicillin. A starter culture (5mL) was incubated overnight at 37°C shaking at 300 rpm. After initial incubation, the starter culture was added to 250 mL of LB broth containing 100 µg/mL ampicillin and allowed to shake at 300 rpm overnight at 37°C. The following DNA purification steps were performed utilizing a Maxi-prep kit from Qiagen and performed per manufacturer instructions. Transfection of the plasmids was confirmed by gene sequencing, which was performed by Angela Schmoldt at the School of Freshwater Science, University of Wisconsin-Milwaukee. Forward and reverse primers were designed for sequencing of both the NF-κB and Renilla plasmid. Table 4 shows the designed primer sequences for amplification of the transfected plasmids. BLAST was used for the comparison and confirmation of transfection.

Table 4: Primers Used for Gene Sequencing

Plasmid Primer	Sequence
NF-κB forward primer	5'CACCTCCCCC TGAACCTGAA AC3'
NF-κB reverse primer	5’CCTCTAGAGGATAGAATGGC3’
Renilla forward primer	5’CGAATTCGAACACGCAGATG3’
Renilla reverse primer	5’CTGCATTCAGTTGTGGTTTG3’

Transfection of Müller glial cells with Luciferase and Renilla Plasmids

To transfect Müller glial cells, 20,000 cells/well were plated in a six-well dish. After three days in culture, cells were approximately at a confluence of 60-70%. Cells were washed twice with 1x phosphate buffered saline (PBS). They were then co-transfected with a NF-κB and Renilla plasmid. Transfection was performed with a cocktail containing 1 mL/well Opti-MEM, 5 µL/well lipofectamine, 1 µg/well NF-κB plasmid and 0.1 µg/well Renilla plasmid (Shelton *et al.* 2007). Transfection was allowed to proceed for 8 hours. Following transfection, the transfection cocktail was aspirated and replaced with DMEM, 5 mM glucose, 2% FBS, 1 % penicillin-streptomycin, 110 mg/L sodium pyruvate for 3-4 hours. Media was again aspirated and replaced with appropriate culture media, high (25 mM) or normal (5 mM) glucose. Cells were maintained in either high or normal glucose conditions for three days and were either treated with 670 nm light or sham treated for 180 seconds daily. At the completion of the treatment, lysates were collected in 1x passive lysis buffer and stored at -80°C until further analysis.

Measurement of NF-κB Transcriptional Activity

A Biotek Synergy HT plate reader was used for detection of luminescence. Luciferase readouts were normalized to Renilla readings. The Renilla sequence provides an internal control because it is constitutively expressed, and not affected by the treatment. If transfection does not occur equally between groups, the Renilla luminescence provides a method for the normalization of data.

Cell Collection and Lysate Processing for ICAM-1 analysis

For cell collection, plates were first washed with 1X PBS and cells were then detached with trypsin. Trypsinization was stopped by adding 8 mL of growth media to the 100 mm plate. Next, cells were centrifuged at 1,000 x g for 5 minutes. The pellet was washed with 1 mL of 1X PBS and centrifuged at 1,000 x g for 5 minutes. Lysis was performed on ice for 20 minutes in 100 µl of 1X RIPA buffer and 1X protease inhibitor cocktail (Thermo Scientific) at a dilution of 1:100. The lysate was then centrifuged at 15,000 x g for 5 minutes to pellet cellular debris. A Pierce™ Bicinchoninic Acid Assay Kit from Thermo Scientific (catalog number 23225) was used, according to manufacturer protocol, to determine protein concentration in the supernatant. Each sample was analyzed at a dilution of 1:5, 1:10, and 1:20 with PBS and absorbance was measured at 570 nm. The standard curve for determining protein concentration ranged from 0 mg/mL to 1 mg/mL.

Assessment of ICAM-1 Levels in Müller glial Cell Culture

A Western blot was used to compare the relative amount of ICAM-1 between treatment groups. 100 µg of cell lysate was analyzed for each sample. Each sample was incubated at 95° C for 15 min with 1x sample buffer (10% glycerol, 2% SDS, 0.01% bromophenol blue, 200 mM Tris-HCl pH 6.8, 20 mM dithiothreitol) to denature proteins. PBS was added to each sample so that each had a volume of 25 µL. Samples were separated on a 12% polyacrylamide Mini-PROTEAN ® TGX Precast Gel (Biorad) along with a protein ladder (Biorad). The gel electrophoresis was performed in running buffer

(0.3% w/v Tris Base, 1.45% w/v Glycine, 0.1% w/v SDS) at 200 volts for 1 hour to separate proteins.

Proteins resolved by electrophoresis were transferred to a polyvinyl difluoride (PVDF) membrane. Prior to transfer, the membrane was soaked in methanol for 2 minutes and then washed with Transfer Buffer (0.3%w/v Tris, 1.45% w/v glycine, and 10% v/v methanol). The gel was sandwiched between 2 sponges and filter papers soaked in transfer buffer and placed into a cassette. Transfer of the proteins proceeded for 16 h at 100 mA and 4°C. Following transfer, the PVDF membrane was blocked with 1% bovine serum albumin (BSA) in 1X Tris-buffered saline (TBS; 0.3% w/v Tris-HCl, 0.88% w/v NaCl) + 0.1% Tween 20 (TBST) for one hour. The membrane was then washed three times for 10 minutes each with TBST. Next, the membrane was cut at the 50 kDa mark to incubate portions with their designated primary antibodies. Membranes were probed overnight with either anti-ICAM (Cell Signaling) at a dilution of 1:500 in TBST with 1% BSA or anti-GAPDH (Cell Signaling) at a dilution of 1:5000 in TBST with 1% BSA. After primary antibody incubation, membranes were washed with TBST 3 times for 10 minutes. A horseradish peroxidase-linked secondary antibody (1:10,000, Jackson Labs) was incubated for 2 hours with the designated ICAM-1 (85 kDa) portion of the PVDF membrane and 1 hour with the designated GAPDH (37kDa) portion of the membrane. For protein detection, SuperSignal™ Chemiluminescent HRP Substrates were mixed at a ratio of 1:1. The membrane was then coated and allowed to rest at room temperature in solution for 90 seconds. Afterward, the membrane was Saran-wrapped and placed in a film cassette. The film was then exposed in a dark room for

various time points and developed with an automated TI-2000 developer. Time of exposure was adjusted to allow for visualization of clear bands. ImageJ software was used to compare protein levels between groups. GAPDH was utilized as a loading control; therefore, resolved ICAM-1 levels were normalized to GAPDH for each group.

Concentration of Sample Media for VEGF Analysis

Media was collected after the fifth day of treatment and stored at -80°C. Media samples were concentrated using 15 mL centrifugal filter spin tubes with a 30 kDa membrane (MilliporeSigma,). 10 mL of media was loaded into the spin column. Tubes were spun at 4,000 x g for 10 minutes to reduce sample size to approximately 350 μL. Concentrated samples were stored at -80°C for future analysis.

Assessment of VEGF Concentration

Concentrated cell media samples were analyzed using an eBioscience Rat VEGF-C Platinum Enzyme-Linked Immunosorbent Assay (ELISA)kit (catalog number BMS626/2) and compared to a standard curve. All manufacturer's protocols were followed while performing this assay. A Bioplex Synergy HT plate reader was utilized to measure absorbance at 450 nm.

Statistics

Samples for all assays were conducted in duplicate and averaged. A one-way analysis of variance (ANOVA) was used for determining the significant difference between

groups. The ANOVA was further followed by a Bonferroni posthoc test. The alpha for statistical analysis was set at a p-value of 0.05.

IV. RESULTS

Rationale

Therapeutic effects of PBM have been demonstrated in a variety of disorders, primarily those in which progression is associated with changes in oxidative stress and inflammation. *In vitro* and *in vivo* research collectively performed by Tang *et al.* (2013) and Saliba *et al.* (2015) indicate that, in models of DR, PBM attenuates the hyperglycemic induction of oxidative stress and reduces leukostasis (Tang *et al.* 2013, Saliba *et al.* 2015). The inhibition of leukostasis correlated with a reduction in the relative level of ICAM-1. Although PBM has been studied in DR by examination of pathological and histological changes, very little is known about the effects of PBM on intracellular signaling events. To better understand the mechanism of PBM, we designed studies to determine its action on the transcription factor NF-κB. Because PBM modulates oxidative stress, it is likely that this diminution of oxidative stress will diminish the activation of NF-κB in a model of DR. The purpose of this experiment is to determine the effect PBM on NF-κB activity in a Müller glial cell culture model of DR. We plan to measure NF-κB activity as well as two downstream proteins that are transcriptionally regulated by NF-κB, ICAM-1 and VEGF (Mamputu *et al.* 2004, Shelton *et al.* 2007).

Part 1. Effects of Photobiomodulation on NF-κB Activity in a Müller glial Cell Culture

NF-κB activity in Müller glial cells cultured in normal (5 mM) glucose or high (25 mM) glucose, that were either sham treated or treated with 670 nm LED for 180 seconds at a dosage of 4.5 J/cm^2 was quantified using a dual luciferase assay. This assay allows for a direct measure of NF-κB transcriptional activity. When cells were cultured in high glucose, there was a significant increase in NF-κB activity in comparison to cells cultured in normal glucose ($n=4$, $p<0.0001$). As shown in Figure 3, a 70% increase in luminescence in the high glucose cultured groups was observed in comparison to those cultured in normal glucose. To analyze Specific Aim 1, the effects of 670 nm LED treatment for 180 seconds at a dosage of 4.5 J/cm^2 on Müller cells cultured in high (25 mM) glucose was additionally analyzed. After treatment for three days with 670 nm LED, the activity of NF-κB in high glucose cultures of Müller glial cells was significantly reduced ($n=4$, $p<0.0001$). As indicated by a p-value of 1 ($n=4$), there was no statistical difference between the degree of NF-κB activation in the normal glucose cultured group receiving sham treatment in comparison to the high glucose group treated with 670 nm PBM. These findings indicate that 670 nm light completely attenuated the activation of NF-κB induced by hyperglycemia. Furthermore, when cells were cultured in normal glucose conditions, treatment with 670 nm light did not appear to affect the activity of NF-κB (Figure 3).

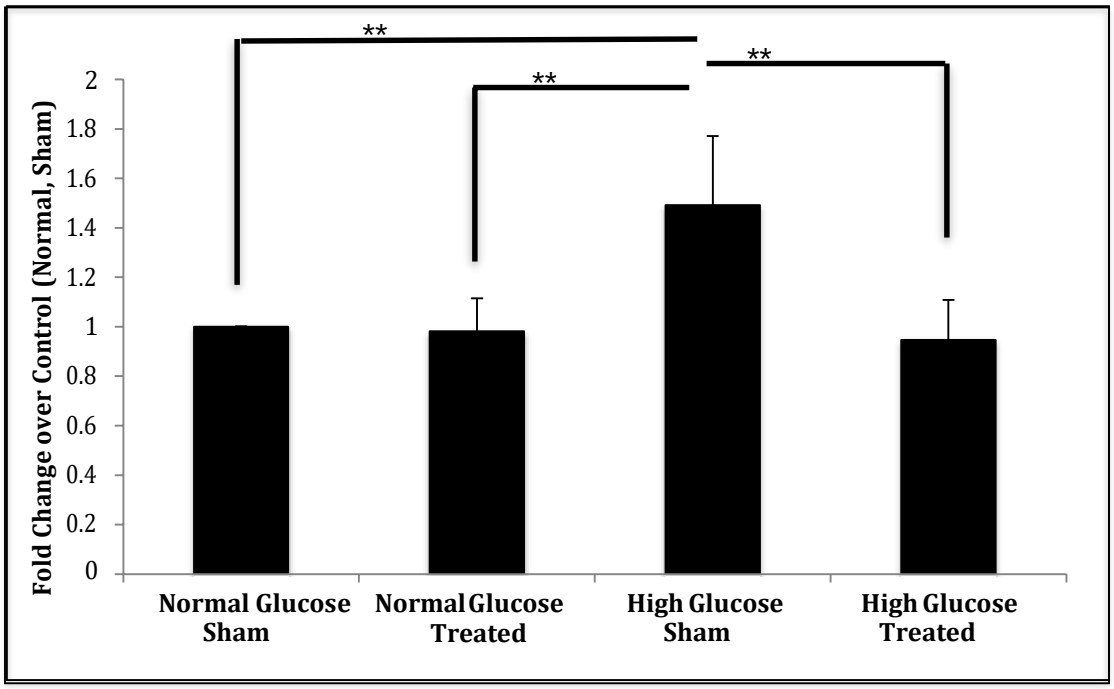


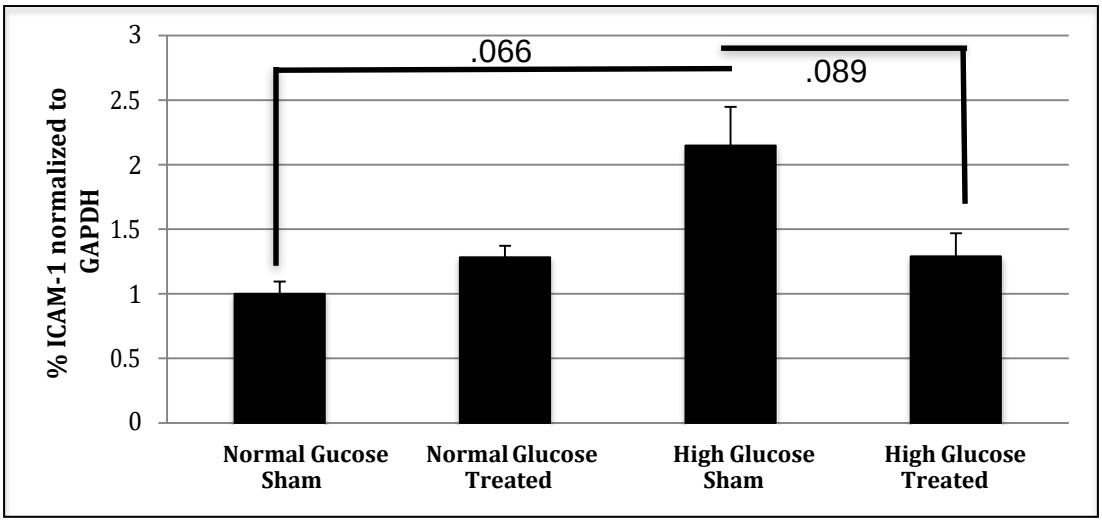
Figure 3: Comparison of NF-κB activity between treatment groups
Bar graph, representing three separate experiments, showing NF-κB activity of Müller glial cells maintained in either normal glucose or high glucose medium. After three days in culture, cells were collected and lysed for analysis of luminescence. Data is presented as a measure of luciferase luminescence normalized to Renilla luminescence (transfection control). Results are presented as a ratio to activity observed compared to that of the normal glucose, sham-treated group. **p<.0001

Part 2. Effects of Photobiomodulation on level of ICAM-1 and VEGF Concentrations

Part 2A: Effects of Photobiomodulation Treatment on ICAM-1 Levels

To analyze the effects of high (25 mM) glucose and treatment with 670 nm LED for 180 seconds at a dosage of 4.5 J/cm^2 on the level of ICAM-1 four sets of cells were plated. These groups were either cultured in normal (5 mM) glucose or high (25 mM) glucose and received either sham or treatment with 670 nm light. After five days of treatment, lysates were analyzed by western blot for quantification of ICAM-1. This is longer than the time frame used for NF-κB activity analysis to provide time for gene transcription and protein translation to occur. As shown in Figure 4, we measured an increase in ICAM-1 concentrations in cell lysates from cells cultured in high glucose compared to those cultured in normal glucose. There was considerable inter-assay variability between replicates in our ICAM-1 assays, and our results did not achieve statistical significance (n=3, p=.066), likely due to small sample size. We then statistically analyzed the effects of 670 nm PBM on the relative level of ICAM-1. Data presented shows that PBM-treated cells cultured in high glucose exhibited a decrease in ICAM-1 levels. We observed a 60% decrease in ICAM-1 concentrations in PBM-treated cells cultured in high glucose compared to sham-treated cells cultured in high glucose. These differences were not statistically significant (n=3, .089). Each experiment was repeated twice. We anticipate that additional experiments will be necessary to observe a statistically significant difference. In the high glucose treated cells, ICAM-1 was

A.



B.

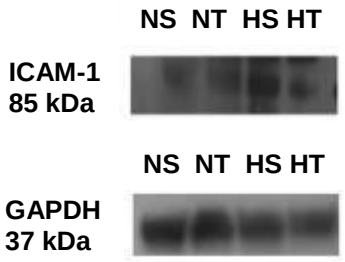


Figure 4: Comparison of respective ICAM-1 levels between treatment groups

(A) Bar graph, representing three separate experiments, showing the percentage of ICAM-1 in relation to GAPDH of Müller glial cells maintained in either normal glucose or high glucose medium, which received either PBM or sham treatment. Cell lysates were collected from Müller glial cells culture for the analysis of ICAM-1 concentrations. Cells either received five days of sham treatment or were treated with 670 nm PBM (n=3) (B) Representative Western blot of ICAM-1 and GAPDH bands for each treatment group (NS: normal glucose, sham-treated; NT: normal glucose, PBM treated; HS: high glucose, sham-treated; HT: high glucose, PBM treated).

reduced back down to levels comparable to both sham and PBM treated normal glucose cultured cells (n=3, p=1). Additionally, between the normal glucose cultured group which received 670 nm light and the normal glucose group, which received sham treatment there was no significant difference in the relative level of ICAM-1 (n=3, p=1). Therefore, in the absence of high glucose treatment with 670 nm LED does not affect the level of ICAM-1.

Part 2B. Effects of 670 nm Photobiomodulation on VEGF concentration in an in vitro model of DR.

VEGF plays a primary role in the progression of DR. Therefore, the inhibition of VEGF signaling has been a primary target for the treatment of DR. To assess the effects of 670 nm LED on VEGF concentrations, Müller glial cells were cultured in either high or normal glucose, with or without 670 nm light treatment. After six days in culture, the effect of high glucose on VEGF concentration was analyzed using a sandwich ELISA. No significant differences in VEGF levels were observed between high glucose and normal glucose medium culture conditions (n=3). Furthermore, 670 nm light treatment did not alter VEGF levels in either high glucose conditions or normal glucose conditions. Collectively, data presented in Figure 5 indicates that in a cell culture model of Müller glial cells, VEGF concentration is not affected by either the concentration of glucose nor 670 nm light treatment.

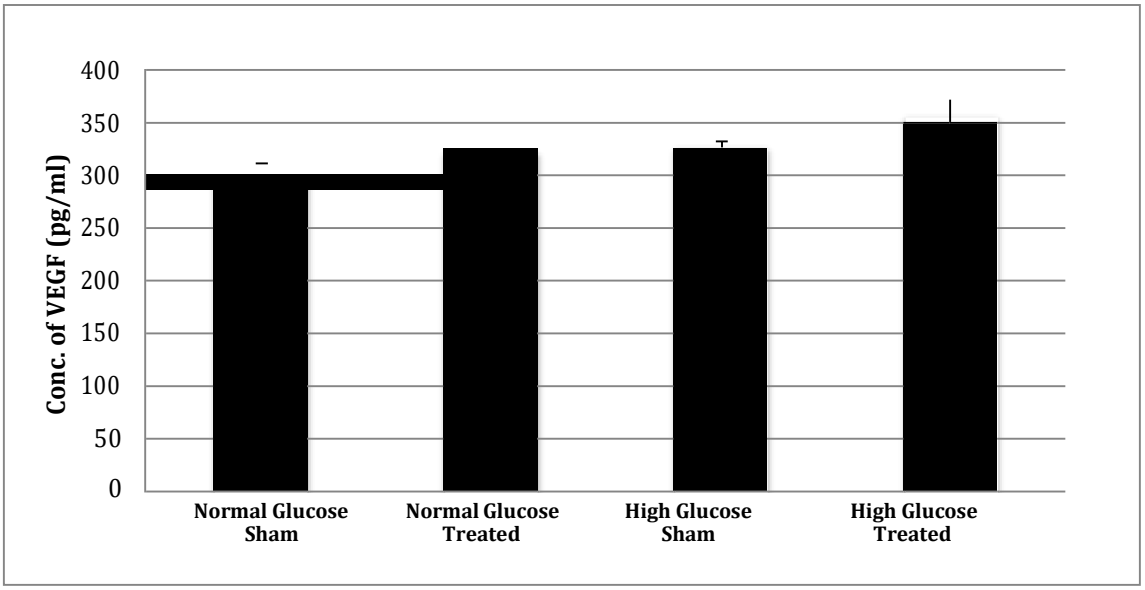


Figure 5: Comparison of VEGF concentrations between treatment groups
Bar graph, representing three separate experiments, showing VEGF concentration of Müller glial cells maintained in either normal glucose or high glucose medium, which received either PBM or sham treated for five days. Conditional media was collected from Müller glial for the analysis of VEGF concentrations. An ELISA was used for detection of VEGF, and concentrations were calculated from a standard curve.

V. DISCUSSION

The objective of this thesis study was to determine the effects of 670 nm light in an *in vitro* model of DR. For this investigation, Müller glial cells cultured in high (25 mM) glucose received 670 nm light at a dose (or fluence) of 4.5 J/cm^2. To analyze the effects of PBM on NF-κB transcriptional activity, transfected Müller glial cells were cultured under high glucose and were either sham-treated or PBM-treated for three days. The data presented shows that treatment with 670 nm LED significantly reduces the over-activation of NF-κB, which occurred as a consequence of exposure to high glucose concentrations. Importantly, NF-κB concentrations in PBM-treated high-glucose-exposed cultures was not different from that measured in cells maintained under normal glucose. Moreover, in comparison to their sham treated, high glucose-exposed counterparts, there was a 60% decrease in ICAM-1 concentrations. Our *in vitro* data on the effects of PBM on ICAM-1 concentrations in high glucose cultured Müller glial cells was comparable to the *in vivo* effects of PBM in diabetic rodents (Tang *et al*. 2013, Saliba *et al*. 2015). Unexpectedly, we observed no change in VEGF concentration under high glucose conditions compared to normal glucose cultured cells. Furthermore, 670 nm light did not affect VEGF concentrations under either glucose condition. Data indicates that in this cell culture model of DR, VEGF is not regulated under similar regulatory function as ICAM-1.

Although NF-κB is known to have diverse regulatory functions, its over-activation has shown to be correlated to an increase in oxidative stress and to be of pathological significance in animal models of DR (Nagai *et al*. 2007). Treatment with 670 nm LED

has been assessed in STZ diabetic rat models and shown to attenuate progression and improve visual acuity (Tang *et al*. 2013). Additionally, PBM decreased oxidative stress in both *in vivo* and *in vitro* studies of retinal ganglion cells, photoreceptors, retinal pigment epithelium cells, and Muller glial cells; which was the cell type also utilized for the present study (Tang *et al*. 2013). How PBM affects intracellular signaling, including NF-κB transcriptional activity and ICAM-1 or VEGF levels, is addressed in a Müller glial cell culture model in this study.

Previous *in vivo* and *in vitro* studies have demonstrated that a hyperglycemic environment induces activation of NF-κB. Pericytes cultured in high (30 mM) glucose for 8 h, 24 h, 48 h, or 1 week exhibited elevated levels of the NF-κB p65 unit in the nucleus (Romeo *et al*. 2002). NF-κB concentrations have also been analyzed in retinal capillaries of diabetic eye donors removed during the nonproliferative stage of DR development (Romero *et al*. 2002). Results from these studies revealed a greater concentration of NF-κB inside the nuclei of pericyte cells from diabetic donors in comparison to pericytes from non-diabetic donors (Romeo *et al*. 2002). This indicates increased translocation of NF-κB to the nucleus, the site where it is functional. However, the translocation of NF-κB to the nucleus is not necessarily suggestive of transcriptional activity (Hayashi *et al*. 1993). To induce DNA binding and transcriptional activity, NF-κB must be reduced by redox regulation (Hayashi *et al*. 1993). In our study, a 70% increase in NF-κB transcriptional activity in the high glucose cultured, sham treated groups was observed in comparison to cells cultured in normal glucose and not receiving treatment (Figure 3). These findings support previous work by Shelton *et al*.

(2007) and Shanmugam *et al*. (2003). Shelton *et al*. reported elevated NF-κB activity in transfected Müller glial cells after 8 hours in high glucose (25 mmol/L) in comparison to cells maintained in normal glucose (Shelton *et al*. 2007). In accordance with this data, transfected THP-1 (human leukemic monocytes) cells incubated under high glucose for three days presented with a five-fold increase in NF-κB activity in comparison to those cultured in normal glucose (Shanmugam *et al*. 2003). However, not all cell types appear to follow this pattern. Endothelial cells cultured in high (30 mM) glucose for 8 hrs, 24 hrs, 72 hrs, and one week had no significant difference in the translocation of NF-κB to the nucleus when compared to cells cultured in normal glucose culture media (Romeo *et al*. 2002). These results suggest that the translocation of NF-κB to the nucleus in response to high glucose appears to be cell type specific.

In addition to the observed increase in NF-κB, ICAM-1 levels were also elevated when Müller glial cells were cultured in high (25 mM) glucose conditions. We measured ICAM-1 levels by Western blot on samples treated for five days, based on a prior study by Chen *et al*. (2012) observing elevated levels of ICAM-1 when cells were cultured in high glucose for a range of one to seven days (Chen *et al*. 2012). After six days in high glucose conditions, a two-fold increase in ICAM-1 concentration relative to levels of GAPDH was observed (Figure 4). Our findings are in agreement with previous studies. Elevated levels of ICAM-1 were, as well, observed in STZ diabetic mice in comparison to non-diabetic control animals (Tang *et al*. 2013, Elmarakby *et al*. 2012). Likewise, in a Müller glial cell culture model of DR, a 4-fold increase in ICAM-1 levels was reported for cells cultured in high (25 mM) glucose in comparison to cells cultured in normal glucose

conditions for five days. Unlike prior studies, our data did not reach significance; however, conducting additional experiments or measuring membrane bound ICAM-1 rather than total protein concentration might enable us to achieve statistically significant changes. Since ICAM-1 induces its function when membrane bound, this may provide a better measure of biological significance.

We postulate that the induction of NF-κB activity, and the increase in ICAM-1 concentrations, observed in the present study as a result of high glucose exposure occurs due to an increase in oxidative stress based on the effects of antioxidants in models of DR (Kowluru *et al*. 2003). Two months post-induction of diabetes in STZ diabetic rats the activation of NF-κB was increased by 60% in retinal samples compared to non-diabetic counterparts (Kowluru *et al*. 2003). Corresponding to this increase in NF-κB activity was a reduction in the antioxidant capacity of superoxide dismutase and an increase in oxidative stress, as measured by the presence of 8-isoprostane in the blood and ratio of oxidized glutathione to reduced glutathione (Kowluru *et al*. 2003). The increase in NF-κB activity and oxidative stress, as well as the reduction in antioxidant capacity of superoxide dismutase, was partially attenuated in diabetic rats which were treated with the antioxidant resveratrol (Kowluru *et al*. 2003). Thus, it is likely that the observed increase in transcriptional activity may be related to a hyperglycemic induction in oxidative stress (Kowluru *et al.* 2003*,* Kim *et al.* 2007*).*

In the progression of DR, the induction of ICAM-1, as a result of NF-κB activation induced by oxidative stress, promotes the movement of leukocytes (leukostasis) and

subsequent death of endothelial cells (Kowluru *et al*. 2003, Joussen *et al*. 2001). Therefore, inhibition of NF-κB, directly or indirectly, may provide a means in which to control the progression of DR. As discussed, one manner in which NF-κB activity can be modulated is through the reduction of oxidative stress (Kowluru *et al*. 2003). In a multitude of models, treatment with light in the NIR range has been shown to diminish oxidative stress (Tang *et al*. 2013, Saliba *et al*. 2015, Silva Macedo *et al*. 2015, Purushothuham *et al*. 2014). Tang *et al*. (2013) determined the effects of PBM on oxidative stress in various cell cultures models of DR. For these studies, retinal glial cells, photoreceptors, Müller glial cells, and retinal pigment epithelial cells were maintained in 30 mmol/L glucose (Tang *et al*. 2013). For cells cultured in high (30 mM) glucose, there was a significant increase in oxidative stress, as indicated by the presence of superoxide when compared to cells cultured in normal (5 mM) glucose media (Tang *et al*. 2013). The effects of hyperglycemic conditions were ameliorated in groups receiving 670 nm LED treatment for four days at a dosage of 5 J/cm^2 (Tang *et al*. 2013).

In STZ-diabetic rats, light treatment was performed at the early onset of DR (7 to 10 days post STZ administration). Animals received 670 nm LED treatment once daily at an irradiance of 25 mW/cm^2 for 270 seconds producing a dose 6.8 J/cm^2 for nine weeks. Treatment with 670 nm LED resulted in decreased superoxide levels in the retina in comparison to non-diabetic controls (Tang *et al*. 2013). Similarly, diabetic mice presented with an increase in retinal superoxide concentrations in comparison to non-diabetic animals, which coincided with enhanced leukostasis and elevated ICAM-1

concentrations within the retinae of diabetic animals (Tang *et al*. 2013). Subsequent studies by Saliba *et al.* (2015) observed similar results to those presented by Tang *et al*. (2013). Animals were either sham treated or were treated with 670 nm LED for ten weeks at a daily dosage of 5 J/cm^2 (Saliba *et al.* 2015). They observed an increase in leukostasis, superoxide, and ICAM-1 levels in diabetic mice when compared to non-diabetic control mice. The increase in leukostasis, superoxide, and ICAM-1 induced by diabetes was ameliorated in groups treated with 670 nm LED (Saliba *et al.* 2015). Additionally, these animals had an improvement in retinal function as measured by ERGs (Saliba *et al.* 2015). Collectively, this data indicates that PBM attenuates oxidative stress, which corresponds to an alleviation of leukostasis as a result of decreased ICAM-1 concentrations. Due to the relationship between oxidative stress, NF-κB, and ICAM-1 in the development of DR, it was likely that this treatment would promote similar effects on NF-κB activity.

To test this, we cultured Müller glial cells under high (25 mM) glucose conditions and then treated the cells with 670 nm LED at a dosage of 4.5 J/cm^2, or sham treated, for three days. Treatment with 670 nm LED promoted a significant reduction in NF-κB activity and a reduction of ICAM-1 levels in a cell culture of Müller glial cells. As mentioned earlier, it is suspected that these observations occur as a result of a reduction in oxidative stress, as shown by Tang *et al.* (2013) and Saliba *et al*. (2015). This theory is supported by work performed by Shelton *et al*. (2007). Under homeostasis, glutaredoxin is considered protective and functions to prevent the irreversible oxidation of proteins. However, in diseases with unresolving oxidative

stress, such as DR, enhanced glutaredoxin activity may disrupt normal redox regulation of transcription factors including NF-κB. Such a state has been observed in Müller glial cells incubated in high glucose (Shelton *et al*. 2007). In Müller glial cells cultured in high glucose, there was a notable increase in glutaredoxin activity, NF-κB transcriptional activity, and an increase in ICAM-1 levels (Shelton *et al*. 2007). Additionally, when glutaredoxin was overexpressed, there was an increase in the nuclear localization of NF-κB (Shelton *et al*. 2007) Conversely, when glutaredoxin activity was knocked down in high glucose cultured Müller cells, there was a significant decrease in ICAM-1 (Shelton *et al*. 2007). These results suggest that the hyperglycemic induction of oxidative stress enhances the glutaredoxin redox regulation of NF-κB. Attenuation of this oxidative stress by PBM, as presented by Tang *et al*., could diminish the redox activity of glutaredoxin and, correspondingly, abate the increased activity of NF-κB, such as that observed in this study (Tang *et al*. 2013). However, since oxidative stress was not directly measured in the current study, further work is needed to compare the effects of PBM on oxidative stress, NF-κB, and ICAM-1.

In regards to prior work performed assessing the effects of PBM on the activity of NF-κB, results presented both in this study and prior studies remain inconclusive. In a model of burn wound healing 904 nm light modulated the activation of NF-κB as measured by western blot (Gupta *et al.* 2015). The injury was induced on Sprague-Dawley rats by an aluminum metal rod heated to 85°C (Gupta *et al.* 2015). Sprague-Dawley rats received treatment for seven days for a duration of 3, 10, or 15 minutes with 904 nm light, or received sham treatment (Gupta *et al.* 2015). NF-κB activity was

significantly reduced for injured animals, which received treatment with 904 nm light in comparison to their sham treated counterparts (Mafra de Lima *et al.* 2009). Conversely, in a model of airway inflammation treatment with PBM increased the activity of NF-κB (Mafra de Lima *et al.* 2009). Airway inflammation was induced in bronchi isolated from male Wistar rats. The bronchi were treated with 650 nm light twice daily for 42 seconds at a dosage of 1.3 J/cm^2 (Mafra de Lima *et al.* 2009). Treatment with 650 nm light reduced constriction to the bronchi when airway inflammation was induced. However, when and NF-κB inhibitors were introduced the beneficial effects of 650 nm light was attenuated. These results suggest that NF-κB activation by PBM is important for therapeutic effects (Mafra de Lima *et al.* 2009). However, due to the diverse role of NF-κB in maintaining cellular function, as well as varying effects observed between wavelengths and dosages further work is needed to better understand the effect of PBM on NF-κB in varying models.

Unexpectedly, we observed that high glucose culture conditions did not significantly alter VEGF concentrations compared to Müller glial cells cultured in normal (5 mM) glucose conditions (data representative of three independent experiments). We were surprised that VEGF was not increased by high glucose in Müller cells, as this does not compare with observations reported by previous studies. Bai *et al*. (2009) have suggested that Müller glial cells are primarily responsible for the increase in VEGF observed in animal models of DR. In conditional VEGF knockout mice, a Cre/lox system was used to determine the significance of Müller glial cell-derived VEGF. When VEGF synthesis was blocked in Müller glial cells of the conditional mice, a 44.5%

decrease in VEGF retinal concentration was observed in comparison to their WT counterparts (Bai *et al*. 2009). However, these results also indicate that more than 50% of VEGF is attributed to additional sources, including neuronal cells and RPE cells. Therefore, a pure Müller cell culture system may not provide substantial concentrations of VEGF to analyze the effect of 670 nm LED treatment. Analysis of VEGF concentrations in aqueous humor samples from diabetic patients, without PDR, revealed very low concentrations of this growth factor, suggesting that it may be challenging to measure VEGF changes above baseline levels (Oh *et al*. 2010). Comparison of VEGF concentrations in the aqueous humor, as well, presented no significant difference between samples collected from non-diabetics, diabetics, or diabetics with severe non-proliferative DR (Oh *et al.* 2010). It was not until patients had developed PDR that a significant increase in VEGF concentration was observed. The difference in VEGF concentration observed between patients with non-proliferative DR and PDR was 30 pg/mL (Oh *et al.* 2010). Therefore, based on findings by Bai *et al*. (2009), only half of the change in concentration was a result of Müller glial cells (Bai *et al*. 2009, Oh *et al*. 2010). Therefore, our model may not be representative of PDR. The changes in the concentration of VEGF may also not be great enough above baseline to determine statistical differences.

Differences between cell culture models and time points selected for analysis may account for the variability observed between our results and previous findings in animals and other cell culture models. For instance, Natarajan *et al*. (1997) cultured primary vascular smooth muscle cells in high glucose (25 mmol/L) and normal glucose (5

mmol/L). Each group of cells were cultured for two passages under selected conditions, and then a northern blot was performed for detection of VEGF mRNA (Natarajan *et al*. 1997). High glucose conditions resulted in a 2.5-fold increase in VEGF mRNA in comparison to cells cultured in normal glucose conditions (Natarajan *et al.* 1997). In primary rMC cultured in high glucose (30 mmol/L), both protein and mRNA levels of VEGF were increased at the time points of 8 hrs, 12 hrs, and 24 hrs (Ya *et al.* 2012). However, levels of VEGF in primary rMC decreased from 24 to 48 hours (Ya *et al.* 2012). Therefore, we may have missed the time point at which VEGF is elevated.

Contrary to these studies, Brooks *et al*. suggest that high glucose alone may not effectively induce VEGF synthesis in Müller glial cells (Brooks *et al.* 2005). Brooks *et al*. (2005) analyzed the effects of pH and glucose on VEGF production under normoxic and hypoxic conditions. After 48 hours, it was observed that when cells were cultured under hypoxic conditions both glucose and pH had a significant effect on VEGF production (Brooks *et al.* 2005). However, if cells were cultured in normoxic conditions for 48 hours, pH and glucose had no measurable effect on VEGF synthesis (Brooks *et al.* 2005).

Comparison of Ya *et al.* (2012) and Brooks *et al.* (2005) protocols indicate two differences, time course of experiments and serum presence. In work performed by Natarajan *et al*. (1997), and Ya *et al.* (2012) media was supplemented with 0.2% BSA or 0.5% BSA, respectively. In our study and Brooks *et al.* medium was supplemented with 2% FBS and 10% FBS, respectively. In these studies, hyperglycemia alone was not

found to induce VEGF (Brooks *et al.* 2005). Both of these serum supplements may contain constitutive growth factors, which could affect results of the assays. FBS, in particular, may contain growth factors unfavorable for analysis of VEGF. In a cell culture of dermal fibroblasts, 10% FBS was found to upregulate expression of pigment epithelium-derived factor, which is known to inhibit the synthesis of VEGF (Man *et al.* 2009, Zhang *et al.* 2006). Differences in serum type and serum concentrations could account for the observable difference. In addition to differences in serum, the time course utilized varied among the studies. A time course analysis, as well as, studies analyzing differences between culture conditions may help clarify the variability observed between the current and prior studies.

Our experimental design also allowed us to examine PBM effects in the presence of normal glucose levels. We show that PBM has no effect on NF-κB or ICAM-1 levels in cells cultured in normal glucose medium (Figure 3 and 4). This suggests that, in the absence of high glucose, PBM does not have an impact on NF-κB transcriptional activity and relative level of ICAM-1. Thus, in regards to DR, treatment with 670 nm LED may only have certain effects while in a hyperglycemic state.

Taken together, we show that 670 nm LED treatment may provide therapeutic effects in models of DR by reducing the increase in NF-κB transcriptional activity and the increase in the level of ICAM-1 induced by high glucose culture conditions. Further research utilizing animal models will help develop an understanding of the effects PBM has on intracellular signaling. As mentioned, signaling through the AGE-RAGE pathway also

plays a role in the development and progression of DR. Animal studies will also allow for the analyzation of treatment with 670 nm LED in advanced stages of DR, and confirm if this treatment will have the same effect on transcriptional activity of NF-κB and changes in the level of ICAM-1.

Conclusion and Future Directions

The aim of this study was to determine the effects PBM using 670 nm light for 180 seconds on the activity of NF-κB, as well as its effects on concentrations of ICAM-1 and VEGF. The hypothesis for this study was that 670 nm light will attenuate the hyperglycemic induction of NF-κB and reduce the concentrations of ICAM-1 and VEGF in a Müller glial cell culture. The data presented in this thesis partially supports the central hypothesis. Data showed that in the presence of high glucose 670 nm light treatment significantly reduced NF-κB activity. Similar results were also obtained for ICAM-1. When Müller glial cells cultured in high glucose were treated for five days with 670 nm LED the level of ICAM-1 detected was 60% lower than that observed in high glucose cultured cells receiving sham treatment. We also analyzed the effects of PBM on NF-κB and ICAM-1 when cells were cultured under normal conditions. Between normal glucose cultured groups, which received 670 nm light treatment and those which did not, there was not a significant difference in either transcriptional activity of NF-κB or the level of ICAM-1. Further studies are needed to support these findings due to ICAM-1 analysis not reaching statistical differences. Performing a time course study for both ICAM-1 and NF-κB would also better allow a direct comparison of the transcriptional activity of NF-κB to the synthesis of ICAM-1.

Currently, our data indicate that VEGF may not be regulated by NF-κB in our model system. However, cell culture analysis for the effects of high glucose on VEGF in previous studies suggests contradictory findings to the data presented (Ya *et al.* 2012, Brooks *et al.* 2005). As suggested above, adjustments to time course and culture conditions may be needed to be made for further VEGF studies.

Translation into an animal model may also provide the best manner in which to study the effects of DR. Performing these experiments in an animal model will allow for examination of changes in the retina, where multiple cell types are present, and cell-cell contact and paracrine signaling may take place. To do this, the treatment protocol (240 seconds daily, 25 mW/cm^2, 6 J/ cm^2) previously established by Tang *et al*. (2013) would be followed, since therapeutic effects, as indicated in improvements in retinal function, have been previously established. Changes in NF-κB translocation would be measure from excised retina samples. Furthermore, ICAM-1 and VEGF concentrations can be measured by an immunoprecipitation assay as described in Tang *et al*. (Tang *et al.* 2013). Addition of a NF-κB inhibitor would also provide a manner to confirm the relation between NF-κB and ICAM-1 in a model of DR.

In conclusion, the work presented in this study does support that treatment with 670 nm light has an inhibitory effect on NF-κB and reduces the synthesis of ICAM-1. This work has begun to provide a mechanism of action for treatment with 670 nm light in our model system. Examining the role of NF-κB with and without PBM in an animal will

allow for further mechanistic insight into PBM. Further elucidation of the mechanism of action for 670 nm light treatment in a model of DR, will assist in the movement of PBM therapy to clinical use.

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