PHOTOMODULATORY EFFECTS ON INSULIN SECRETING BETA CELLS

by

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Presented to the Faculty of Graduate School of

The University of Texas at Arlington in Partial Fulfilment

of the Requirements

for the degree of

MASTER OF SCIENCE BIOMEDICAL ENGINEERING

THE UNIVERSITY OF TEXAS AT ARLINGTON

May 2021

Acknowledgments

I would like to thank my supervising professor Dr. Michael Cho for his professional supervision and guidance throughout this project. I would also like to thank Dr. Caleb Liebman and Dr. Edidiong Inyang for taking their time to teach me and guide me though the lab experimentations and research development. I am also grateful for my fellow researchers, Noemi Salgado and Annie Phillips, for being there whenever I needed help. Financial support from Drs. Alfred R. and Janet H. Potvin is gratefully acknowledged. I would also like to thank my parents and friends that have helped me throughout my path at the University of Texas at Arlington.

May 07, 2021

Abstract

PHOTOMODULATORY EFFECTS ON INSULIN SECRETION IN BETA CELLS

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Diabetes mellitus is a chronic metabolic disease characterized by increased blood glucose levels and affects more than 8% of people worldwide (Rutter et. al, 2015). Typically, diabetes is divided into two large subcategories: type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is an autoimmune disorder caused by the attack of insulin secreting cells by t-cell mediated inflammatory response (Khrraoubi & Darwish, 2015). Currently, islet transplants offer a clinical treatment for restoration of glucose control. However, the current procedure may not be optimal as many transplanted islets do not survive because of the stresses induced during the process (Miki et. al, 2017). Photobiomodulation (PBM) has shown to have antioxidant capabilities in different cell types (Hamblin, 2017). Because a reduction of stress in islets may lead to improved functionality, PBM could enhance insulin secretion. Using mouse insulinoma β-cells as a model, the current study is aimed at demonstrating the capability of PBM to increase the protective antioxidant activity and to validate an enhanced insulin secretion. Finally, we tested the hypothesis that the rates of photoenergy delivered to the cells modulate the PBM-induced effects in β-cells.

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Chapter 1 Introduction

1.1 Diabetes Mellitus

Diabetes mellitus is a chronic metabolic disease mainly characterized by increased blood glucose levels. The hyperglycemia is caused by insulin inaction, insulin deficiency, or a combination of both (Tuomi, 2005). More than 8% of people worldwide are affected by diabetes mellitus and this number is expected to double within the next 20 years (Rutter et. al., 2015). It is believed that the increase in diabetes cases is caused by the worldwide increase in sedentary lifestyles. The possible complications for diabetes mellitus include stroke, cardiovascular disease, blindness, and kidney failure (Rutter et. al., 2015 & Khan et. al., 2015). According to a review done by Kharroubi and Darwish (2015), genetic and nongenetic factors are involved in diabetes development and specific diagnosis is required for risk assessment and complication predictions.

1.1.1 Type 1 Diabetes

Type 1 diabetes is caused by the absolute loss of insulin secretion ability (Rutter et. al., 2015). More specifically, type 1 diabetes (T1D) is an autoimmune disease in which beta cells are destroyed by T-cell mediated inflammatory response (Kharroubi & Darwish, 2015). Beta - cell destruction leads to a decreased ability to produce insulin. This is due to the fact that beta - cells are solely responsible for insulin production and secretion. A person with T1D produces autoantibodies against pancreatic islet cells, which are responsible for blood glucose regulation. The specific types of autoantibodies created by the patient are islet cell autoantibodies, autoantibodies to insulin (IAA), glutamic acid decarboxylase, protein tyrosine phosphatase, and zinc transporter protein (Kharroubi &

Darwish, 2015). Genetic factors play an important role in T1D predisposition and development; however, environmental factors can also affect T1D. For example, viral factors, viral infections, low vitamin D levels, prenatal exposure to pollutants, early infant nutrition, and insulin resistance caused by childhood obesity can lead to T1D advancement. It is common for T1D to develop suddenly with symptoms such as increased thirst, frequent urination, extreme hunger, unexpected weight loss and extremely low energy levels (Mayo Clinic, 2021). Currently, T1D patients manage their blood glucose levels by regularly administering insulin. Different kinds of insulin are available for treatment and vary in how quickly blood sugar is lowered, how long the insulin works inside the body, and when insulin is at its maximum strength (Center of Disease Control and Prevention, 2021). In addition to autoimmune T1D there are two other kinds of T1D, idiopathic T1D and fulminant T1D. Idiopathic T1D is less severe than autoimmune T1D and is of unknown origin. Fulminant T1D is characterized by rapid beta cell destruction that results in an almost complete inability to produce insulin (Kharroubi & Darwish, 2015).

1.2 Beta Cells

Pancreatic beta cells are solely responsible for insulin release in humans and many other animals. Insulin release is triggered by increased blood glucose concentration, hormones, and nervous stimuli (Kulkarni, 2004). The release is directly proportional to the blood glucose level (Rutter et. al., 2015). Insulin release happens in two phases. The first phase, referred to as the rapid and transient phase, consist of a high concentration of insulin release lasting 3-5 minutes (Rorsman & Renstrom, 2003). It is

then followed by second phase, referred to as the sustained phase, in which insulin is secreted at a sustained lower rate lasting about 20 minutes (Rorsman & Renstrom, 2003). The two-phase mechanism is important because it ensures that enough insulin is released to regulate blood glucose levels, while preventing hypoglycemia which can be deadly (Rutter et. al., 2015).

1.2.1 Inside the Cell During Insulin Release

For insulin secretion to occur, glucose must first enter the beta cell via glucose transporter 2 (GLUT2) and is transported by hexokinase IV. Hexokinase IV is not allosterically regulated in beta cells. This allows for glucose to enter the cell without acknowledging any kind of feedback inhibition from glucose-6-phosphate. The glucose is then converted to ATP through glycolysis, the citric acid cycle, and oxidative phosphorylation (Tokoph, 2019) (Gerber & Rutter, 2017). The amount of ATP produced is proportional to the amount of glucose in the bloodstream because of the nature of hexokinase IV. When there is a high concentration of glucose in the bloodstream, ATP production increases causing a closure of the K^+ efflux channel. The loss of K^+ efflux causes a change in the membrane potential (depolarization), which then opens the voltage dependent Ca^{2+} channel, increasing Ca^{2+} influx (Rorsman & Renstrom, 2003). The increased Ca^{2+} inside the cell triggers the exocytosis of insulin from insulin granules. It is important to note that insulin release is a graded mechanism. Meaning that the amount of insulin released by the cell is directly proportional to the glucose levels of the bloodstream (Tokoph, 2019) (Gerber & Rutter, 2017). Low glucose in the

bloodstream produces low insulin release levels, and high glucose in the bloodstream produces high insulin release levels.

1.2.2 Beta Cell Antioxidant Capacity

The process of insulin secretion causes an increase in reactive oxygen species (ROS) via oxidative phosphorylation inside beta cells (Gerber & Rutter, 2017). More specifically, as blood glucose concentrations increase, ATP production inside beta cells increases which in turn produces more ROS. Oxidative phosphorylation accounts for 1-4% of ROS production (Miki et al., 2017). Hypoxia, ischemia and cytosolic enzymes also produce ROS in beta cells (Chang et.al., 2015). Chronic oxidative stress can impair beta cell function at the specific mechanisms needed for insulin synthesis and secretion (Jung et. al., 2017). For this reason, oxidative stress is a common feature of T1D. As a primary defense against oxidative stress, cells have antioxidant enzymes; however, beta cells have been reported to have lowest levels of antioxidant enzyme expression and activity (Miki et. al., 2017).

1.2.3 Role of SOD2 in Beta Cells

Antioxidant enzymes such as catalase, selenium dependent glutathione peroxidase (GPX1), and Mn-superoxide dismutase (SOD2), are present in beta cells but have low activity when compared to other cell types (Lei & Vatamaniuk 2010). SOD2 and catalase work in tandem to reduce oxidative stress. SOD2 converts mitochondrial superoxide (O_2^-) into hydrogen peroxide (H_2O_2), then catalase converts hydrogen peroxide into water (H_2O) (Lei & Vatamaniuk 2010). A study performed by Kang et al.

(2014) analyzed glucose stimulated insulin secretion (GSIS) in two different groups of mice. Both groups were fed a high fat diet however one group had wild type SOD2 expression (sod2+/+) while the other had heterozygous deletion of SOD2 expression (sod2+/-). The research determined that the reduction in SOD2 impaired the beta cell's GSIS capacity further elucidating the importance of SOD2 in beta cell function. A different study performed by Jung et al. (2017) demonstrated that increasing SOD2 levels in beta cells protected them from death, allowing for sufficient insulin secretion in the diabetic model mice.

1.3 Islet Transplant

Islet transplantation is performed to restore glycemic control in T1D patients after a failed pancreas transplantation. Many events need to occur before a patient receives an islet transplant. These events are: (1) patient experiences pancreatic failure, (2) patient receives a pancreas or kidney transplant that restores normal glucose levels, (3) glycemic control is lost, (4) islets are transplanted, and (5) glucose levels return to post pancreatic transplant levels (Gamble et. al., 2018). An islet transplant is preferred over a secondary pancreas or kidney transplant because the patient's increase in age can cause surgical complications. Additionally, islet transplants have shown to delay the progression of retinopathy and nephropathy possibly justifying the chronic use of immunosuppressants (Bruni et. al., 2014). A large problem with islet transplantation is cell death during isolation, purification, and infusion. During these steps islet cells experience ischemia, chemical stress, and mechanical stress which all result in oxidative stress. The increase in oxidative stress causes loss of beta cell functionality

(Miki et. al., 2017). Most times, more than one islet transplant is required to restore glycemic control making the procedure less than optimal. Although the procedure is currently less than optimal, additional research has shown that culturing islets for 7 to 65 hours before transplantation has led to better outcomes. Mainly because the additional time allows for preparation of the patient and decreasing immunogenicity of the allograft (Froud et. al., 2005). Because of the success of culturing islets before transplantation, this study analyzes the effect of photobiomodulation in beta cells for up to 3 days.

1.4 Antioxidant Capabilities of Photobiomodulation

Photobiomodulation (PBM) has been studied for almost 50 years. Because of the variety of results observed, it is important to consider the biphasic dose response produced by cells when exposed to PBM. High levels of light can have inhibitory effects, while low levels of light can have stimulating effects. It has been shown that at adequate doses PBM can reduce ROS levels in oxidatively stressed cells. A review done by dos Santos et. al. (2017) outlined various studies in which PBM reduced oxidative stress after muscle injury. The review concluded that PBM reduced oxidative stress markers and increased antioxidant substances such as superoxide dismutase (SOD) and catalase, which are both present in beta cells. A current problem with PBM studies is the large range of PBM parameters used. In one review alone, wavelengths ranged from 650 nm to 1200 nm, power ranged from 5 mW to 100 mW, and fluence ranged from 5 to 180 J/cm^2 (Hamblin, 2017). A specific study performed by Zare et. al. (2019)

showed that PBM can improve survival, doubling time, and proliferation in diabetic mesenchymal stem cells *in vitro*.

The detailed mechanisms by which PBM interacts with cells is yet to be fully understood. However, a consensus has been achieved of several proposed mechanisms by which PBM interacts with cells. A review done by de Freitas & Hamblin (2016), summarized the various mechanisms that have been analyzed in recent years. It is apparent that cytochrome C oxidase (CCO) in the electron transport chain of mitochondria is perhaps the most important chromophore involved in initiating the cascade of effects caused by PBM. Many schematics have been developed to interpret the intracellular effects of PBM. As illustrated by Ao et. al. (2017; Figure 1), PBM can increase the mitochondrial respiration and generate reactive oxygen species (ROS) transiently.

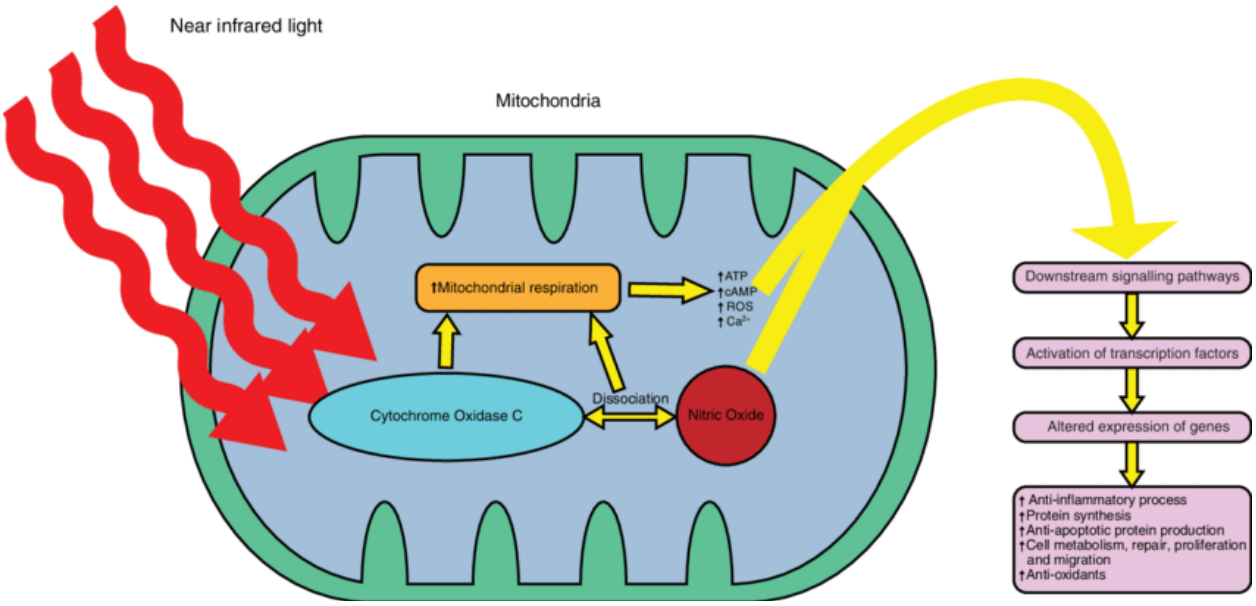

Figure 1. Diagram of Mechanisms of PBM.

The illustration was adopted from a previous published work of Ao et. al. (2017). It depicts potential coupling mechanisms in response to PBM using near infrared photostimulation. While multiple signaling pathways are suggested in this schematic diagram, this thesis is designed to probe antioxidant effects by PBM and an important downstream effect of enhanced insulin secretion in β-cells.

It has been demonstrated that a fluence of 9 J/cm^2 can reduce oxidative stress in a type 1 diabetic kidney model (Lim et. al, 2009). Additionally, Hawkins and Abrahamse (2006) noticed DNA damage when using a fluence of 10 J/cm^2 or higher. A common trend in previous studies analyzed was a decrease in oxidative damage and an increase in antioxidant enzymes such as SOD and catalase. Because of the success seen by Lim et al (2009) in a diabetic kidney model, a fluence of 9 J/cm^2 will be used throughout this study.

CCO is complex IV of the electron transport chain; it is responsible for oxidizing cytochrome C by transferring the electron to dioxygen species (Ahmad et. al., 2020). There are 4 redox centers on CCO, of particular interest is the dimer copper A (Cu_A). Cu_A has a strong absorption of near infrared wavelengths when oxidized (Bale et al, 2016). Previous studies have reported that red (600-700 nm) and near infrared wavelengths (770-1200 nm) interact with CCO and have maximal penetration depth when compared to other wavelengths of light (Hamblin, 2017). More specifically, at 810 nm in wavelength, light absorbance by CCO is optimal (Bale et al, 2016) (Figure 2). When CCO is irradiated by red and near infrared light nitric oxide is dissociated causing an increase in ATP production, enzyme activity, and oxygen consumption (Hamblin, 2017). The increase in cellular metabolism after the dissociation of nitric oxide is due to the fact that nitric oxide competes with oxygen at CCO reducing the rate oxygen consumption (Taylor & Moncada, 2009). Along with the selection of the fluence of 9 J/cm^2, the wavelength of 810 nm was chosen to be used in this study for those reasons.

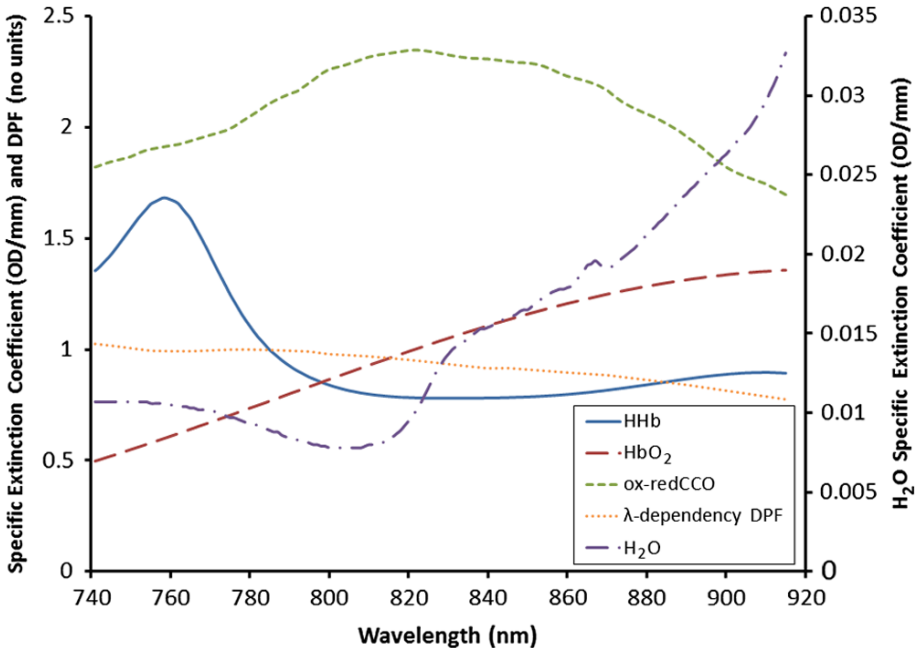

Figure 2. Specific Extinction Coefficients.

Absorbance spectra of molecules such as CCO, hemoglobin and water are shown. For this study, it is important to note that from 800 nm to 810 nm CCO has the highest absorbance.

Source: Bale et. al, (2016)

1.5 Possible Influence of Utilizing Different Power Densities

Photobiomodulation studies typically focus on fluence and wavelength. Few studies have been performed to determine if power density is also an important parameter to consider during PBM, especially when it comes to irradiating beta cells. Gál et. al. (2009) compared the effects of different power densities on healing skin wounds, for example. The study determined that higher densities were more effective; however, the highest power density used was 15 mW/cm^2. A review was performed to analyze possible effective PBM parameters. The review was divided into different categories. Because beta cells are considered to have a high number of mitochondria, we are only

interested in that specific category (Wollheim & Maechler, 2002). Power densities in this review ranged from 0.8 mW/cm^2 to 1.75 W/cm^2, spanning more than 3 decades of the intensity parameter. It was determined that cells with a high number of mitochondria respond to lower power densities (Zein et. al, 2018). Because of this conclusion, power densities of 150 mW/cm^2 and 300 mW/cm^2 were analyzed in this study. Moreover, since the fluence of 9 J/cm^2 is kept constant, the exposure time was varied 60 to 30 sec and thereby allowing to test whether the rates at which photoenergy are delivered affects the PBM-induced physiology in β-cells.

1.6 Aim of this Study

Given the numerous studies showing possible antioxidant enhancement in different cell types, this study aims to understand and explore the mechanisms occurring in pancreatic beta cells after PBM using different power densities *in vitro*. To do this, cell proliferation, antioxidant enzyme expression, and GSIS were analyzed. The study is performed in an effort to create preliminary results for the possible irradiation of pancreatic islets before islet transplantation in an attempt to create more optimal procedure outcome.

Chapter 2 Methods

2.1 Cell Culture

The mouse insulinoma b-cells (BTC-6) were purchased from ATCC (CRL-11506, Manassas, VA). Trypsin was purchased from Sigma-Aldrich (St. Louis, MO). The cells were grown in T-25 flask using a density of 20,000 cell/cm^2 in 84% high glucose Dulbecco's Modified Eagle Media (DMEM)(Sigma-Aldrich, St. Louis, MO), 15% fetal bovine serum (FBS) (Atlanta Biologics), and 1% penicillin-streptomycin (PS) (Sigma-Aldrich, St. Louis, MO). Cells were dissociated using trypsin and seeded in petri dishes on 22 mm by 22 mm glass slides. Once seeded in petri dishes the cells were maintained in 84% low glucose DMEM (Sigma-Aldrich, St. Louis, MO), 15% FBS, and 1% PS. Cells were sub-cultured about once per week. The cells would grow inside the incubator with the temperature set at 37°C, with 5% CO_2.

2.2 Laser Exposure

An 810nm laser was used for all experimentation. Both control and experimental petri dishes were wrapped in sterilized parafilm to prevent contamination during laser exposure. 70% ethanol was used to sterilize the parafilm. The process of wrapping the petri dishes was performed inside a cell culture hood. After being wrapped, all petri dishes were moved to a dark room where the 810 nm laser is located. For the first experimental group each petri dish was exposed to the laser for 60 seconds at a power density of 150 mW/cm^2, yielding a fluence of 9 J/cm^2. For the second experimental group, each petri dish was exposed to the laser for 30 seconds at a power density of

300 mW/cm^2, yielding a fluence of 9 J/cm^2. The control petri dishes were kept in the same dark room while experimental groups were exposed to laser.

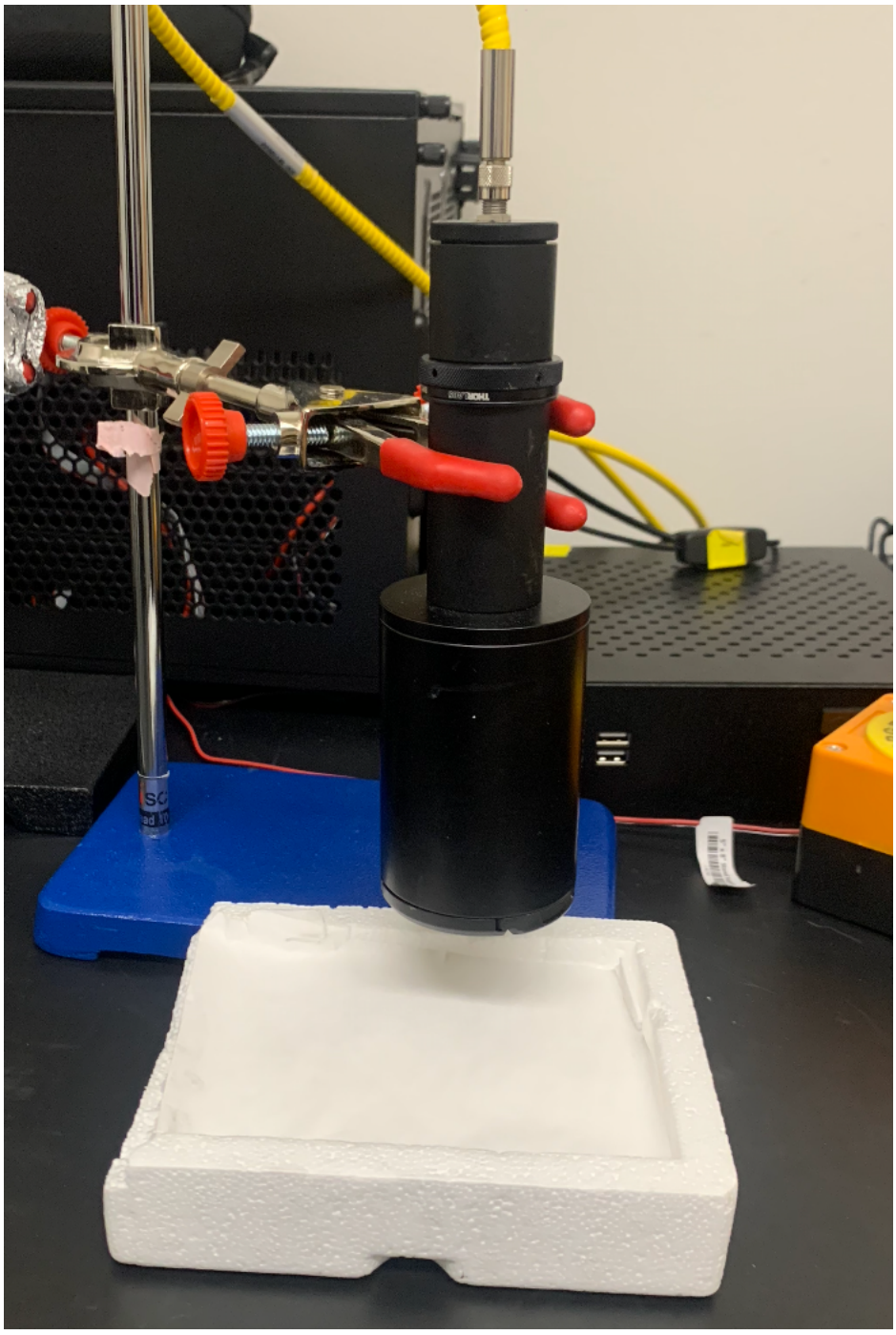

Figure 3. Laser Exposure Representation.

A common concern with laser exposure is temperature rise in either media or tissue. Dr. Cho's laboratory documented temperature change over time in media while being exposed to the maximum power density of the exposure system (380 mW/cm^2) at a wavelength of 810 nm. During the first minute of exposure, the change in media temperature was less than 1 °C, which is the within the suggested threshold for minimal thermal effect (McColloch et. al, 2020). Given that the power density used during this study is less than 380 mW/cm^2 and that the greatest time of exposure is 1 minute, it is determined that the parameters used in this study are unlikely to cause thermal effects.

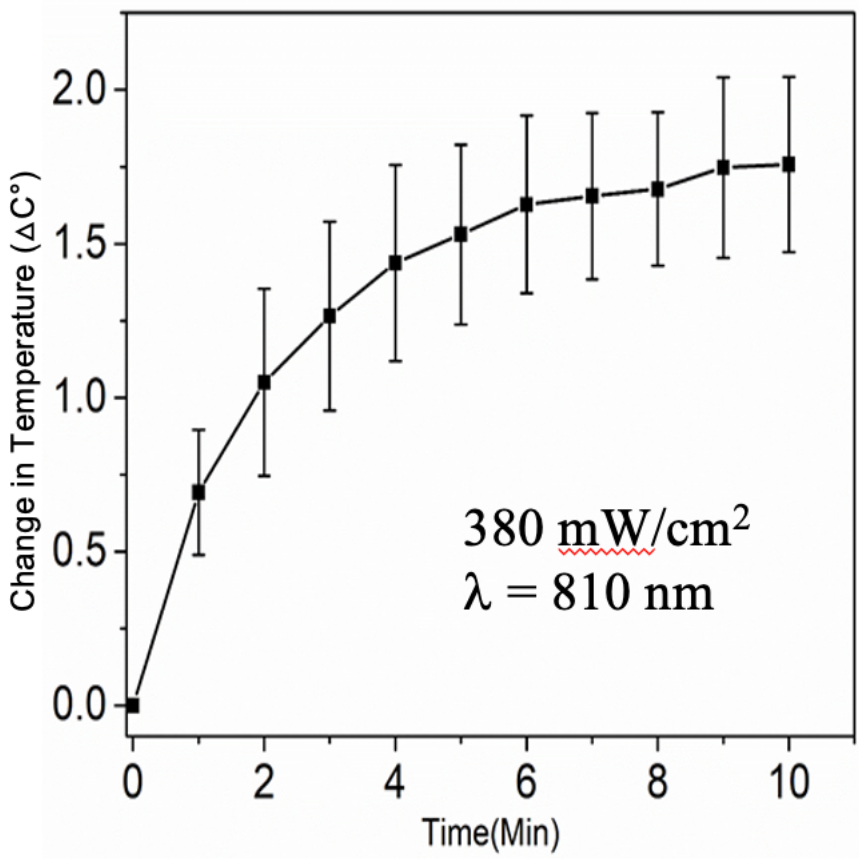

Figure 4. Change in Media Temperature During PBM.

The figure above represents the change in temperature of cell media of 2 mL. Temperature was measured using a thermistor and recorded at 1 min intervals. [Adopted from the work of Dr. Caleb Liebman in Cho laboratory]

2.3 Superoxide Fluorescent Microscopy – live cell imaging

For live cell imaging, cells were stained using MitoSox (5 μM, Thermo-Fisher, Waltham, MA), 1 drop/mL NucBlue. This stain was used to image mitochondrial superoxide and nuclei respectively. Hank's balanced salt solution (HBSS) (H8264, Sigma, St. Louis, MO) was used throughout this procedure. Cells were stained for 30 minutes at 37°C in 5% CO2. For imaging, cells were mounted on an imaging chamber containing HBSS. The pattern used for acquiring images can be seen in Figure 5. Typically, the pattern resulted in 9 images being taken for each sample.

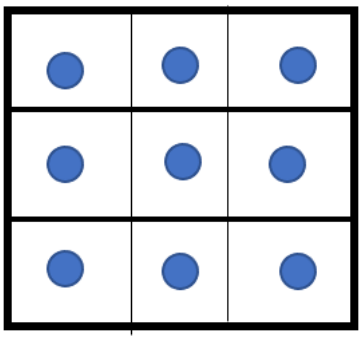

Figure 5. Grid Pattern Used to Acquire Cell Images.

The blue circles represent the approximate locations where each image was acquired.

2.4 Image Analysis – Superoxide Images

Fluorescent images were analyzed using NIS-Elements software that was developed by Nikon. Regions of interest (ROI) were created around individual cells to calculate average intensity per cell in each image. Segmentation of each cell was performed by NIS-Elements image processor. Background intensity was subtracted from each individual value generated from the RIOs.

2.5 Immunofluorescent Microscopy

Cell were rinsed with phosphate buffered saline (PBS). The cells were then fixed using 4% paraformaldehyde for 15 minutes. Once again, the cells were rinsed with PBS. Subsequently, the cells were permeabilized with 0.1% Triton-X 100 solution for 10 minutes. Cells were rinsed thrice, and then non-specific binding was blocked using 2.5% bovine serum albumin (BSA) (Sigma Aldrich St. Louis, MO) solution for 30 minutes. Primary antibodies (1:250) was used to bind SOD2 (Invitrogen Rockford, IL PA5-30604) for 5 hours. The secondary fluorescence staining was performed for 60 minutes at room temperature using anti-rabbit antibodies (1:500 dilution, Invitrogen

Rockford, IL A11008), was washed again, and mounted for imaging. Image acquisition was performed at the locations schematically described above (see Figure 5).

2.6 Image Analysis – SOD2 Images

Image J was used to analyze SOD2 intensity per cell. The cell numbers were first counted based on NucBlue images. Then Image J was used to analyze the total intensity of the SOD2 stained image with appropriate background subtraction. The raw values generated by the software were then used for mathematical calculation of intensity per cell. The total SOD2 intensity would be divided by the cells counted in the NucBlue image resulting in mean intensity per cell. Once a mean value was generated at each of the 9 locations, average of the 9 values would be calculated to represent the SOD2 expression in a given sample. Typically, more than 300 to 800 cells were examined per each sample.

2.7 Glucose Stimulated Insulin Secretion

Cells were seeded at 30,000 cell/cm^2 before experimentation. Cells were rinsed and then incubated in Kreb's Ringer buffer (KRB) with 0.1% BSA (118.5 mM NaCl, 2.54 mM CaCl2, 1.9 mM KH2PO4, 4.74 mM KCL, 25 mM NaHCO3, 1.19 mM MgSO4, 10 mM HEPES, pH 7.4). After being washed, cells were incubated for 60 minutes in KRB. Incubation was followed by a 15-minute exposure to 3.0 mM glucose solution to induce insulin secretion. A concentration of 3.0 mM was chosen for glucose challenge because characterization of the BTC-6 cell line determined that maximal glucose stimulation was seen at the concentration of 2.8 mM (Piotout et al., 1995). Approximately 1 mL of 3.0

mM glucose solution was collected and frozen to later be analyzed using a mouse insulin ELISA kit from Thermofisher. Cells were also frozen for CyQuant (Thermofisher) analysis that would be used to normalize insulin secretion per cell.

2.8 CyQuant Cell Proliferation Analysis

Cells used for insulin secretion were frozen and stored at -20°C. To prepare for analysis cells were lysed using CyQuant Lysis Buffer. 200 uL of lysed cell solution were added to a dark sided 96 well plate. Each sample was analyzed 3 times by having triplicates of the solution within the well plate. CyQuant stain was added to each well at a 2x concentration according to the manufacture protocol. Additionally, a standard curve was created (Figure 6) to determine the actual number of cells in each sample.

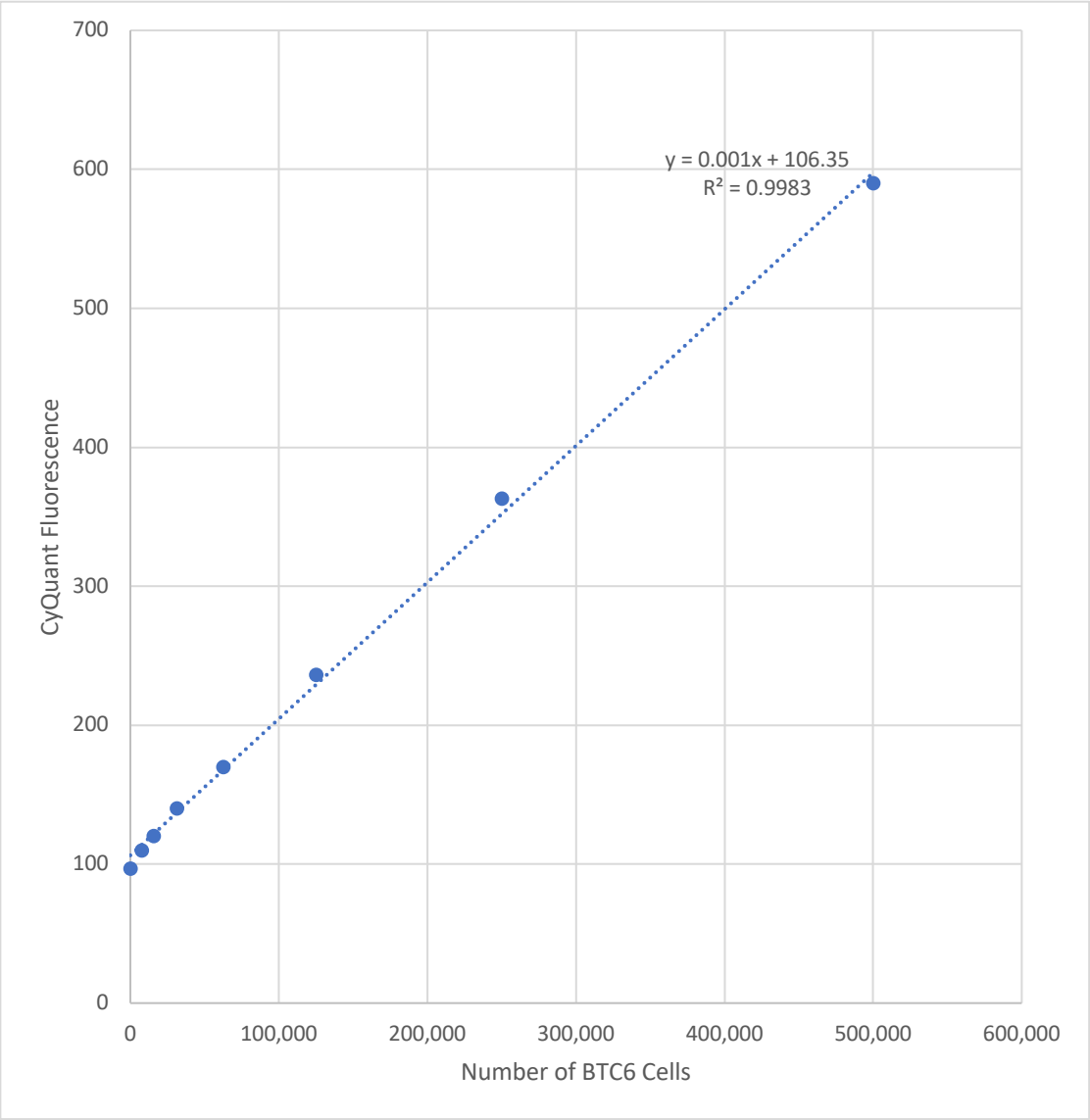

Figure 6. CyQuant Standard Curve for βTC-6 Cell Line.

A linear relationship was established between the CyQuant fluorescence and the cell numbers up to 500,00 cells.

2.9 Statistical Analysis

For each experiment, the experimental values for each PBM parameter were compared to the control individually using a one-sided unpaired t-test. A one-sided t-test was

preformed because the data was expected to follow a normal distribution and show an increase in one direction. The data was considered statistically significant if $p < 0.05$.

2.10 Group Naming

Throughout the study, experimental groups will be referred to using abbreviations formatted as the following: # of days, the letter D followed by the power density used. If the group is a control, it will be followed by the letter C. For example, the group that was irradiated for 2 days using a power density of 300 mW/cm^2 will be referred to as 2D300. Actual PBM treatment was carried out once per day. The group that was used as a control for 1 day of PBM exposure will be referred to as 1DC.

Chapter 3 Results

3.1 Cell Proliferation

Cell Proliferation was analyzed using CyQuant Cell Proliferation analysis. Representative images of b-cells for 3 day experiments are shown in Figure 7. Based on image analysis, for day 1 and day 2 there is no change in cell proliferation when comparing the control cells to the cells exposed to the laser light. For day 3, the number of cells in the 3D150 group increased by approximately 100,000 cells (~ 30% increase) when compared to the control, and the 3D300 group decreased by approximately 50,000 cells (~ 15% decrease) when compared to the control, suggesting the laser intensity may have altered the cell proliferation. However, changes in cell count were not determined to be statistically significant when analyzed using a one-sided unpaired t-test (Figure 8).

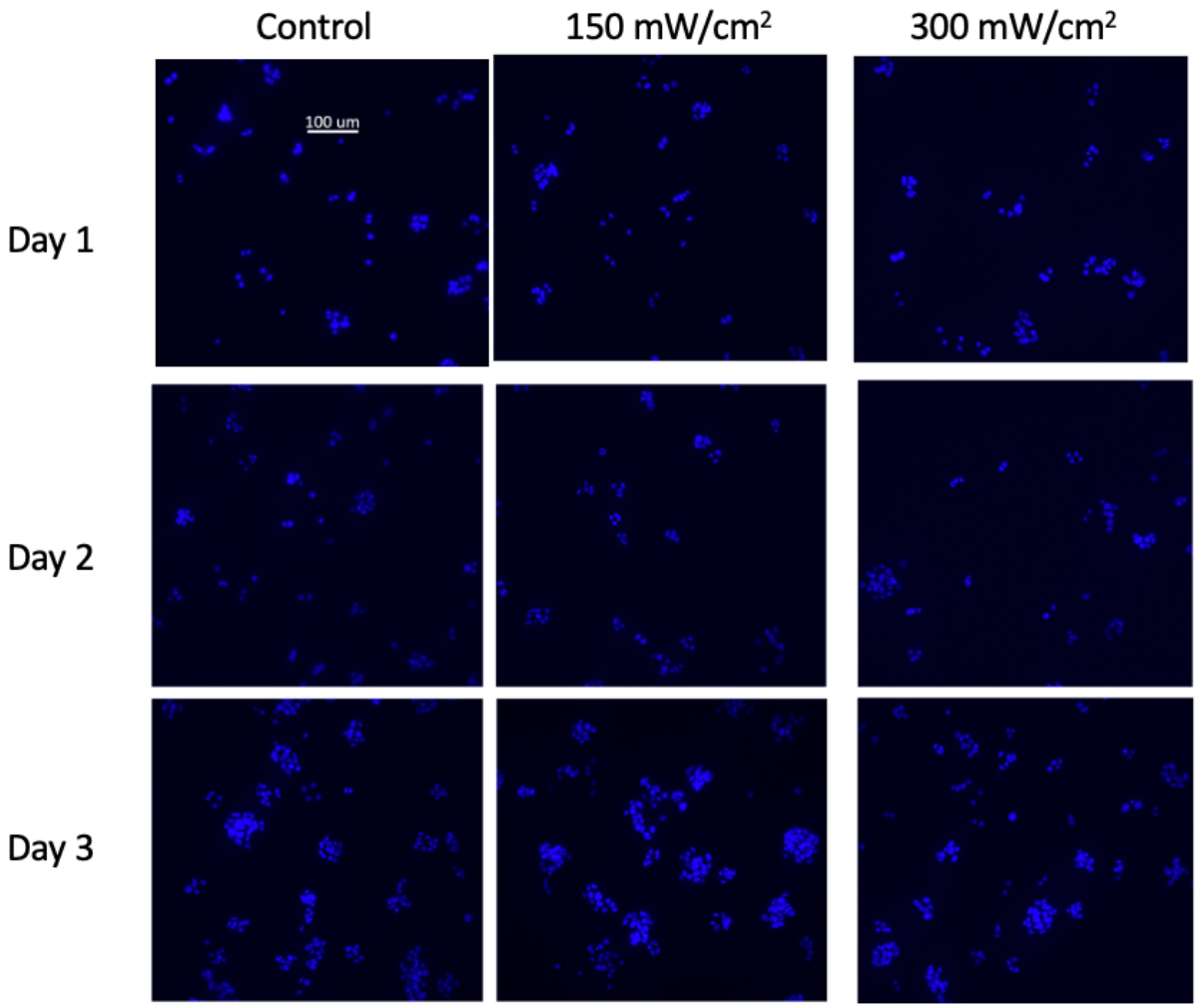

Figure 7. Representative Cell Images for Cell Proliferation.

Cells were labeled with NucBlue to identify the nuclei. These images were used to determine the number of cells. A 20x microscope objective was used to record the image. Bar = 100 μm.

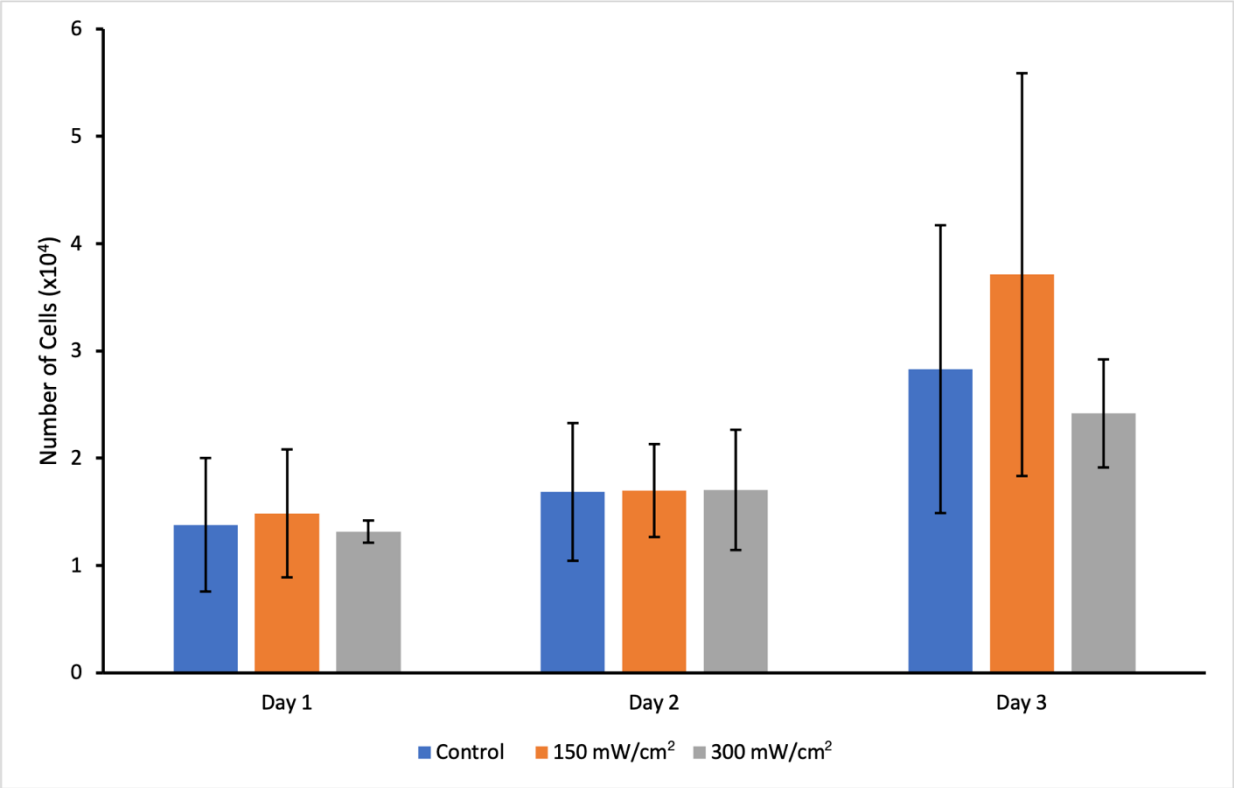

Figure 8. Cell Proliferation using CyQuant Analysis.

Data represents the number of cells counted on sample and laser light exposure. Experiments were divided into 3 groups per day (control, 150 mW/cm^2, and 300 mW/cm^2), and the results presented as mean ± standard deviation (n=8). While no statistically significant change is observed when comparing the PBM groups to their corresponding control, at day 3 the cells appeared to have proliferation under all 3 experimental conditions.

3.2 Superoxide Analysis

MitoSox superoxide staining was performed to analyze superoxide levels in the BTC6 cells 24 hours after PBM exposure (Figure 9). The fluorescent images indicate that changes in the superoxide level may not be significant. Data analysis of the images confirmed the initial visual inspection. Over the 3-day monitoring time, the laser intensity

did not modulate the superoxide level (Figure 10). Large margins of error indicate that the superoxide level recorded may represent a snapshot but no statistically significant changes are correlated with the laser intensity (Figure 10).

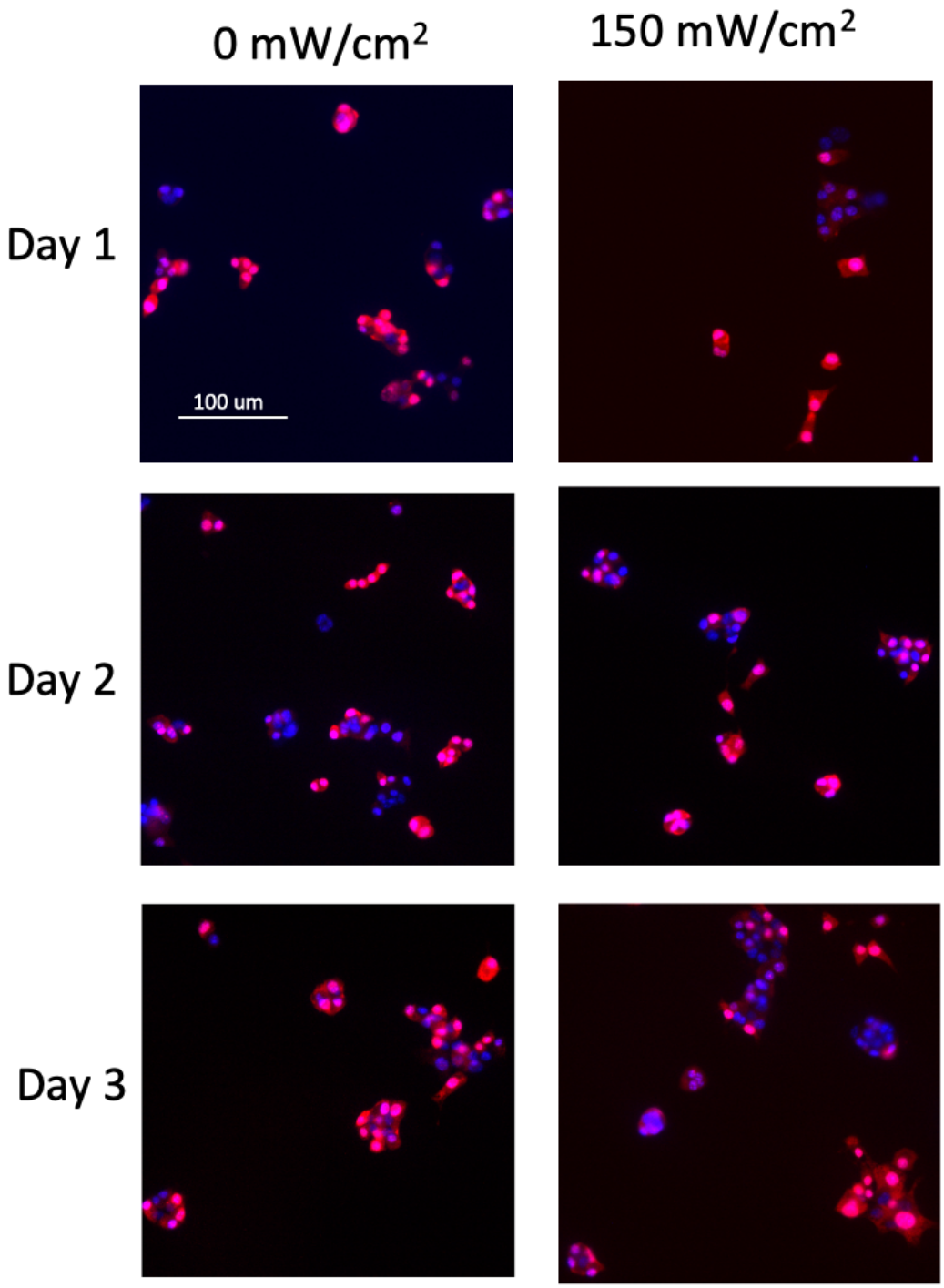

Figure 9. Superoxide Representative Cell Images.

The images above were taken at a 20X magnification. The scale bar in the first images represents 100 μm and all images are at the same scale. The superoxide is stained red, and the nuclei are stained blue.

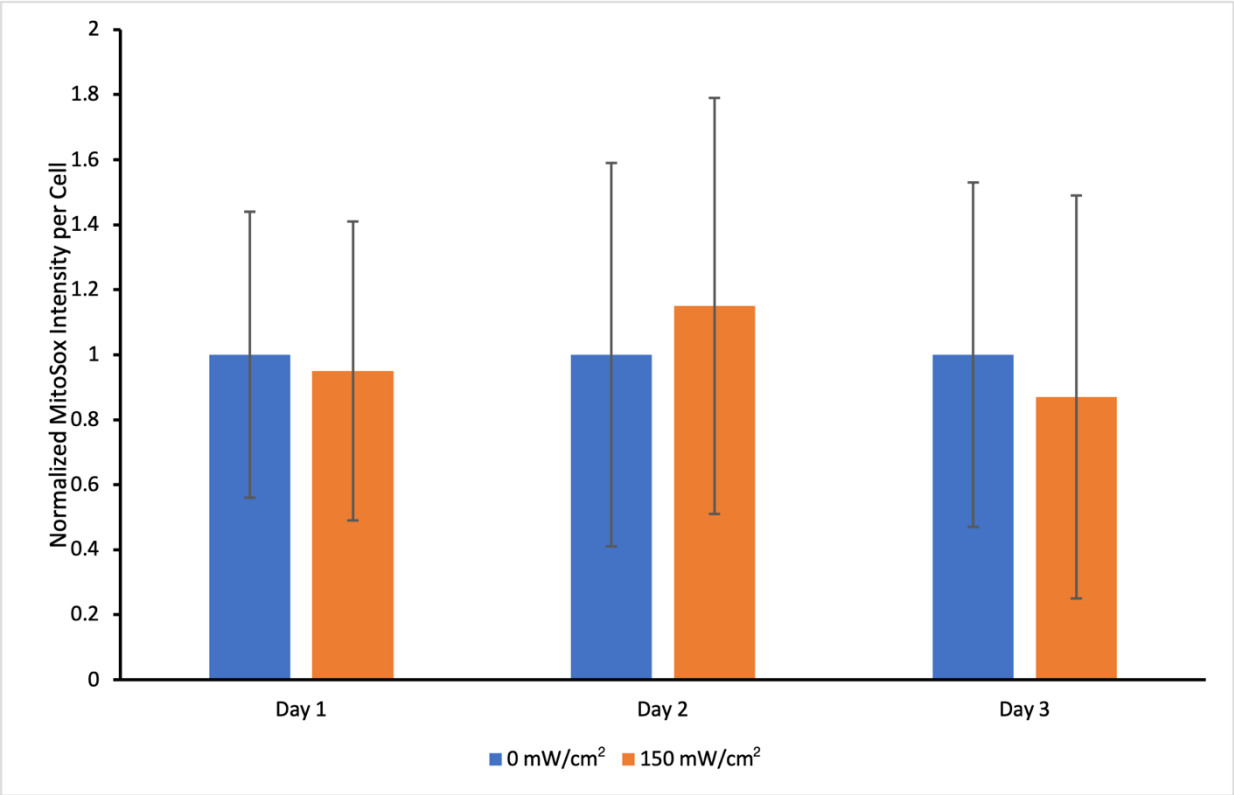

Figure 10. Superoxide in BTC-6 Cell Line After Multiple Doses of PBM using 150 mW/cm^2 Laser Intensity.

Data represents the superoxide level per cell detected 24 hours after laser light exposure and are presented as mean ± standard deviation. No statistically significant change is observed when comparing the PBM group to its corresponding control group. n > 300 cells.

3.3 SOD2 Analysis

Immunofluorescence staining was used to detect SOD2 expression in BTC-6 cells 24 hours after PBM exposure. The composite panel of images in Figure 11 show representative images of antioxidative enzyme SOD2 expression in control cells and in the cells treated with either 150 or 300 mW/cm^2. There appears to be upregulation of SOD2 expression in the treated cells. Image analysis shows that the less intense laser

treatment (150 mW/cm^2) significantly upregulated the SOD2 expression, and the additional treatment for two more days actually reduced the enzyme expression (Figure 12). In contrast, the more intense laser treatment (300 mW/cm^2) also upregulated the SOD2 expression when compared to the control cells, but such an increase was not statistically significant. Moreover, two more days of treatment with this higher intensity down-regulated the SOD2 expression. If the SOD2 expression were to be a marker for PBM-induced antioxidant activity, use of the lower light intensity (150 mW/cm^2) for one treatment was determined to reach the maximal effect.

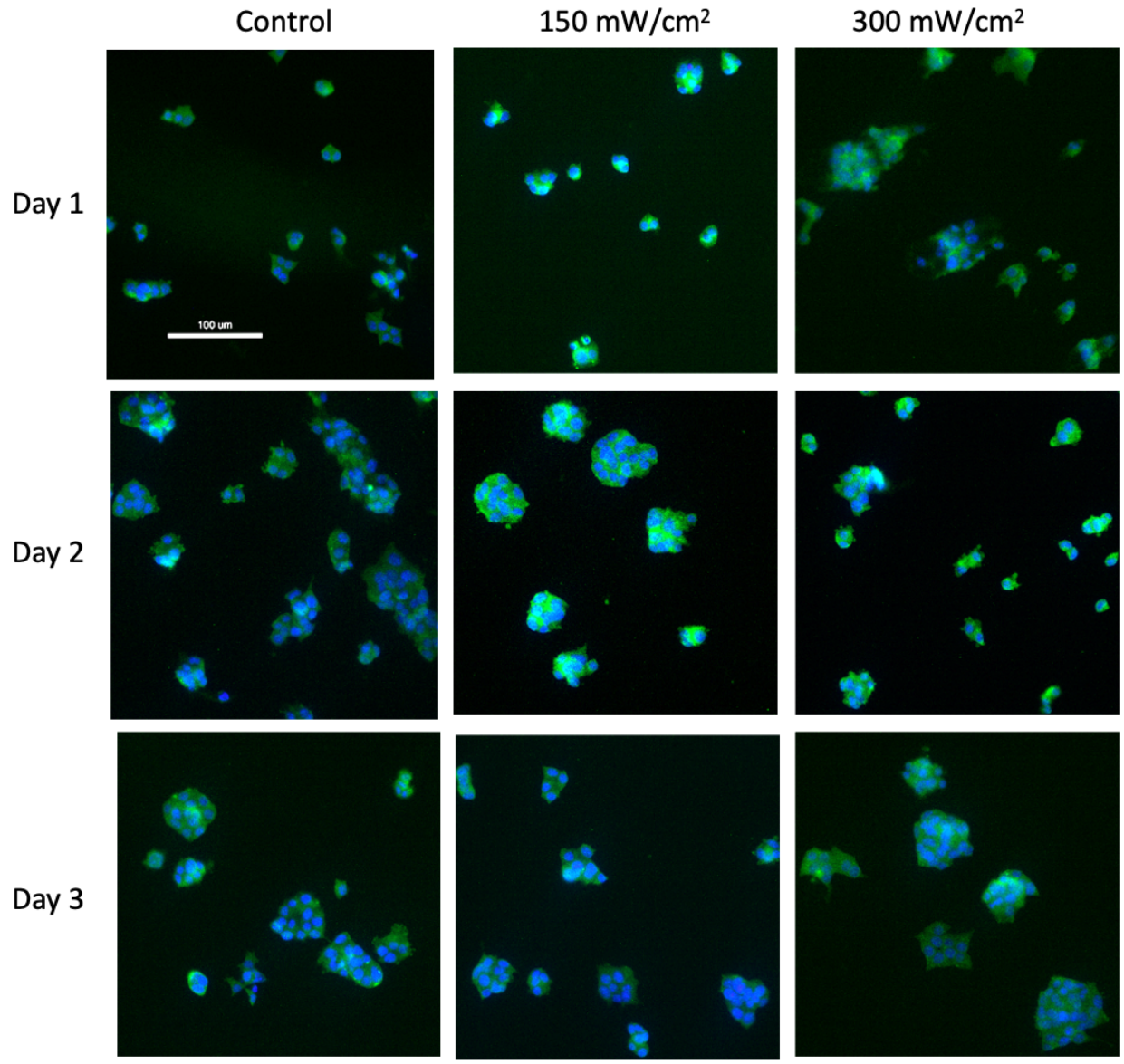

Figure 11. Representative SOD2 Cell Images.

The images above were taken at a 20X magnification. The scale bar in the first images represents 100 μm and all images are at the same scale. SOD2 is stained green, and the nuclei are stained blue.

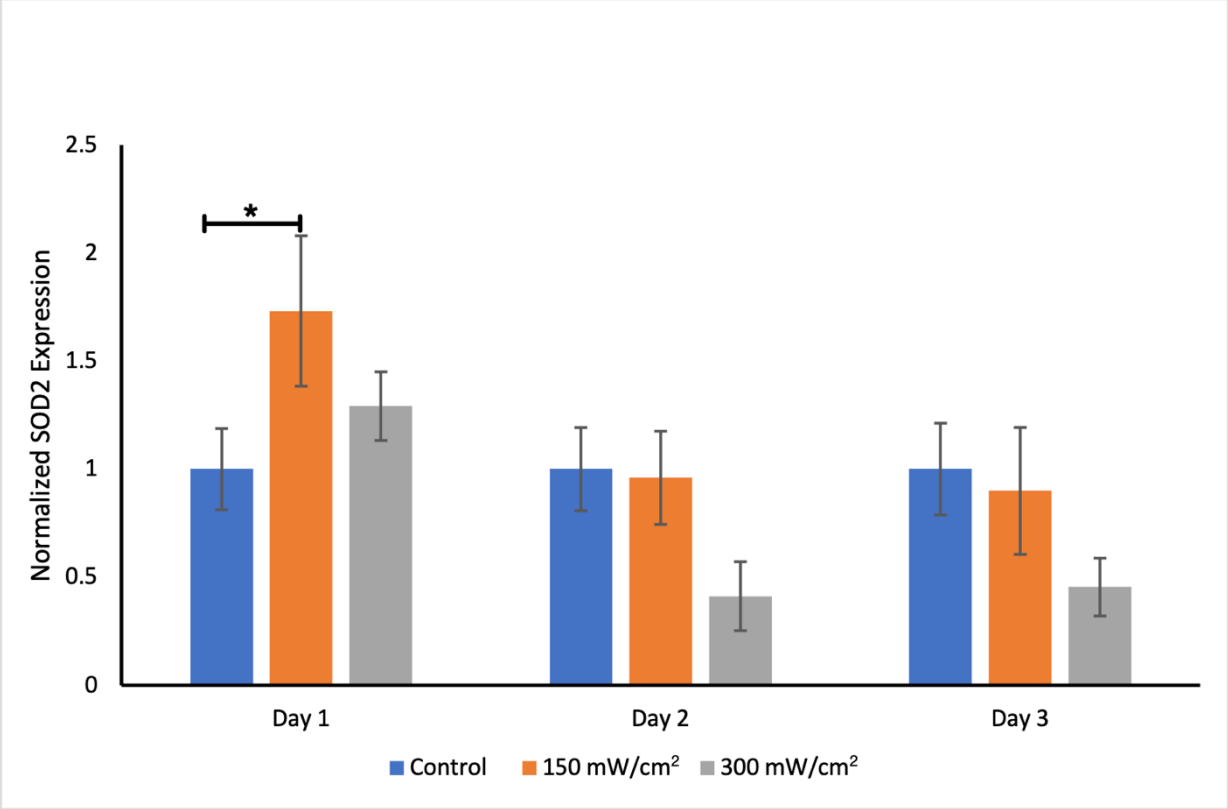

Figure 12. Immunostaining Fluorescence of SOD2 After Multiple Doses of PBM.

*Data represent SOD2 expression 24 hours after different doses PBM for 3 days and are presented as mean ± SEM (n=5). * indicates $p < 0.05$*

3.4 Glucose Stimulated Insulin Secretion

An enzyme-linked immunosorbent assay (ELISA) was used to determine insulin secretion in BTC-6 cells after different doses of PBM. The raw values produced by the assay were converted to micro-international units per cell (uIU/cell) using a standard curve and the cell number counted during the CyQuant analysis. Therefore, the data shown in Figure 13 represents the insulin secretion normalized by the cell count. Control and treated cells were challenged with 3 mM glucose and the subsequent

insulin release was measured as described in the Methods Section. Noticeable and statistically significant increases in insulin secretion were observed after one PBM treatment. Interestingly, unlike the SOD2 expression, use of the higher laser intensity for a shorter exposure time produced nearly a 6-fold increase in insulin secretion. A trend has been established in the analysis that two more days of PBM treatment diminished the insulin secretion from the cells. In fact, at day 3 the insulin secretion was indistinguishable from that measured in control cells. Implication is that one PBM treatment was sufficient to reach the maximal insulin secretion. Moreover, this trend is opposite of what was observed with the SOD2 expression analysis, indicating a possible negative correlation between increased SOD2 expression and reduced insulin secretion.

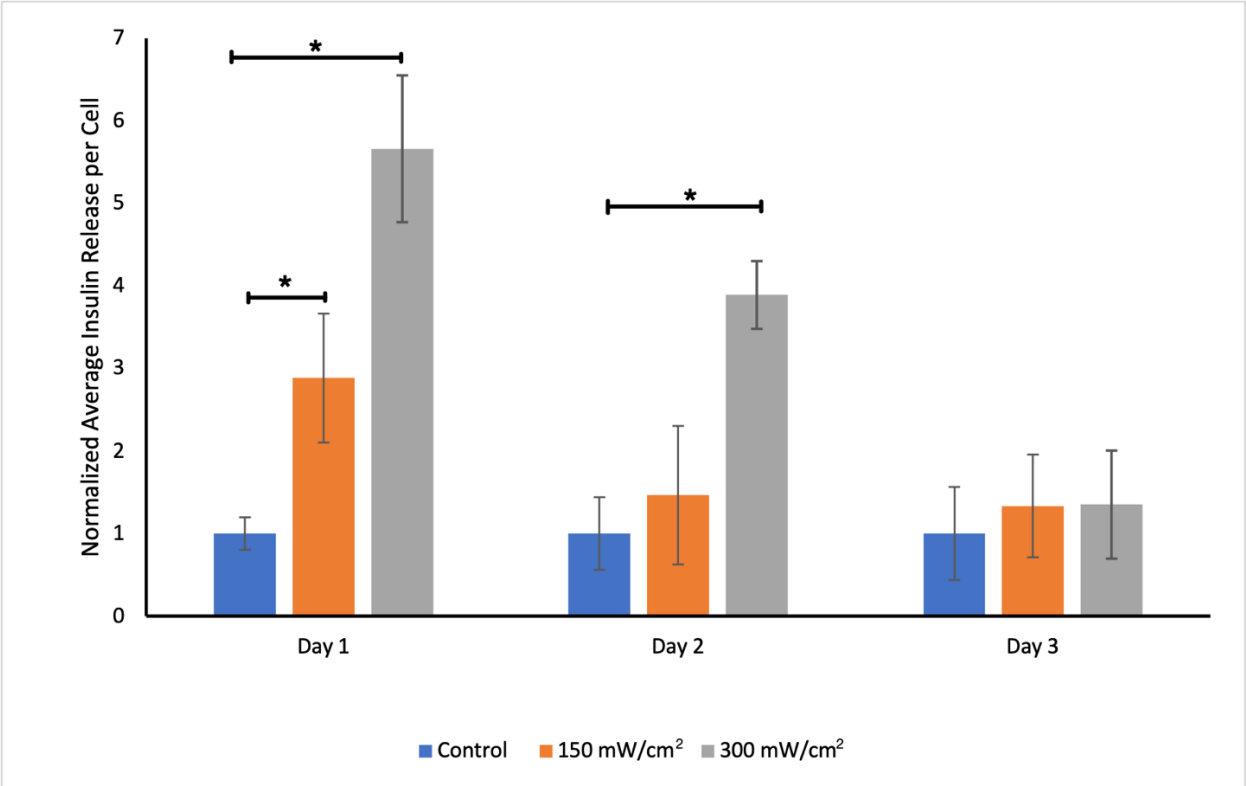

Figure 13. Insulin Secretion After Multiple Doses of PBM.

*Data represent insulin secretion 24 hours after PBM at different power densities for 3 days and are presented as mean ± standard deviation (n=8). * indicates $p < 0.05$*

Chapter 4 Discussion

4.1 Cell Proliferation

Cell proliferation was analyzed due to an observation during experimentation. While analyzing cells under the microscope, there seemed to be more cells in petri dishes that had undergone PBM. Even though the BTC6 cell line is derived from a pancreatic tumor, it seemed reasonable to analyze proliferation and compare the irradiated cells to the non-irradiated cells (control). Recent studies have shown that photobiomodulation is capable of enhancing cell proliferation in multiple cell types. Specifically, a study comparing blue (415 nm), green (540 nm), red (660 nm) and near infrared (810 nm) light concluded that red and near infrared light enhanced cell proliferation in human adipose stem cells (Wang et. al., 2017). A different study conducted by Jere et. al. (2018) analyzed proliferation of diabetic wound fibroblast cells. Again, PBM showed to increase cell proliferation by increasing ATP. The PBM parameters used for this experiment did not have any effects on proliferation. The number of cells in each group is approximately the same at each day. A possible reason for this could be a lack of PBM parameter optimization. Additionally, because the cells are derived from a pancreatic tumor, the cells may already be proliferating rapidly. More so, a study determined that PBM does not require CCO to enhance proliferation (Lima et. al., 2019). The wavelength used in this experiment, 810 nm, was chosen to specifically target CCO and could therefore be another reason why proliferation was not affected.

4.2 Superoxide Analysis

Superoxide levels after PBM have been analyzed by several research groups, such as Huang et. al. (2011), Sharma et. al. (2011), and Zupin et. al. (2019). It was determined that PBM produces a biphasic response in ROS production (Sharma et. al., 2012). More specifically, when an extremely low fluence (0.03 J/cm^2) and extremely high fluence (30 J/cm^2) were used to irradiate cortical neurons, ROS levels increased significantly. When a fluence of 10 J/cm^2 was used to irradiate the cells, ROS levels decreased (Sharma et. al., 2012). Similar results were seen by Huang et. al. (2011) who developed a 3D representation of the biphasic dose response that they predict would be seen *in vitro* (cortical neurons) and *in vivo* (traumatic brain injury mouse model) after PBM. The model took into consideration both time of exposure and power density. The purpose of the current experiment was to determine how PBM affected ROS levels in BTC6 cell line after irradiating the cells at a fluence of 9 J/cm^2 using different power densities (150 mW/cm^2 for 60 s or 300 mW/cm^2 for 30 s). Due to the large standard deviations for all values found in both the control and experimental groups, the results were inconclusive. Differences in data acquisition and analysis could explain our contradicting results against those published by other laboratories (Huang et. al., 2011). While other investigators typically measured the superoxide levels immediately following PBM exposure, we were examining a longer time effect after waiting 24 hours. Indeed, a published work from the Cho laboratory (McColloch et. al., 2019) demonstrated an increase in the superoxide level in stem cells if the measurements were performed immediately after the PBM exposure. Taken together, the production of superoxide is

mediated by PBM, but sustained ROS production appears to require continuous PBM exposure.

4.3 SOD2 and GSIS

It has been demonstrated that PBM can increase SOD2 in muscle tissue *in vivo* (Ribeiro et. al., 2016, De Marchi et. al., 2012). Ribeiro et. al. (2016) determined that oxidative stress seen after a muscular injury would delay recovery and decided to test how PBM before and/or after injury would affect the healing process. Their study determined PBM reduced stress markers by increasing antioxidant enzymes, such as SOD2, during the muscle repair process. De Marchi et. al. (2012) also showed the effects of PBM on humans. Treating volunteers' quadriceps and hamstrings with an 810 nm light before testing their running ability, exercise was used to generate oxidative stress within the muscle and was expressed as fatigue. The study determined that PBM increased SOD2 activity when compared to the control/non-irradiated group. Based on these studies, we formulated a hypothesis that the antioxidant SOD2 expression should increase in β-cells to facilitate the cell protective mechanism.

Referring to Figure 12, the 1D150 group displayed the greatest increase in SOD2 expression when compared to its corresponding non-irradiated/control group. Although a slight increase is also seen in the 1D300 group, this increase is not considered statistically significant. For the remainder of the exposure days, the SOD2 expression remained unchanged in control cells, whereas the 2D150 and 3D150 groups showed the expression levels that were reduced to the control value. Such a reduction using the

higher power density of 300 mW/cm^2 actually demonstrated significant decreases in the SOD2 expression. One postulate may be formulated in which the initial increases in ROS generations at day 1 are transient but upregulated the antioxidant activity. Additional PBM treatments are no longer required nor needed to protect the cells against oxidative stress. A naïve expectation was the SOD2 upregulation would enhance insulin secretion. However, an apparent negative relationship between the SOD2 expression and insulin secretion was observed (Figure 12 and Figure 13). For example, while exposure of light with an intensity of 300 mW/cm^2 induced a statistically insignificant increase in SOD2, it enhanced more than a 5-fold increase in insulin secretion. This trend was unexpected because increased SOD2 indicated a higher antioxidant capacity which, in turn, should have indicated improved cell function. This finding is interesting and appears to contradict the current paradigm of the relationship between antioxidant enzymes and insulin secretion. A simple explanation would be as follows. Insulin secreting cells are known to have more robust capability of converting O_2^- into H_2O_2, than for converting H_2O_2 into H_2O (Gurgul et. al., 2004). The constant conversion of superoxide into H_2O_2, can lead to increased H_2O_2 interaction with the protein thiol groups, which can further lead to the formation of a highly reactive oxygen radical HO (Newsholme et. al., 2019). Lortz et. al. (2005) investigated SOD2 over expression and its effects on insulin secreting cells. Cell proliferation and cell function were analyzed in cells with both reduced and increased SOD2 expression. The study determined that an over expression of SOD2 without the over expression of catalase can lead to toxic levels of H_2O_2 within the cells, therefore decreasing cell proliferation and cell function. The negative relationship between SOD2 expression and insulin

secretion may be explained by an expression of SOD2 by PBM using 150 mW/cm^2 for 60 sec, indicating that O_2^- was transformed into H_2O_2 at a much faster rate leading to possible H_2O_2 toxicity. This H_2O_2 toxicity could be one of the main causes that decreased insulin production, when compared to the higher power density (300 mW/cm^2). Lortz et. al. (2013) studied the possible protective effects of catalase overexpression in insulin secreting cells. Their study determined that an overexpression of catalase protects insulin secreting cells from both ROS and proinflammatory cytokines. These findings further demonstrate the prediction that overexpression of SOD2 without the overexpression of catalase can create a toxic cell environment leading to reduced cell function. It is important to note however, that after one exposure at either power density, was sufficient to induce measurable increases in insulin secretion.

On the other hand, SOD2 has been shown to have no effect on GSIS, but its upregulation is capable of protecting cells from oxidative stress (Pi et. al., 2007), indicating that an increase in the insulin secretion is likely caused by other mechanisms such as PBM-enhanced ATP production (Hamblin, 2018). Although the coupling mechanisms are yet to be fully established PBM could be used as a pre-treatment for islets before transplantation. Further investigation is required to determine to which extent, if any, SOD2 is involved in the insulin secretion.

Few studies could be formulated to elucidate potential explanations. Similar to the results of Kang et. al. (2014), cells with knockout SOD2 expression could be exposed to PBM using the parameters used in this thesis. Insulin levels would be analyzed, and if

they remain the same, then one might conclude that SOD2 does not impact insulin secretion. Additionally, hydrogen peroxide levels could be analyzed following a PBM exposure to determine if continuous accumulation inside the cells could be a plausible explanation.

Finally, this study established the feasibility of optimizing the PBM parameters. Although only 2 sets of such parameters were tested, the results suggest that such optimization likely demands additional experiments and analyses. Protective antioxidant effects are better stimulated by delivering photoenergy at a lower light intensity for a longer duration (e.g., 150 mW/cm^2 for 60 sec), and yet insulin secretion is better facilitated by a higher intensity for a shorter duration (300 mW/cm^2 for 30 sec). This apparent paradox suggests that there must be multiple signaling pathways in the β-cell that can be modulated by light stimulation. As an example, the PBM-induced ROS generation can activate the cellular defense mechanisms in the cytoplasm and the nucleus (Hamblin, 2017). In addition, secretion of vesicles containing insulin is highly dynamical and critically depends on ATP (Koster et. al, 2005). These intracellular processes are presumed to participate in intricate feedback loops that are differentially activated or inhibited by various stimulatory modalities and simulation parameters. Establishment of a molecular paradigm that encompasses the cross-talk circuitry is indeed challenging. However, potential clinical translation of light-based islet treatments will require a detailed understanding of multiple signaling pathways

4.4 Future Studies

Based on the findings of this study, future works could be directed in the following directions:

1. Investigating the effects of the parameters used in this study on the catalase expression to determine if both SOD2 and catalase overexpression are required for optimal cell function.
2. Determining if any long-term effects are seen in cell physiology after PBM-induced responses have subsided. This would help determine if PBM has lasting enhanced cell function or such effects are transient.
3. Investigating different laser power densities to determine whether there is an optimal range for beta cell stimulation.
4. Simulating the isolated islets from donors before transplantation and test cell function thereafter. This could be achieved by exposing the islets to laser light, simulate the islet transplant, followed by glucose challenge.

Optimization of the PBM parameters may depend on the aim of potential phototherapy. Specifically, if the objective is to stimulate CCO in the mitochondria, a wavelength of 810 nm could be beneficial. This is because, as the wavelengths increase, the photoenergy readily interacts with water inside the cell (Hamblin, 2017). The success reported by Lim et. al., (2009) showed that using a fluence of 9 J/cm2 could possibly be optimal for diabetic cell stimulation. However, other studies have indicated success when treating diabetic cells in vitro using lower fluence values. For example, Zare et al., (2020) reported decreased apoptosis rates, increased proliferation rates, and lower

population doubling times when treating diabetic bone marrow mesenchymal stem cells using fluences of 0.5 J/cm^2, 1 J/cm^2, and 2 J/cm^2. Based on the results of this thesis, the power density used could either be increased or decreased depending on the intended effects in response to PBM. In contrast, if the objective is to increase SOD2 expression to protect the cells from oxidative stress, lower power densities should be considered. Additionally, if the goal is to increase insulin secretion, the power density could be increased. Regarding the frequency of treatment, Ahmadi et al., (2020) reported success in laser treatment of diabetic stem cells every other day. A range of the PBM parameters and dosage indicates a complex problem that requires systematic studies before clinical translation can be contemplated.

Chapter 5 Conclusion

The physiological benefits of PBM have been made evident by many researchers over the last 50 years. Currently, most research focuses on changing the PBM fluence. But it is important to note that many different variables can be changed during light exposure, including irradiance. This study is an incremental but useful step towards deciphering the subcellular responses in the insulin secreting cells after PBM and, therefore, opens the door towards optimization of PBM. More extensive studies are required to determine which irradiance would be optimal for islet treatment prior to transplant in T1D patients. The greatest problem currently facing the islet transplant is the large number of islets required to reach insulin independence. This study focused on increasing the cell function using PBM to enhance the insulin secretion. It is interesting to note that, for the specific parameters used in this study, the largest increase in insulin secretion was observed after only one dose of PBM, and it appears to be power density-dependent. We postulate that a greater increase in GSIS at the higher power density is likely due to an enhanced SOD2 expression up to a threshold to provide protective effects. Determination of the threshold would be a critical step toward clinical translation.

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