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Cytochrome C: A Potential Early Biomarker of Diabetic Retinopathy

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CYTOCHROME C; A POTENTIAL EARLY BIOMARKER OF DIABETIC RETINOPATHY

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ABSTRACT

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Diabetic retinopathy is a prevalent ocular disease which is diagnosed in patients who experience progression of diabetes mellitus over time. Generally, patients remain undiagnosed due to lack of knowledge that they are diabetic or a delay in diabetic retinopathy symptom appearance. Ophthalmologists and eye specialists often obtain diagnosis of diabetic retinopathy once the patient begins to show progressed symptoms of the disease. Previous experiments have been performed to increase our knowledge of diabetic retinopathy and early biomarkers of the disease. Several studies have determined the effects of diabetic retinopathy and apoptosis with cytochrome c presence using bovine retinal cells and rat models. The purpose of this thesis is to understand and analyze the effects of high glucose levels, which is a common concern for the diabetic patient, on human retinal pericyte cells and how the presence of cytochrome c in the mitochondria or cytosol of the cell could be used as an early indicator for diagnosis of diabetic retinopathy by using TUNEL, heme staining and Western Blot methods.

DEDICATION

This thesis is dedicated to my mother, Ruth and grandparents, Rosario and Onesimo Villarreal, along with friends and colleagues who always believed me to be capable of accomplishing anything I set my heart to.

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CHAPTER I

INTRODUCTION

Diabetes mellitus is among the most prevalent diseases in the United States. According to the American Diabetes Association, 34.2 million Americans suffered from diabetes in 2018. Failure to maintain blood sugar levels balanced can result in detrimental effects imposed on all organ systems. Diagnosis of diabetes mellitus generally does not occur until symptoms begin to make an appearance or affect the individual's everyday life. One disease that often results from diabetes mellitus is diabetic retinopathy, which is characterized for the damaging effects it imposes on the blood vessels of retinal tissue. Since patients often do not seek professional assistance until symptoms are present, it is often the case that the retinal blood vessels are irreversibly damaged by the time diagnosis takes place. Symptoms of diabetic retinopathy include blurred vision, floaters, trouble telling the difference between colors and seeing a dark circle in the central point of vision. Diabetic retinopathy progresses in four stages. These four stages include mild, moderate, and severe non-proliferative retinopathy and proliferative retinopathy. Effects of diabetic retinopathy may be slowed through treatments with anti-VEGF injections, laser treatments or eye surgeries, therefore early detection of the ocular disease is crucial in preventing its progression.

When a patient develops diabetes mellitus and the disease causes diabetic retinopathy, the chances of apoptosis occurring in retinal cells highly increases. Apoptosis is commonly known as "cell mediated-death" or "programmed cell death" (van den Oever, I. A, et al. 2010). What

this means is that the cell will kill itself off in a suicide-like manner. Apoptosis may occur naturally or may be induced by outlying factors. Balanced levels of apoptosis are crucial for the elimination of cell waste. Although apoptosis is needed for homeostatic control by the body, previous studies have shown that hyperglycemic conditions, induced by diabetic retinopathy, result in a higher incidence of apoptosis, ultimately becoming detrimental to the retina.

Diabetes mellitus has also been found to play a key role in the release of cytochrome c from the mitochondria of cells. Cytochrome c has proven to be an important factor in ATP synthesis. In humans, it is a 104 amino acid peptide chain which has a single heme group (Eleftheriadis, T., et al. 2016). The heme protein has a molecular weight of approximately 12,000 Daltons or 12 kDa (Eleftheriadis, T., et al. 2016). High amounts of cytochrome c release from the mitochondria into the extracellular space of the cell pose as an indicator of damages to the mitochondria of a cell, leading to cell death (Eleftheriadis, T., et al. 2016). Previous studies have shown that once cytochrome c is released, it binds to Apaf-1, known as protease activating factor-1 (Eleftheriadis, T., et al. 2016). Apaf-1 proceeds to oligomerize and binds to adenosine triphosphate (ATP), forming an apoptosome (Shakeri, R., et al. 2017). Cytochrome c's role in adenosine triphosphate (ATP) synthesis lies in its ability to take electrons from complex III and transfer them into complex IV. After the apoptosome is formed, the apoptosome then undergoes proteolysis, protein degeneration, and activates procaspase 9, which in turn also activates caspases 3, 6 and 7 (Eleftheriadis, T., et al. 2016) (Shakeri, R., et al. 2017). Caspases 3, 6 and 7 have been found to execute apoptosis by damaging the cell (Eleftheriadis, T., et al. 2016). In summary, the process results in elevated occurrences of retinal pericyte loss, a staple of diabetic retinopathy.

Previous studies of the effects of cytochrome c release from the mitochondria into the extracellular space of cells have been conducted in various fields, with focus on different organs and parts of the body. Studies involving the measurement of the levels of cytochrome c in the spleen, splenocytes, known as white blood cells (of different types) located in the spleen, were observed after a period of heat-shock, which induces necrosis and apoptosis (Eleftheriadis, T., et al. 2016). High levels of cytochrome c in the extracellular space of cells were recorded. When studying patients after cardiac arrest, it was proven that cytochrome levels dramatically increase after the event (Eleftheriadis, T., et al. 2016). Studies which analyze the effects of cytochrome c release in the liver and kidneys have also proven that cytochrome c is a good biomarker in detection of liver injury (drug induced hepatotoxicity studies) and acute kidney injury (Eleftheriadis, T., et al. 2016). In acute kidney injury, high levels of cytochrome c were also present in the urine of the patients (Eleftheriadis, T., et al. 2016). As far as cancer studies, it has been proven that high levels of cytochrome c were present in patients with much more aggressive tumors, although it also proved that after chemotherapy, high levels of cytochrome c were a good signal (Eleftheriadis, T., et al. 2016). One similar finding in all studies is that more cellular tissue damage equals higher levels of cytochrome c. More studies are being conducted to try to better understand the effects of cytochrome c presence.

The purpose of this study is to identify whether cytochrome c can be deemed an effective biomarker in detecting early diabetic retinopathy through TUNEL assays, heme staining and Western blotting. In experiment 1, human retinal pericyte cells were cultured in differing glucose concentrations (5.5 mM and 30 mM). The purpose of this experiment is to identify how human retinal pericyte cells are affected in hyperglycemic conditions when compared to euglycemic conditions. In experiment 2, apoptosis incidence was measured and compared in human retinal

pericytes treated with differing glucose concentrations (5.5 mM and 30 mM) using TUNEL assay. The purpose of this experiment was to investigate whether there could be a possible connection between hyperglycemia and an increase in apoptotic cell presence. In experiment 3, cytochrome c levels of human retinal pericyte cells present in whole cell lysates and cytosolic isolated fractions, treated under normal and hyperglycemic conditions using Western blotting and heme staining methods were compared to confirm the effectiveness of potentially identifying cytochrome c as a biomarker of diabetic retinopathy. Originally, the plan was to compare cytochrome c levels of human retinal pericyte cells present in the mitochondrial and cytosolic isolated fractions of human retinal pericyte cells treated with normal and hyperglycemic conditions, but the mitochondrial fraction was not successfully isolated. Further studies will be conducted.

I propose that cell viability will be negatively affected by treatment with higher glucose concentrations, such as those seen in diabetic retinopathy patients. In turn, the effects of the disease imposed on the HRP cells also result in an increase in apoptosis incidence. All in all, I believe cytochrome c will prove to be an effective biomarker.

CHAPTER II

REVIEW OF LITERATURE

Diabetic Retinopathy

Diabetic retinopathy has been found to be a major, leading cause of blindness is adults. The effects of diabetic retinopathy are gradual, with the disease progressing over time. Therapies and treatments, as well as management of the diabetes itself, have proven to slow progression of the disease (Garcia-Medina, J. J., et al. 2020). The retina is made up of cells which include pericytes and endothelial cells (known as vascular cells), microglia, which include Muller cells and astrocytes, neurons, such as photoreceptors and ganglion cells and microglia (Garcia-Medina, J. J., et al. 2020). Diabetic retinopathy has been proven to have negative effects on these cells in divergent ways. Of the cells mentioned, scientists have conducted many studies with focus on pericytes and loss thereof (Garcia-Medina, J. J., et al. 2020). Pericytes have proven to regulate vascular flow through contraction-like movements (Garcia-Medina, J. J., et al. 2020). These pericytes diminish and are lost when kept under constant hyperglycemic conditions. As an increase in pericyte loss occurs over time, microaneurysms begin to appear and capillary occlusion takes place, leading to acellular capillary formation (Garcia-Medina, J. J., et al. 2020). As diabetic retinopathy progresses and goes untreated or poorly treated, hard exudates, edema, cotton wool spots, hemorrhages, among other detrimental effects also appear, leading to the progression of non-proliferative diabetic retinopathy to proliferative diabetic retinopathy (Garcia-Medina, J. J., et al. 2020). When the homeostasis of mitochondria is disrupted, the

mitochondrial DNA becomes damaged and leads to detrimental effects (Kowluru, R. A. 2019). With time, diabetic retinopathy begins to show symptoms which were not present in the initial stages of the disease. These symptoms include microaneurysms, vision distortion and gradual vision loss, as well as retinal deposits which can be observed in images of the retina (Kowluru, R. A. 2019). As symptoms and the disease reach a high point, total vision loss is observed in the diabetic patient. This is due to the occurrence of retinal detachment after neovascularization and hemorrhages appear and are present for some time, along with all other symptoms (Kowluru, R. A. 2019). A link between the increase of oxidative stress and progression of the disease proves to be prominent in many major studies of the disease (Kowluru, R. A. 2019).

Oxidative stress is studied and considered among one of the major causes of diabetic retinopathy progression. It has been found, through experimentation and extensive research, that oxidative stress plays a pivotal role in reactive oxygen species formation (Cecilia, O. M., et al. 2019). Inflammatory responses are activated when hyperglycemic conditions are constantly present (Cecilia, O. M., et al. 2019). Cellular degradation then takes place, oxygen becomes present in inadequate amounts for proper tissue functions to be carried out and these effects ultimately lead to creation of reactive oxygen species (Cecilia, O. M., et al. 2019). Hyperglycemia, ROS, inflammation, pryoptosis, autophagy, neurodegeneration, oxidative stress-related genetics and antioxidant supplements all play a role in the destruction or reparation/supplementation (in the case of antioxidant supplements) (Cecilia, O. M., et al. 2019). Scientists propose that the way to slow down or halt the progression of diabetic retinopathy is to use multi-target therapies, lower oxidative stress using various strategies, consume antioxidant supplements and manage diabetes and blood glucose levels overall (Cecilia, O. M., et al. 2019).

Sinclair, M. D., et al. hypothesized that diabetes and the complications that are tied to the disease arise from common pathophysiologies (Sinclair, M. D., et al. 2019). Pathophysiologic mechanisms include inflammation and immune regulation, such as reactive oxygen species, inflammatory cytokines and chemokines, aberrant growth factor signaling, and vascular dysfunction, genetic, environmental factors and epigenetic changes, and insulin resistance and abnormal metabolic environment (Sinclair, M. D., et al. 2019). Early recognition of the presence diabetic retinopathy is crucial and treating the disease early-on may slow down or stop the effects on the retina and vision. Current treatments and approaches to treat diabetic retinopathy include addressing oxidative stress and epigenetic changes, treating inflammation and neurodegeneration and overall regulation of the way lipids are metabolized (Sinclair, M. D., et al. 2019). Therapies include laser treatments, vitrectomy, cryotherapy, intravitreal steroids, implants and inserts such as Ozurdex and Iluvien and intravitreal injections of anti-VEGF antibodies, Eylea, Lucentis, Macugen and Avastin (Sinclair, M. D., et al. 2019).

Apoptosis

Apoptosis refers to the programmed cell death which becomes activated due to metabolic stress, protein oxidation, accumulation of proteins and mutations in the deoxyribonucleic acid sequence of the organism (Santucci, R., et al. 2019). The process is crucial to the development and growth of living organisms and exists naturally to eliminate unwanted or damaged cells, such as pre-cancerous or virus-infected cells. It has been proven that interaction of cytochrome c with the phospholipid cardiolipin is important for the release of cytochrome c from the mitochondria (Santucci, R., et al. 2019).

Cytochrome c

Cytochrome c, discovered in the 29th century by Charles A. MacMunn, operates by taking electrons and transporting them to the respiratory electron transport chain (Kulikov, A. V., et al. 2012). This process is crucial in cellular respiration as it sustains production of adenosine triphosphate (ATP) (Kulikov, A. V., et al. 2012). This ATP provides the energy required by cells to carry out important functions and cellular processes (Kulikov, A. V., et al. 2012). Cytochrome c is a class 1 c-type cytochrome (Santucci, R., et al. 2019). In the case of human cytochrome c, the macromolecule is single chained and is composed of 104 residues (Santucci, R., et al. 2019). As previously mentioned, the heme protein has a molecular weight of approximately 12,000 Daltons or 12 kDa (Eleftheriadis, T., et al. 2016). The native conformation consists of three major and two minor alpha helixes which are connected by secondary structure motifs (Santucci, R., et al. 2019). The heme is covalently bonded to the polypeptide with Cys 14 and lies within a crevice of the cytochrome c heme protein (Santucci, R., et al. 2019). Alternative conformations of cytochrome c exist. Uses of cytochrome c include mediation of electron transfer in the respiratory chain, aid in disposal of reactive oxygen species present within its environment and it plays a major role in apoptosis (Santucci, R., et al. 2019).

During the 1990's, approximately 1996, Xiaodong Wang and colleagues from the Department of Biochemistry in Emory University School of Medicine, Atlanta, Georgia, discovered that proteolytic processing and caspase cascade activation occur as a result of cytochrome c presence (Kulikov, A. V., et al. 2012). Proteolytic processing and cascade of caspases are required for the process of apoptosis to be successfully carried out (Kulikov, A. V., et al. 2012). When cytochrome c moves into the cytosol of the cell, it binds to Apaf-1, causing Apaf-1 oligomerization, the formation of an apoptosome and deleterious effects on the cell

(Kulikov, A. V., et al. 2012). This causes a caspase cascade and the death of the cell (Kulikov, A. V., et al. 2012). This is called apoptosis. For apoptosis to be induced, cytochrome c must remain unmodified as unmodified holocytochrome c (Kulikov, A. V., et al. 2012).

Cytochrome c exists in all living organisms, including plants (`Zhang, M., et al. 2017). When present in the inner membrane of the mitochondria, cytochrome c aids in many molecular processes, but has been found to play an especially important role in cellular respiration, functioning in the electron transport chain (`Zhang, M., et al. 2017). The release of cytochrome c into the cytosol causes detrimental effects, which lead to disturbances within the cell, ultimately causing cell death and the development of many diseases, like diabetic retinopathy in this case (`Zhang, M., et al. 2017). The process begins with the release of cytochrome c from the inner membrane of the mitochondria, exiting through the outer mitochondrial membrane and into the cytosol where it then binds to IP3 receptors, found in the endoplasmic reticulum. This, in turn, causes a calcium concentration increase and an increase in the amount of cytochrome c being released (`Zhang, M., et al. 2017). The result is presence of cytotoxic levels of cytochrome c in the cell, which causes a caspase cascade of caspases 9, 3 and 7, and ends with the death of the cell (`Zhang, M., et al. 2017). The exact mechanism and the process of cytochrome c release from the mitochondria has been studied for years and will be studied for many more to come.

Scientists hypothesize that phosphorylation of cytochrome c controls the mitochondrial electron transport chain flux and the process and triggering of apoptosis (Kalpage, H. A, et al. 2014). Phosphorylation refers to an addition of a phosphoryl group to a molecule. The addition of the phosphoryl group to a molecule is critical for optimal growth, development and regulation of cell processes, such as apoptosis (Kalpage, H. A, et al. 2014). Phosphorylation sites of cytochrome c heme protein are tissue specific. These phosphorylation sites include Tyrosine 97

(heart), Tyrosine 48 (liver), Threonine 28 (kidney), Serine 47 (brain), Threonine 58 (kidney) (Kalpage, H. A, et al. 2014). Scientists have concluded that the discovery of these phosphorylation sites of cytochrome c provide greater insight into what is already known about cytochrome c and its role in apoptosis (Kalpage, H. A, et al. 2014).

Through research and experimentation, the role of mitochondria has proved to be important and have connection with cytochrome c release and increased occurrences of cellular apoptosis (Gogvadze, V., et al. 2006). Several proteins have also been found to play a principal role. Bcl2 proteins, located in the outer mitochondrial and nuclear membrane, as well as in the endoplasmic reticulum, is an anti-apoptotic protein, inhibiting apoptosis (Kluck, R. M., et al. 1997). There are multiple pathways in which cytochrome c is permeabilized and released from the outer membrane of the mitochondria. The mechanism begins with mitochondrial permeability transition (MPT) induction, causing the pores of the inner membrane to open and cause osmotic swelling, which ultimately leads to rupture of the outer membrane of the mitochondria and release of proteins (Gogvadze, V., et al. 2006). A second mechanism includes Bcl2 family proteins, which have proven to halt the release of cytochrome c when overexpressed in apoptotic cells (Gogvadze, V., et al. 2006). In short, Bcl2 family proteins prevent cytochrome c from being released from the mitochondria and ultimately prevent apoptosis (Gogvadze, V., et al. 2006). Several studies have also been conducted to understand the importance of Bax and Bak and their interaction with mitochondria, which leads to release of cytochrome c (Gogvadze, V., et al. 2006). It is important to understand how all of these factors and mechanisms work together, or against each other, in patients with diabetic retinopathy in order to find better treatments and gain a greater insight of the disease itself.

As mentioned, detection of cytochrome c in the cytosol could potentially lead to several applications in clinical diagnosis or therapies. Previous studies have shown that high levels of cytochrome c are often observed in patients who suffer from heart disease, liver disease and autoimmune diseases (Santucci, R., et al. 2019). Researchers are still unsure about the introduction of the cytochrome c test into the clinical setting, as many more studies must still take place. It is important to understand and keep performing experiments which will increase our knowledge of biomarkers, in this case cytochrome c, and their use in diagnosis or early detection of diseases. Further studies which investigate the purpose and role of the mitochondria in the process of apoptosis must also be performed, as the role of mitochondria is still not entirely understood.

CHAPTER III

METHODOLOGY

Human Retinal Pericyte Cultures

Complete classic media (Cat: 4Z0-500; Cell Systems) was prepared. Serum containing basal medium was supplemented with 10% FBS, 1% antibiotic and culture boost (4CB-500) for a total of 500mL of complete classic media. Once CCM was prepared, HRP P6 (Cat: ACBRI-183 V. Cell Systems, Kirkland, WA.) cells were thawed and added into a 15mL conical, along with 5mL of complete classic media and centrifuged at 900 RPM for 10 minutes at 4 degrees Celsius. While HRP cells were centrifuging, the plate was coated with 1mL of attachment factor then removed. 10mL of complete classic media were added on to the plate. The tube was taken out of the centrifuge, media was aspirated, and 1 mL of media was added into the conical and the pellet was re-suspended. Once re-suspended, 1mL was taken from the conical and added onto the plate, coating the plate as evenly as possible. Seeding into 12-well plates for cell counts took place during passage 8. Seeding into the 16-chamber slide (Thermo Fischer Scientific LOT 1288395-3007) for the TUNEL assay took place during passage 9. Plates used for cytochrome c release western blot experiments were starved for 24 hours and treated at passage 9. HRP cells were 85-90% confluent at every split.

Glucose Treatments

Glucose treatments of 5.5 mM, 18.5 mM and 30 mM were prepared accordingly for treatment of HRP cells. Appropriate amounts of glucose were added into DMEM -D-glucose (no

glucose) supplemented with 1% AB and sterilized. The glucose treatments were used during cell count, TUNEL, western blot and heme stain experiments to determine treatment effects on HRP cells. HRP cells were treated for 24, 48 and 72 hours prior to collection for cell count experiments. Treatment for 24 hours was done for TUNEL experiments. For Western blot and heme stains, HRP were starved for 24 hours and then treated with euglycemic and hyperglycemic glucose treatments for a total of 5 days.

Cell Counts

To conduct cell counts, 15 uL of the media and cell pellet mixture were re-suspended with 15 uL of trypan blue stain (1:1 addition) (Ref: 15250-061). 15uL of the mixture was then placed on a cell counting chamber slide (Countess, Thermo Fisher Scientific) and slide was loaded on to the automated cell counter (Countess II FL, Cat. AMQAF1000) and viable and non-viable cell counts were obtained.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

To conduct the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, a fluorescein in situ cell death detection kit (Sigma Aldrich Cat No. 11684795910) was used to quantify apoptosis of 5.5, 18.5- and 30-mM glucose treated human retinal pericytes. 5,000 cells were seeded into each well. Duplicate wells were used for each treatment. After 24 hours of treatment, the cells were fixed. To fix, the media was taken out of the wells and the wells were left to dry at room temperature for an hour. Once dried, 150 uL of PFA were added into each well and left at room temperature for an hour. After the hour, the slides were rinsed with PBS three times, carefully, making sure not to disturb the cells. 150 uL of PBS were then

added into the wells and stored in the 4-degree Celsius refrigerator to be stained the following day. Once ready to stain, 100 uL of permeabilizing solution were added into the wells

and resuspended, on ice, for two minutes. Once permeabilized, the TUNEL reaction mixture was prepared, along with the negative and positive control. To prepare the TUNEL reaction solution, 100 uL of label solution were taken out from the purple top (label solution) container. 50 uL of the enzyme solution were added to the remaining 450 uL in the label solution vial to obtain a total of 500 uL of TUNEL reaction mixture. Once the mixture was prepared, the slides were rinsed with PBS two times, making sure that the area around the sample was dry after the last PBS wash. 50 uL of TUNEL reaction mixture were added into the wells. The cells were then incubated for an hour at 37 degrees Celsius in the dark. After the incubation, the slides were rinsed with PBS three times. The chamber was then manually pulled off from the slide. 6 drops of vectashield mounting medium with DAPI were added onto the slide and the slide was covered with a 16 well cover slide and sealed with nail polish. The slide was then kept in the dark in the 4-degree Celsius refrigerator and left there to view the following day. Images were captured using the Leica microscope and Leica X image capture software.

Total Cytochrome c Expression

To collect whole lysates of euglycemic and hyperglycemic treated HRP cells, cells were collected by trypsinization, centrifuged, and washed three times with PBS. After the supernatant was removed, the pellet that remained was resuspended in 100 uL mixture of RIPA lysis buffer and protein inhibitor (1:1000) and kept on ice for 20 minutes. The Eppendorf containing the cell pellet, RIPA and PI mixture was then centrifuged for 20 minutes at 13,000 RCF, 4 degrees Celsius. The supernatant was collected and subjected to BCA protein assay to detect and quantify protein amounts collected according to manufacturer's instructions.

Cytosolic Fraction Isolation

To compare the amount of cytochrome c released from the mitochondria into the cytosol of euglycemic and hyperglycemic treated HRP cells, cytosolic protein lysates were obtained by using cytosol extraction buffer. Cells were first scraped and collected, centrifuged, and washed three times with PBS. The supernatant was removed. The remaining cell pellet was resuspended in 100 uL of cytosol extraction buffer. The cytosol extraction buffer was prepared using 220 mM mannitol, 58 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCL, 5 mM EDTA, 2 mM DTT and protease inhibitors (1:100) (Bianchi et al., 2003; Cheng et al., 2011; Lu et al., 2003). Once resuspended in the cytosol extraction buffer, the Eppendorf vials were kept on ice for 45 minutes. After the 45 minutes, the contents were added into a glass Dounce homogenizer and homogenized by 20 strokes. The homogenates were collected and centrifuged at 14,000 RCF for 15 minutes, 4 degrees Celsius. The supernatant was collected and subjected to BCA protein assay to detect and quantify protein amounts collected according to manufacturer's instructions.

Western Blot and Cytochrome c Release

To identify and compare cytochrome c presence in the whole cell lysates and cytosolic fractions of euglycemic and hyperglycemic treated human retinal pericyte cells, a western blot was performed. Whole cell lysates and cytosolic fraction homogenates of euglycemic and hyperglycemic treated HRP cells were prepared as previously mentioned and protein concentrations were determined through use of BCA kit, according to protocol. Once determined, Eppendorf vials containing appropriate amounts of protein, lysis buffer and Laemmli (4X) SDS reducing sample buffer were heated to 97 degrees Celsius for 5 minutes. Equal amounts (15 uL) of each sample were loaded onto 4-20% polyacrylamide gels, along with protein ladders. Once loaded, the gel was run at 120 volts for approximately an hour to an hour

and a half. Once complete, the gel was transferred onto a nitrocellulose membrane. After transferring, the membrane was washed with TBST for 5 minutes. Ponceau S staining was used to detect protein bands on the membrane. The membrane was then washed with TBST two additional times for 5 minutes and blocked for one hour at room temperature in 10 mL of TBST and non-fat dry milk mixture. Once blocking was complete, the membrane was washed with TBST 3 times for 5 minutes each time and cytochrome c was probed using the cytochrome c antibody after overnight incubation in 4-degree Celsius refrigerator. During the following day, the membrane was washed with TBST 3 times for 5 minutes each time and incubated for an hour at room temperature in the secondary antibody (anti-rabbit IgG; R&D Systems Catalog #HAF008) and milk mixture. Protein bands were visualized using chemiluminescence methods.

Heme Staining

For the heme staining experiment, whole cell lysate and cytosolic fraction samples of euglycemic and hyperglycemic treated human retinal pericyte were loaded onto a 4-20% polyacrylamide gel. The gel was run at 200 volts for approximately an hour and a half to two hours. Once the gel was running, the TMBZ staining solution was prepared. 6.3 mM of TMBZ (0.04542 grams) were added to 30 mL of methanol. Once prepared, the conical was covered with foil and vortexed. TMBZ solution was stored at 4 degrees Celsius until ready for use. The 0.5 M sodium acetate solution was then prepared by mixing 1.4356 grams of sodium acetate and 35 mL of distilled or deionized water. Acetic acid was added to adjust the pH (desired pH=5). Once run on gel was complete, the gel was rinsed using water for 5 minutes. The previously prepared 6.3 mM TMBZ solution (6.3 mM TMBZ in 30 mL methanol) was immediately mixed with the 0.25 M sodium acetate solution (35 mL) and the gel submerged in the mixture for approximately 30 minutes, mixing the solution every 10-15 minutes at room temperature in a dark room. 300 uL of

30% hydrogen peroxide were added. The stains became visible after approximately 10 minutes. The gel was viewed using a white screen and gel scanner.

Experiment 1: HRP Cell Viability

To complete experiment 1, human retinal pericyte cells were cultured in complete classic media, as mentioned. Once confluent, the cells were split onto 12 well plates with 100,000 cells per well. Regular media changes were required to maintain healthy cells. Once confluent, cells were treated with 5.5 or 30 mM glucose. Treatments were prepared as mentioned. The HRP cells used for this experiment were treated for 24, 48 or 72 hours. Cell counts were then performed by trypan blue staining and the use of an automated cell counter. Once cell counts were obtained, the data was plugged into SPSS software and statistical analysis was performed.

During initial experiments for cell counts, 24 well plates were used but it was found that the cells did not grow efficiently enough for effective cell counts so 12 well plates were used instead. Another change that was made after repeating the cell counting experiments, was the amount of trypsin used to lift the cells. Initially, 500 uL were used to lift the cells but it was found that this amount of trypsin was excessive and the number of dead cells was much higher than those that were live. After discovering that the amount of trypsin being used could be too high, 80-100 uL of trypsin were used for detachment instead. This method gave improved readings. The amount of time that trypsin was kept in the 4 degree fridge instead of the -20 degree fridge additionally gave different results.

Experiment 2: TUNEL Assay to Identify Apoptotic Cells

For experiment 2, TUNEL assay was used to identify apoptotic human retinal pericyte cells. Apoptosis, as mentioned, is a form of programmed cell death. TUNEL staining is known for its ability to visualize and quantify apoptotic cells. Briefly, HRP cells were grown on 16-chamber

slides with 5,000 cells per well. Treatments included glucose concentrations of 5.5. 18.5 and 30 mM, prepared as mentioned. Each treatment was added in duplicate wells and cells were treated for 24 hours. TUNEL protocol was followed according to manufacturer instructions and the slides were viewed and imagining was captured using a LEICA microscope. To quantify apoptotic cells, the Image J cell counter tool was used. Data collected was plugged into SPSS software and TUNEL positive percentage totals for each glucose treatment were computed.

Experiment 3: Western Blotting and Heme Staining to Determine Effectiveness of Cytochrome c as a Biomarker of Diabetic Retinopathy

To complete experiment 3, western blot and heme stain methods were used. After various attempts and using several kits to try to obtain cytosolic and mitochondrial fractions of glucose treated HRP cells, it was found that comparing the total cell lysates for total cytochrome c expression and isolating cytosolic fractions would be an efficient start. Total cell lysates were obtained using RIPA lysis buffer and protein inhibitor cocktail. Cytosolic fraction isolation was done with the use of an in lab made cytosolic extraction buffer. To prepare the cytosolic extraction buffer 220 mM mannitol, 58 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCL, 5 mM EDTA, 2 mM DTT and protease inhibitors (1:100) were combined.

Once total cell lysates and cytosolic fractions were obtained, a BCA protein assay was conducted according to manufacturer protocol to determine how much protein was found in each sample. The protein samples were then used for western blotting, following traditional Western blot methods, as mentioned. 10 ug of each protein samples were added into the wells. An internal loading control, which serves to compare signals between lanes and proves that the amount of protein loaded into each well is equal, was not used. In the future, when repeating these experiments, I intend to use GAPDH or beta actin as loading control. Cytochrome c probing was done with cytochrome c antibody. Protein samples were also used for heme staining

experiments, as mentioned. It was found that the longer the gel was allowed to soak, the better the stain intensity became. A total of 1-2 hours has proven to work best. Protein bands for both Western blot and heme staining experiments were captured using a Western blot and gel imaging scanner (Thermo Fischer; Invitrogen iBright 1500). Image J was used to identify differences in protein bands.

CHAPTER IV

FINDINGS

Experiment 1: Hyperglycemic Conditions Significantly Affect Cultured Human Retinal Pericytes When Compared to Euglycemic Conditions

According to the World Health Organization, the normal value range for fasting blood glucose in a patient are 3.9 mmol/L to 5.6 mmol/L, with levels above 7 mmol/L establishing a diabetes diagnosis. Experiment 1 focused on culturing human retinal pericyte cells in euglycemic and hyperglycemic glucose levels to understand and be able to identify how human retinal pericyte cells are affected when treated with hyperglycemic conditions, which mimic conditions for diabetic patients, as opposed to those treated with euglycemic conditions. To compare the effects of normal and high glucose conditions on HRP viability, confluent cells were treated for 24, 48 and 72 hours with DMEM (-D Glucose, +L-Glutamine, -Sodium Pyruvate) prepared with 1% antibiotic and either 5.5 mM (euglycemic) or 30mM (hyperglycemic) glucose. By treating human retinal pericytes with differing glucose treatments (5.5 mM and 30 mM) it can be hypothesized that those treated with close to normal or euglycemic levels of glucose, 5.5 mM, will thrive and function optimally and those treated with hyperglycemic levels of glucose, 30 mM, are expected to be significantly affected and declines of normal cell behavior and apoptosis will be observed. Figure 1 shows differences in total viable cells/mL of 5.5 mM and 30 mM glucose treated HRP at 24, 48 and 72 hours. Human retinal pericytes that were treated with 5.5 millimolar glucose media had a viable cell average of 77% after 24 hours, 73.67% after 48 hours

and 74.33% after 72 hours. Human retinal pericytes treated with 30 millimolar glucose media, on the other hand, had a viable cell average of 60.33% after 24 hours, 57% after 48 hours and 57% after 72 hours.

A two-way ANOVA was used to show statistical significance between glucose concentrations, treatment time points and interaction between glucose concentration and treatment time point. It was concluded that significant increases are observed as glucose concentrations increase; $F(1,12) = 1.20$, $p<0.0001$, and as treatment time increases; $F(2,12) = 13.07$, $p=0.001$. No significance was found between the interaction between glucose concentration and treatment time point; $F(2,12) = 0.5976$, $p=0.5657$. Tukey's multiple comparison test showed significance between 24 vs. 72-hour treatment times for euglycemic concentration **$p=0.0036$ and between 24 vs. 72-hour treatment times for hyperglycemic concentration *$p=0.0278$.

Experiment 2: Apoptosis/Cell Death and TUNEL Staining

When cells undergo DNA fragmentation, the ends of the DNA strands are broken. Through the TUNEL assay, these DNA breaks are detected and can be used to quantify the percent of apoptosis occurring within cells. The TUNEL assay method has been used in numerous studies. These studies have shown that treatments with higher glucose concentrations will ultimately lead to obtaining higher measurements of apoptosis presence within the cells. Experiment 2 focused on detecting and quantifying apoptosis in human retinal pericytes treated with differing glucose amounts (5.5 mM, 18.5 mM and 30 mM) using TUNEL assay. When viewing the slide using the Leica X microscope, it was evident that as glucose concentrations increased, not only was there a significant change in the morphology of the human retinal pericyte cells, but areas where apoptosis occurred within the cell appeared to be more frequently

observed. Percentage calculations were done by counting the total number of DAPI stained nuclei and dividing it by the total amount of DAPI stained nuclei which showed apoptosis (bright green-blue colored glow) once the images were merged. Table 1 shows counts of DAPI stained nuclei and apoptotic cells (merged image). These calculations were used to obtain an apoptosis percentage for each of the glucose treatments. Figure 2 shows the percentage of apoptosis measured in 5.5 mM, 18.5 mM and 30 mM glucose treated HRP cells. The average percentage of apoptotic cells for 5.5 mM glucose treated HRP was 26%. The average percentage of apoptotic cells for 18.5 mM glucose treated HRP was 32%. The average percentage of apoptotic cells for 30 mM glucose treated HRP was 43.5%. A one-way ANOVA was performed to determine significance between differences in increasing glucose concentrations. Significance was found in the presence of TUNEL positive HRP cells as percentage of TUNEL positive HRP cells increased as glucose concentrations increased; $F(2,6) = 5.503$, $p=0.043$.

Experiment 3: Cytochrome c as a Potential Biomarker of Diabetic Retinopathy

As mentioned, diabetic retinopathy is a leading cause in blindness among adults and is most often diagnosed once progression of symptoms of the disease have become established. It is crucial to find methods to diagnose the disease before these symptoms begin to affect the patient's day to day life and activities or become so pronounced that there is no way to reverse these detrimental effects and all that is left to do is slow them down. Countless studies have been conducted using differing cell lines (in the laboratory) and human test subjects (in clinical research studies) to try to determine the best course of action to gaining understanding of the disease and pathways which lead to diagnosis, symptom appearance and progression and treatment advances/discoveries. Experiment 3 focused on determining whether there is a potential link or correlation between cytochrome c levels present in whole cell lysates when

compared to the cytochrome c levels present in the cytosol of human retinal pericyte cells treated with euglycemic and hyperglycemic conditions using Western blotting and heme staining methods. Benzidine staining procedures using sodium dodecyl sulfate polyacrylamide gels have been utilized to detect levels of heme-associated activity. The bands which appeared on the heme stained polyacrylamide gels possess a blue hue and may be photographed and scanned for further analyzation.

Results of Western blot and heme staining experiments were captured using chemiluminescence and gel scanning. Figure 4 shows cytochrome c detection in the isolated cytosolic fractions and whole cell lysates of euglycemic and hyperglycemic treated HRP cells. When preparing treatments and loading controls, 4 HRP plates grown in CCM without treatment were to be used as control samples but plates were contaminated. Due to time constraints, the loading control was not loaded onto gels for western blotting or heme staining experiments. Lack of control sample meant that ratios for cytochrome c release could not be quantified. To compensate for lack of control sample, ImageJ software was used to detect the area of the western blot bands, along with the intensity of the bands. With the areas of each band obtained, it can be concluded that there was a 1:1 difference between 5.5 mM cytosolic fraction bands and 30 mM cytosolic fraction bands and a 3:1 difference between 5.5 mM whole cell lysate bands and 30 mM whole cell lysate bands. When comparing between cytosolic fractions and whole cell lysates, there was a 6:1 difference between 5.5 mM whole cell lysate bands and 5.5 mM cytosolic fraction bands and a 2:1 difference between 30 mM whole cell lysate bands and 30 mM cytosolic fraction bands. Averages of triplicate and duplicate samples were taken to reach these conclusions. Band intensities can be observed in Table 2. As for heme staining experiments, Figure 5 depicts the blue hue of the bands, along with the exposed gel showing protein presence.

Location of the bands on the gel correlate with cytochrome c bands captured during Western blot experiments. Determining whether cytochrome c could potentially be deemed a biomarker of diabetic retinopathy will require repetition of experiments, but the results presented could benefit and serve as a contribution to the knowledge we currently possess.

CHAPTER V

DISCUSSION

Retinal pericyte dropout is widely recognized as a staple of diabetic retinopathy. In this study, I investigate the effects of euglycemic and hyperglycemic conditions on human retinal pericyte cells and the presence of apoptosis after treatment with stated conditions. The possibility of whether cytochrome c presence in the cytosol, in comparison to the mitochondria, of treated cells could serve as a means to recognize and potentially highlight the probability of disease development at earlier stages was also explored. The experiments conducted in this study are the first to collectively explore cytochrome c as a biomarker for diabetic retinopathy using HRP. The possibility of using heme staining of human retinal pericyte mitochondrial and cytosolic fractions to detect presence of cytochrome c was also introduced.

Before exploring these ideas, it was important to understand the behavior of human retinal pericytes in hyperglycemic and euglycemic conditions. Cell viability measurements using the trypan blue exclusion method serve as a key indicator of the effects of different conditions on HRP cells, along with many other cell lines. The dye detects damaged cells. A previous study by Vidro, E. K. et al. showed that when HRP cells were cultured in 18 mM (high glucose) for 5 days, cell viability was reduced by 32% (Vidro, E. K. et al. 2019). Vidro, E. K. et al. went on to also expose HRP cells to 33- and 40-mM glucose concentrations, which conclusively showed a 47% and 64% reduction in viability, respectively (Vidro, E. K. et al. 2019). This study used the trypan blue exclusion method to account for viable and nonviable HRP cells. As hypothesized,

and consistent with previous studies, hyperglycemic conditions proved to significantly affect cultured human retinal pericyte cells in comparison to euglycemic conditions.

Another method used in this study, which detected fragmented DNA and assessed apoptotic HRP cells, was the TUNEL assay. In a previous study by Trudeau et al., bovine retinal cells were treated with normal (5 mM) and high glucose (30 mM) for 7 days and analyzed for apoptosis using TUNEL assay. The results showed that when HRP were treated with high glucose, mitochondrial morphology and metabolism were compromised and an increase of TUNEL positive cells was observed, 5.6 ± 2.6 TUNEL-positive cells/1000 cells compared with 13.6 ± 3.5 TUNEL-positive cells/1000 cells in normal; $P = 0.007$ (Trudeau, K. et al. 2011). The results gathered in this study demonstrate that hyperglycemic conditions imposed on human retinal pericytes not only showed a significant reduction in viability after 24, 48 and 72 hours of treatment but also a significant increase in total percentage of apoptosis. These findings were consistent with previous reports.

Once the behavior of human retinal pericytes in hyperglycemic and euglycemic conditions was understood after cell viability and apoptosis measurements were analyzed in this study, the possibility of cytochrome c as a biomarker of diabetic retinopathy could be further investigated. Previous studies have shown molecular mechanisms of diabetic retinopathy begin with diabetes and hyperglycemia which lead to changes in mitochondrial morphology and cause mitochondrial fragmentation, further causing a release of cytochrome c from the mitochondria into the cytosol, which in turn activates apoptosis (Roy, S. et al. 2019). When this happens, acellular capillaries form and retinal pericytes are lost (Roy, S. et al. 2019).

C-type cytochromes are hemeproteins, containing a covalently attached heme at the center. Kowluru, R. A. and Abbas, S. N. conducted a study in 2003 where retinal pericytes were

isolated from diabetic rats and treated with normal and high glucose treated cells for 1, 3, 5 and 10 days. The results showed that cytochrome c cytosolic contents significantly increased by fourfold when treated with high glucose after 5 days but did not show significant differences when compared to cells treated with normal glucose before day 3 (Kowluru, R. A., & Abbas, S. N. 2003). ECL (enhanced chemiluminescence) detection reagents and TMBZ staining have also been used to detect c-type cytochromes in the past, using bovine hearts, but not in connection to human retinal pericytes and diabetic retinopathy (Vargas, C. et al. 1993).

In this study, no significant cytochrome c presence differences were found between 5.5 mM and 30 mM cytosolic fractions of glucose treated HRP cells. Significant cytochrome c presence differences were found between 5.5 mM and 30 mM whole cell lysates of glucose treated HRP cells. These results did not show what I had initially hypothesized. Previous studies using other cells lines have shown that a significant increase is often observed when comparing whole cell lysates, and mitochondrial and cytosolic fractions, with there being a higher concentration of cytochrome c in samples treated with higher glucose concentrations. Although significance was not found or proved in specific aim 3 experiments, which involved the use of western blotting and heme staining methods, repeating the experiments could provide more reliable results and prove the effectiveness of cytochrome c as a biomarker of diabetic retinopathy. Identifying cytochrome c as a biomarker of diabetic retinopathy could potentially lead to the use of methods which detect cytochrome c in human samples for use in animal studies and clinical interventions.

Potential changes that can be made or key notes to keep in mind in order to improve the results which were obtained throughout these experiments, include but are not limited to, finding better/easier methods to quantitate TUNEL results, using GAPDH or beta actin as loading

control during Western blotting, as well as having a control sample in order to compare bands, allowing heme gels to be submerged in the TMBZ, methanol and sodium acetate mixture for 1-2 hours instead of 5-30 minutes, allowing stain intensity to increase for a longer period of time (+30-45 minutes) after the addition of 30% hydrogen peroxide and finding more effective methods to quantify heme stain bands. In future experiments, I propose to find a way to efficiently separate mitochondrial and cytosolic fractions of glucose treated cells for Western blotting and heme staining experiments.

Diagnosis of diabetic retinopathy most commonly occurs after continuous progression of the disease, at which point symptoms are presented and have advanced in severity. The conclusions presented in this study could prove to be essential, as finding a non-invasive or minimally invasive method to diagnose the disease at an earlier time point could lead to treatment of the disease before further progression takes place. Researchers are also still not certain about the introduction of cytochrome c as a biomarker, as many more studies must still be conducted. Detection of cytochrome c in the cytosol could lead to several applications in clinical diagnosis or therapies. To test a patient's cytochrome c levels, a sample of blood, or urine, may be evaluated. Previous studies in the biomedical research industry, specializing in ocular diseases, have also been conducted with rat models and bovine retinal cells, but cytochrome c release from the mitochondria has not been proven as an effective biomarker for the early detection of diabetic retinopathy in humans.

It is crucial to continue to perform experiments which will increase our knowledge of biomarkers, such as in this study with cytochrome c, and their use in diagnosis or early detection of diseases such as diabetic retinopathy.

REFERENCES

Bianchi, P., Seguelas, M.H., Parini, A., Cambon, C., 2003. Activation of pro-apoptotic cascade by dopamine in renal epithelial cells is fully dependent on hydrogen peroxide generation by monoamine oxidases. J. Am. Soc. Nephrol. 14 (4), 855–862.

Cecilia, O. M., José Alberto, C. G., José, N. P., Ernesto Germán, C. M., Ana Karen, L. C., Luis Miguel, R. P., ... & Adolfo Daniel, R. C. (2019). Oxidative stress as the main target in diabetic retinopathy pathophysiology. Journal of diabetes research, Vol 2019, Article ID 8562408.

Cheng, B., Maffi, S.K., Martinez, A.A., Acosta, Y.P., Morales, L.D., Roberts, J.L., 2011. Insulin-like growth factor-I mediates neuroprotection in proteasome inhibition-induced cytotoxicity in SH-SY5Y cells. Mol. Cell Neurosci. 47 (3), 181–190

Eleftheriadis, T., Pissas, G., Liakopoulos, V., & Stefanidis, I. (2016). Cytochrome c as a Potentially Clinical Useful Marker of Mitochondrial and Cellular Damage. Frontiers in immunology, 7, 279.

Garcia-Medina, J. J., Rubio-Velazquez, E., Foulquie-Moreno, E., Casaroli-Marano, R. P., Pinazo-Duran, M. D., Zanon-Moreno, V., & Del-Rio-Vellosillo, M. (2020). Update on the Effects of Antioxidants on Diabetic Retinopathy: In Vitro Experiments, Animal Studies and Clinical Trials. *Antioxidants (Basel, Switzerland)*, *9*(6), 561.

Gogvadze, V., Orrenius, S., & Zhivotovsky, B. (2006). Multiple pathways of cytochrome c release from mitochondria in apoptosis. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1757(5-6), 639-647.

Kalpage, H. A., Wan, J., Morse, P. T., Zurek, M. P., Turner, A. A., Khobeir, A., ... & Sanderson, T. H. (2020). Cytochrome c phosphorylation: control of mitochondrial electron transport chain flux and apoptosis. The International Journal of Biochemistry & Cell Biology, 121, 105704.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., & Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science, 275(5303), 1132-1136.

Kowluru, R. A. (2019). Mitochondrial stability in diabetic retinopathy: lessons learned from epigenetics. Diabetes, 68(2), 241-247.

Kowluru, R. A., & Abbas, S. N. (2003). Diabetes-induced mitochondrial dysfunction in the retina. Investigative ophthalmology & visual science, 44(12), 5327-5334.

Kulikov, A. V., Shilov, E. S., Mufazalov, I. A., Gogvadze, V., Nedospasov, S. A., & Zhivotovsky, B. (2012). Cytochrome c: the Achilles' heel in apoptosis. Cellular and molecular life sciences: CMLS, 69(11), 1787–1797.

Lu, S., Hoestje, S.M., Choo, E., Epner, D.E., 2003. Induction of caspase-dependent and – independent apoptosis in response to methionine restriction. Int. J. Oncol. 22(2), 415–420

Roy, S., Kim, D., & Sankaramoorthy, A. (2019). Mitochondrial structural changes in the pathogenesis of diabetic retinopathy. Journal of clinical medicine, 8(9), 1363.

Santucci, R., Sinibaldi, F., Cozza, P., Polticelli, F., & Fiorucci, L. (2019). Cytochrome c: An extreme multifunctional protein with a key role in cell fate. International journal of biological macromolecules, 136, 1237-1246.

Shakeri, R., Kheirollahi, A., & Davoodi, J. (2017). Apaf-1: Regulation and function in cell death. Biochimie, 135, 111–125. https://doi.org/10.1016/j.biochi.2017.02.001

Sinclair, M. D., Hayward, S., Schwartz, M. D., & Steven, S. (2019). Diabetic Retinopathy-An Underdiagnosed and Undertreated Inflammatory, Neuro-Vascular Complication of Diabetes. Frontiers in Endocrinology, 10, 843.

Trudeau, K., Molina, A. J., & Roy, S. (2011). High glucose induces mitochondrial morphology and metabolic changes in retinal pericytes. Investigative ophthalmology & visual science, 52(12), 8657-8664.

Van den Oever, I. A., Raterman, H. G., Nurmohamed, M. T., & Simsek, S. (2010). Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus. Mediators of inflammation, Vol 2010, Article ID 792393.

Vargas, C., McEwan, A. G., & Downie, J. A. (1993). Detection of c-type cytochromes using enhanced chemiluminescence. Analytical biochemistry, 209(2), 323-326.

Vidro, E. K., Gee, S., Unda, R., Ma, J. X., & Tsin, A. (2008). Glucose and TGFbeta2 modulate the viability of cultured human retinal pericytes and their VEGF release. *Current eye research*, *33*(11), 984–993.

`Zhang, M., Zheng, J., Nussinov, R., & Ma, B. (2017). Release of Cytochrome C from Bax Pores at the Mitochondrial Membrane. Scientific reports, 7(1), 2635.

APPENDIX

APPENDIX

TABLES AND FIGURES

Concentration	DAPI stained nuclei	Merged (Apoptotic Cells)	Average
5.5 mM	78	18	23.08%
5.5 mM	54	17	31.48%
5.5 mM	89	21	23.60%
18.5 mM	91	34	37.36%
18.5 mM	102	26	25.49%
18.5 mM	63	21	33.33%
30 mM	62	25	40.32%
30 mM	64	34	53.13%
30 mM	51	19	37.25%

Table 1. Experiment 2; Counts of DAPI Stained Nuclei and Apoptotic Cells (Merged). Average % included.

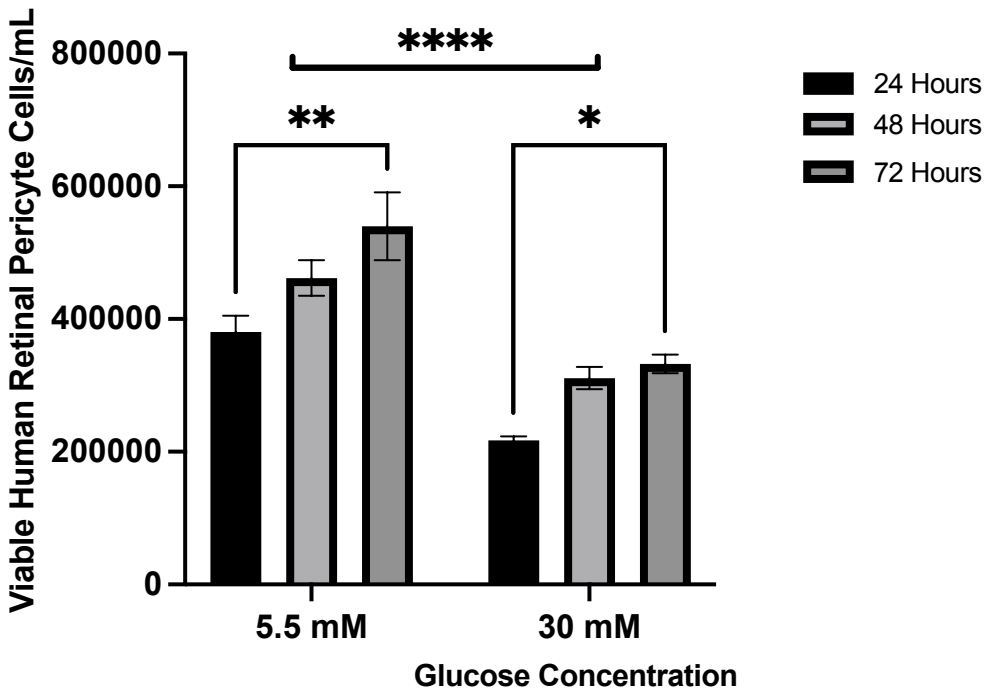

Figure 1. Experiment 1; Viability Measurements of HRP using Trypan Blue Staining. Human retinal pericyte cells grown in complete classic media and treated with euglycemic (5.5 mM) and hyperglycemic (30 mM) media for 24, 48 and 72 hours. Data shown represents the mean ± SEM. N=3. Viable human retinal pericyte cells were counted using automated cell counter (Countess II FL, Cat. AMQAF1000) and trypan blue stain. HRP treated with euglycemic (5.5 mM) media had an average of 77% after 24 hours, 73.67% after 48 hours and 74.33% after 72 hours. HRP treated with hyperglycemic (30 mM) media had an average of 60.33% after 24 hours, 57% after 48 hours and 57% after 72 hours. When comparing euglycemic and hyperglycemic concentrations, significant differences were found in viable cells/mL; $F(1,12) = 61.2$, ****$p<0.0001$. When comparing 24-, 28- and 72-hour treatment time points, significant differences were found in viable cells/mL; $F(2,12) = 13.07$, ***$p=0.001$. Glucose concentration and treatment time interaction did not have a significant effect on number of viable HRP cells/mL; $F(2,12) = 0.5976$, $p=0.5657$. Tukey's multiple comparison test showed significance between 24 vs. 72-hour treatment times for euglycemic concentration **$p=0.0036$ and between 24 vs. 72-hour treatment times for hyperglycemic concentration *$p=0.0278$.

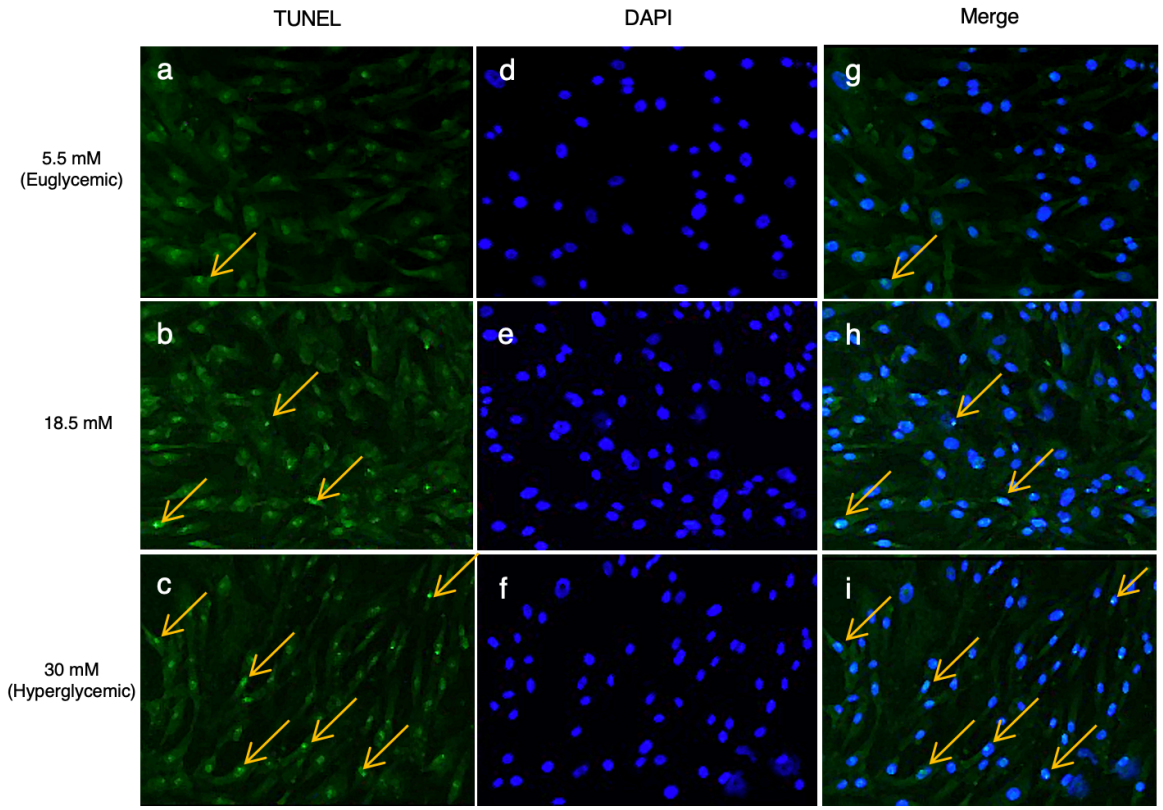

Figure 2. Experiment 2; TUNEL Stains of HRP Treated with Increasing Concentrations of Glucose. Glucose treated HRP TUNEL staining (a, b, c) with respective DAPI stain (d, e, f) and merged image (g, h, i). Human retinal pericyte cells grown in complete classic media and treated with DMEM media containing 5.5 mM (a, d, g), 18.5 mM (b, e, h) and 30 mM (c, f, i) glucose concentrations for 24 hours. Yellow arrows (g, h, i) show apoptotic cells. Increasing glucose concentrations lead to increase in observed number of TUNEL positive cells.

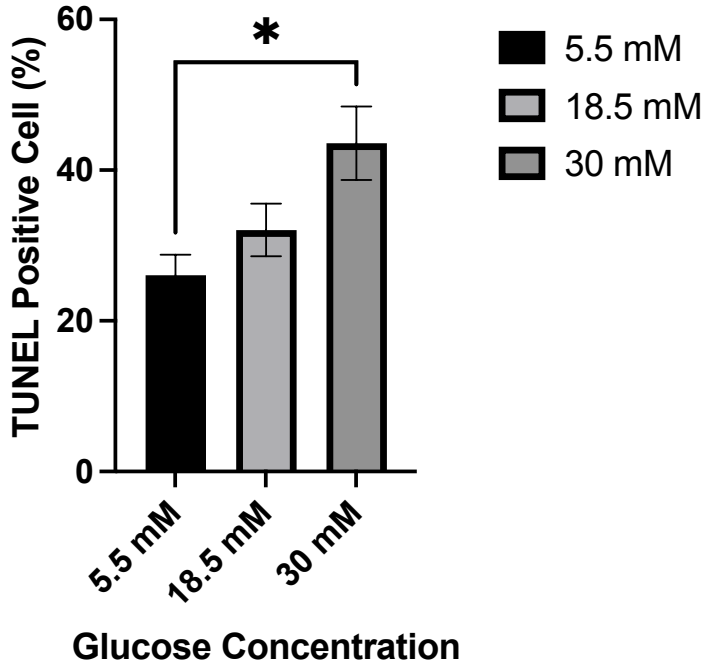

Figure 3. Experiment 2; TUNEL Positive Cell Percentages by Increasing Glucose Concentration. Human retinal pericyte cells grown in complete classic media and treated with DMEM media containing 5.5 mM, 18.5 mM and 30 mM glucose concentrations for 24 hours. The average percentage of apoptotic cells for 5.5 mM glucose treated HRP was 26%. The average percentage of apoptotic cells for 18.5 mM glucose treated HRP was 32%. The average percentage of apoptotic cells for 30 mM glucose treated HRP was 43.5%. A one-way ANOVA was performed to determine significance between differences in increasing glucose concentrations. Significant increases are observed in TUNEL positive cell percentages as glucose concentrations increase; $F(2,6) = 5.503$, $p=0.0439$. *$p<0.05$. When comparing apoptosis in HRP cells treated with 5.5 and 30 glucose, an approximate 20% increase in incidence was detected. The increase could potentially support findings in experiment 1, accounting for viable cell decreases.

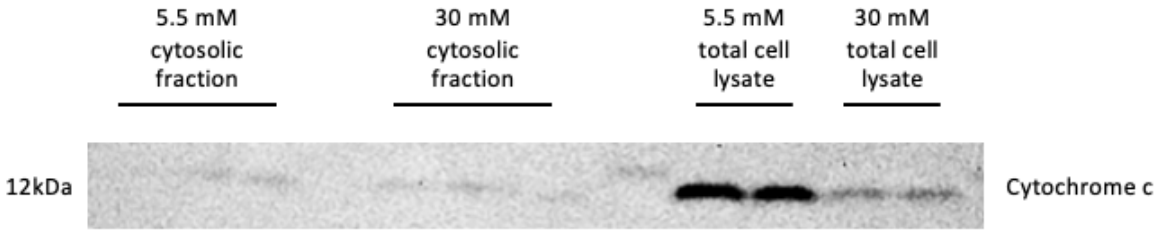

Figure 4. Experiment 3; Cytochrome c Detection in the Cytosol and Whole Cell Lysates of Glucose Treated HRP Cells. Human retinal pericytes were starved for 24 hours and treated with 5.5 mM and 30 mM glucose for 5 days. Cytochrome c presence was determined in the cytosolic fractions and whole cell lysates through Western blot analysis. Cytochrome c has a molecular weight of approximately 12,000 Daltons or 12 kDa. Triplicates of 5.5 mM and 30 mM cytosolic fractions and duplicates of 5.5 mM and 30 mM total cell lysates were loaded onto the gel. 10 ug of each protein sample were loaded. Lack of control sample meant that ratios for cytochrome c release could not be quantified. To compensate for lack of control sample, ImageJ software was used to detect the area of the western blot bands, along with the intensity of the bands. A 1:1 difference between 5.5 mM cytosolic fraction bands and 30 mM cytosolic fraction bands and a 3:1 difference between 5.5 mM whole cell lysate bands and 30 mM whole cell lysate bands. When comparing between cytosolic fractions and whole cell lysates, there was a 6:1 difference between 5.5 mM whole cell lysate bands and 5.5 mM cytosolic fraction bands and a 2:1 difference between 30 mM whole cell lysate bands and 30 mM cytosolic fraction bands. Averages of triplicate and duplicate samples were taken to reach these conclusions. No significant cytochrome c presence differences were found between 5.5 mM and 30 mM cytosolic fractions of glucose treated HRP cells. Significant cytochrome c presence differences were found between 5.5 mM and 30 mM whole cell lysates of glucose treated HRP cells. These findings are not consistent with initial hypothesis or previous study findings. Typically, higher glucose concentrations yield higher presence of cytochrome c in the cytosol of the cell.

Band	Band Intensity
1	613.5
2	587.4
3	553.7
4	510.7
5	543.0
6	550.1
7	889.2
8	884.6
9	719.2
10	737.8

Table 2. Experiment 3; Western Blot Band Density. According to Image J, the band densities do not have units but instead can be used to compare relativeness to standard band.

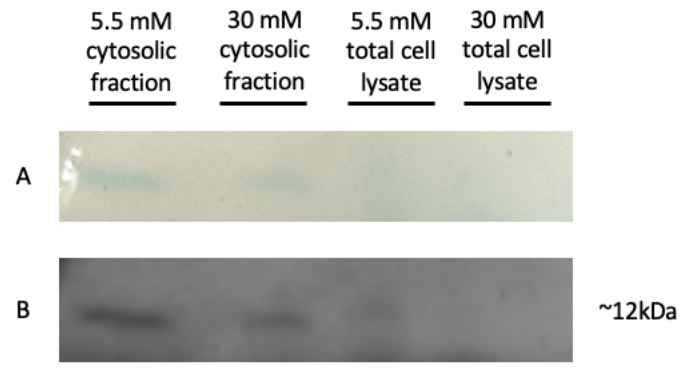

Figure 5. Experiment 3; Heme Stains of Euglycemic and Hyperglycemic Treated HRP Cells. Samples of 5.5 mM and 30 mM glucose treated HRP cytosolic fractions and 5.5 mM and 30 mM glucose treated HRP total cell lysates loaded and ran on SDS-polyacrylamide gels and captured using gel scanner. 10 ug of protein loaded in each well. (A) Heme stain possesses blue hue often seen in c-type cytochrome staining experiments after TMBZ stains. (B) Protein present in gels captured by gel scanner correlate with cytochrome c band location captured in Western blot experiment.

BIOGRAPHICAL SKETCH

Ileana Georgina Villarreal, born on November 5, 1994, started school in Houston, Texas at Pyburn Elementary when she was 5 years old. She attended Pyburn Elementary until the completion of second grade, then moved to Nuevo Leon, Mexico where she lived with grandparents for a year. While living there, she completed third grade in primary school. Ileana moved back to the United States with her mother where she attended Sam Houston Elementary to complete fourth and fifth grade. For grades sixth through eight, she attended Cesar Chavez Middle School and then went on to complete ninth grade at La Joya High School and earned her high school diploma from Premier High School of Palmview in 2012 as class Salutatorian and was accepted to UT Pan-American that same year. Ileana earned a Bachelor of Science Degree from UTRGV in Biology in 2018 and a Master of Science degree in Biology in August 2022.

During her time as an undergraduate student, she was hired to work as a clinical research coordinator at Valley Retina Institute in McAllen, Texas. After a year of conducting clinical research studies, she decided to apply to the graduate biology program and focus on my studies. During her time as a graduate student, she was employed in Dr. Tsin's laboratory as a graduate lab assistant, where she accepted an assistantship position during Summer 2020. Ileana became a teaching assistant for UTRGV in 3 biology 2 sections during spring 2021 while employed by Renaissance Laboratories as a lab assistant.

Ileana's current research focuses on diabetic retinopathy, with emphasis on the use of cytochrome c as a biomarker for early detection of the disease. Her personal email is vileana94@gmail.com.