Sodium Iodate Response in ARPE-19 Cells:

An *In Vitro* Model of Age-Related Macular Degeneration

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Abstract

With the rise in aging populations worldwide, diseases of the eye like age-related macular degeneration (AMD) will continue to rise and the need to investigate ways to slow down the progression of the disease are at an all-time high. AMD exists in two forms, the slower and less invasive dry form and the more aggressive wet form which can lead to a rapid loss of vision in patients diagnosed. This study aims to investigate the role of oxidative stress and the ability to use natural antioxidant compounds as a means of reducing oxidative stress and mitigating further damages in AMD. This study also aims to further investigating the role of gene knockdowns in cytoprotection of human retinal pigment cell line (ARPE-19 cells) pre-treated with natural antioxidant compounds. A wild-type ARPE-19 cells (WT) was used as an *in vitro* model of AMD to investigate the use of antioxidant compounds to mitigate oxidative stress damages. Resveratrol, pterostilbene, lutein, punicalagin, loganin, chebulagic acid, beta-carotene, and zeaxanthin were investigated in the cell culture model as a means of offering cytoprotection to ARPE-19 cells exposed to blue light and sodium iodate (SI), both used as inducers of oxidative stress. Blue light exposure of the ARPE-19 cells resulted in increased oxidative stress. SI exposure resulted in a significant ($p<0.05$) decreases in cell viability as well as a significant ($p<0.05$) increase in intracellular oxidative stress when utilized at concentrations above 15 mM. From initial testing with the 6 compounds using cell viability assay, lutein, zeaxanthin, and beta-carotene showed the most

promising potential of cytoprotection from SI induced oxidative stress and were selected as the main compounds of interest to study its effect on markers of oxidative stress, antioxidants (Manganese-dependent superoxide dismutase (MnSOD) and catalase) and cell death (caspase 3) . Results indicated that lutein and zeaxanthin pre-treatment prior to exposure to SI resulted in a significant ($p<0.001$) protective effect on maintain cell viability as well as a significant ($p<0.001$) reduction in intracellular oxidative stress when compared to the SI treatment alone cells on ARPE-19 WT cells. Zeaxanthin pre-treatment resulted in a significant ($p<0.05$) increase in MnSOD protein expression at lower SI concentrations (15 mM) and significant ($p<0.05$) decreases at higher SI concentrations (17.5 mM). Catalase protein expression was significantly ($p<0.001$) decreased when compared to SI alone control cells. CRISPR Cas-9 Knockdown cells for survivin (BIRC5) and sirtuin 1 (SIRT-1) gene were used to investigate its role in the cellular response of lutein, zeaxanthin, and beta-carotene when exposed to SI. Comparing the results of WT cells to the BIRC 5 and SIRT-1 knockdown cells, it was evident that the cytoprotective role of lutein is not mediated by either of these pathways. The decrease in cell viability and an increase in oxidative stress observed with zeaxanthin treatment of BIRC5 KO cells indicated that the beneficial effect of zeaxanthin is mediated through the BIRC5 pathway. A relatively modest protection by beta-carotene in the SIRT-1 and BIRC5 KO cells when compared to the ARPE-19 WT cells suggested that the beneficial effects of beta-carotene is independent of the survivin

and sirtuin pathways. This study investigated the possible protective effect of various classes of bioactive antioxidant compounds. From the experiments conducted, lutein, zeaxanthin, and beta-carotene were found to offer cytoprotective effects and mitigated SI induced oxidative stress in ARPE-19 cells. The results from this work supports the further evaluation of the underlying mechanisms of carotenoids in the prevention of ARPE-19 cell damage.

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Abbreviations

4-HNE	4-Hydroxynonenal/ 4-hydroxy-2-noneal
A2E	N-retinyl-N-retinylidene ethanolamine
AMD	Age-Related Macular Degeneration
AREDS	Age-Related Eye Disease Study
AREDS2	Age-Related Eye Disease Study 2
BIRC5	Baculoviral IAP Repeat Containing 5 (Survivin)
CCK-8	Cell Counting Kit-8
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CMH2-DCFDHA	2',7'-Dichlorodihydrofluorescein Diacetate
DMEM-f12	Dubecco's Modified Eagle Medium-f12
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
GPx-1	Glutathione Peroxidase 1
H_2O_2	Hydrogen Peroxide
KO	Knockout
LED	Light Emitting Diode
MnSOD	Manganese-dependent Superoxide Dismutase (SOD2)

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NaCl	Sodium Chloride
PAM	Protospacer Adjacent Motif
PBS	Phosphate Buffered Saline
PCB	Printed Circuit Board
PCR	Polymerase Chain Reaction
PDT	Photodynamic Therapy
pRGC	Photosensitive Retinal Ganglion Cells
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelial
SDS	Sodium dodecyl sulfate
SEM	Standard Error of the Mean
sgRNA	single guide-RNA
SI	Sodium Iodate ($NaIO_3$)
SIRT-1	Sirtuin-1
TBST	Tris-buffered Saline with Tween-20
Tm	Melting Temperature
VEGF-A	Vascular Endothelial Growth Factor A
VIEW	VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet AMD
VISION-1	VEGF Inhibition Study in Ocular neovascularization

WT	Wild Type

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Chapter 1: Introduction

1.1 Introduction

AMD is a progressive eye disease of the macula, resulting in a blurred central vision field (Colijn et al. 2017; Moutray and Chakravarthy 2011). Two forms of AMD are presently known, dry AMD is slower progressing than wet AMD, or exudative AMD (Bressler 1988; Hernández-Zimbrón et al. 2018). Typically, treatments for the dry form include nutritional supplementation, vitamins, and regular monitoring for transition to the more advanced and aggressive wet form of AMD (Bressler 1988; Jager 2008). Dry AMD is characterized by the formation of small acellular polymorphous debris called drusen deposits in between the RPE cells and Bruch's membrane (Jager 2008; Crabb et al. 2002). Drusen deposits can range from one deposit up to several hundred (Bressler 1988). Wet AMD is characterized by the formation of abnormal blood vessels which tend to be weak and leak fluid under the macular resulting in a change in macular shape resulting in vision loss (Kovach et al. 2012).

AMD is a multifactorial disease where environmental stressors, genetics, and lifestyle can play a role in the development of both forms (Jager 2008). One main stressor that has been noted to be a driving force behind its development is oxidative stress (Beatty et al. 2000). The retinal pigment epithelial (RPE) cells involved in the visual pathway are prone to oxidative stress build up due to their high oxygen consumption and chronic exposure to photooxidative stress (Jabbehdari and Handa 2021). Studies have highlighted the possibility that short wavelength, blue light, has the

ability to penetrate to the back of the eye and damage the RPE cells and induce increases in oxidative stress (Kuse et al. 2014; Algvere, Marshall, and Seregard 2006). High mitochondrial levels in the RPE cells results in large metabolic activity which can increase reactive oxygen species (ROS) within the eye exponentially in the presence of mitochondrial dysfunction (Jabbehdari and Handa 2021; Flohé 2016). Although oxidative stress is thought to be a key player in AMD progression, numerous other pathways both known, and unknown are likely playing a major role in the development of the disease.

SIRT-1 is a protein that has been the center of recent research due to its involvement in numerous biological pathways with focus on longevity and cell survival. SIRT-1 has been found to be involved in diabetic nephropathy and regulation of lipid and glucose metabolism (Hase et al. 2021; W. Wang et al. 2019). SIRT-1 is also known to be heavily involved in the regulation of inflammation as it relates to neurodegenerative diseases (Sharma et al. 2012). With this understanding of SIRT-1's involvement in ocular disease, it is an attractive potential target in prevention of progression of AMD (Tan et al. 2020).

BIRC5 (also known as Survivin) has been identified as a major target of many cancer signaling pathways (Cao et al. 2019). It is primarily involved in cell proliferation and immune response related signaling pathways (Jiang et al. 2021; J. Wang et al. 2021). BIRC5 has been identified as a regulator of autophagy, mitosis, apoptosis, migration

and invasion in many cancers (Adinew et al. 2022; T. Lin et al. 2019). Although the BIRC5 pathway is not fully understood, its involvement in cell proliferation and immune response pathways makes it a possible mechanism of action for ocular diseases like AMD.

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 system has been widely used to efficiently and accurately generate knockout cells in various cell types allowing for the construction of experimental model of diseases and the identification of potential therapeutic targets for these diseases (Chandrasekaran et al. 2022; Hart et al. 2015; Inoue et al. 2023; H. Wang et al. 2017; Yichen Wang et al. 2014; Yongping Zhang et al. 2017). Using CRISPR-cas9 in ARPE-19 cells will allow for the investigation of how these pathways of SIRT-1 and BIRC5 play a role in the antioxidant response to mitigate oxidative stress and protect cell viability.

SI has been shown to be a reliable model in studies of RPE damage and cellular therapies within the eye (Kannan and Hinton 2014; Hanus et al. 2016). Exposure to SI has been shown to induce oxidative stress and apoptosis in RPE cells, closely resembling the pathophysiological conditions of AMD (X.-Y. Zhang et al. 2016).

Antioxidant supplements have been a major area of investigation for slowing down the progression of dry AMD. The Age-Related Eye Diseases Study (AREDS) demonstrated the importance of antioxidant vitamins and minerals in reducing the risk of AMD progression (Chew et al. 2022). Lutein and zeaxanthin, natural bioactive

compounds present in leafy green vegetables, have gained attention for their potential role in preventing vision loss from AMD (Chew et al. 2022; Bernstein et al. 2016; Bernstein and Arunkumar 2021). Other bioactive antioxidant compounds such as resveratrol, pterostilbene, chebulagic acid, loganin, punicalagin, and beta-carotene offer strong antioxidant properties that may be useful in mitigating oxidative stress within the eye of patients with AMD.

This study aims to investigate the protective effects lutein, beta-carotene, zeaxanthin, loganin, chebulagic acid, punicalagin, resveratrol, and pterostilbene have on ARPE-19 cells. SI will be used as an oxidant allowing for linear increase in oxidative stress. The ARPE-19 cells will be pre-treated with antioxidant compounds and subsequently exposed to SI prior to measuring markers of cell viability, intracellular ROS, antioxidant response, and apoptosis. To further investigate the role of SIRT-1 and BIRC5 pathways in AMD, CRISPR-Cas9 ARPE-19 cells will be used to allow for a further understanding of how these pathways are involved with the protective effects of antioxidant compounds exposed to oxidative stress caused by SI. The overall goal of this study is to further understand the link between antioxidants and prevention of cell damage due to oxidative stress accumulation similar to that seen in AMD.

1.2 Rationale

This study aims to investigate the role of carotenoids (lutein, zeaxanthin, and beta-carotene) as possible supplements to mitigate oxidative stress within eye disease. The AREDS study showed promising results on antioxidant vitamins and minerals in reducing the risk of AMD progression. Our reason for selecting these proven carotenoids with other classes of natural antioxidant compounds for comparison was to investigate the possibility of stilbenoids (resveratrol and pterostilbene), tannins (chebulagic acid and punicalagin), or iridoid glycoside (loganin) having a stronger protection on retinal cells from oxidative stress than carotenoids. To further investigate the antioxidant response in ARPE-19 cells, BIRC5, an inhibitor of apoptosis pathway, and SIRT-1, a cell survival and longevity pathway, were further examined through the use of CRISPR-Cas9 gene knockdown cells. The BIRC5 pathway has been identified as a regulator of autophagy, mitosis, apoptosis, migration and invasion in many cancers and diseases and may be involved in the progression of AMD (Adinew et al. 2022; T. Lin et al. 2019). SIRT-1 has been implicated in ocular diseases such as AMD by means of reducing oxidative stress and ROS within the eyes (M. Zhou, Luo, and Zhang 2018). It is our hope to further understand the involvement between the selected groups of compounds and the pathways of interest to expand the knowledge of antioxidant therapeutics in mitigation of oxidative stress in AMD.

Chapter 2: Literature Review

2.1 The Eye

The human eye is a complex organ which has many parts working together with the goal of providing vision. The front part of the eye is composed of the cornea, iris, pupil, and lens which work collectively to help focus the inbound light waves entering the eye onto the back portion of the eye, the retina. When light waves enter the eye, the cornea bends the rays, directing them to pass through the circular pupil. The iris acts as a regulator by opening and closing to allow the optimal amount of light to enter the eye. The light then passes through the lens which further bends the rays and changes their shape to focus them on the retina (Figure 2.1). The retina is a very thin layer of cells on the back side of the eye which contains millions of light sensing nerve cells called rods and cones (Figure 2.2).

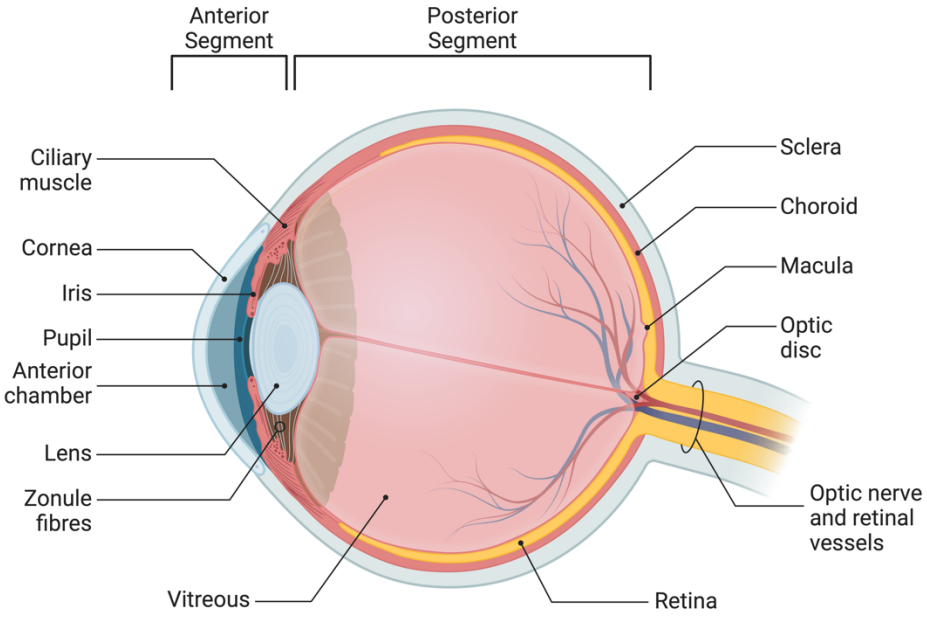

Figure 2.1 - Anatomy of the Human Eye.
The basic structure of the human eye. The anterior segment includes the cornea, anterior chamber, and iris. The posterior segment of the eye includes the lens, vitreous, retina, choroid, sclera, and optic nerve. The macula is the center of the fovea and is responsible for central vision. Adapted from "Anatomy of the eye", by BioRender.com (2024).

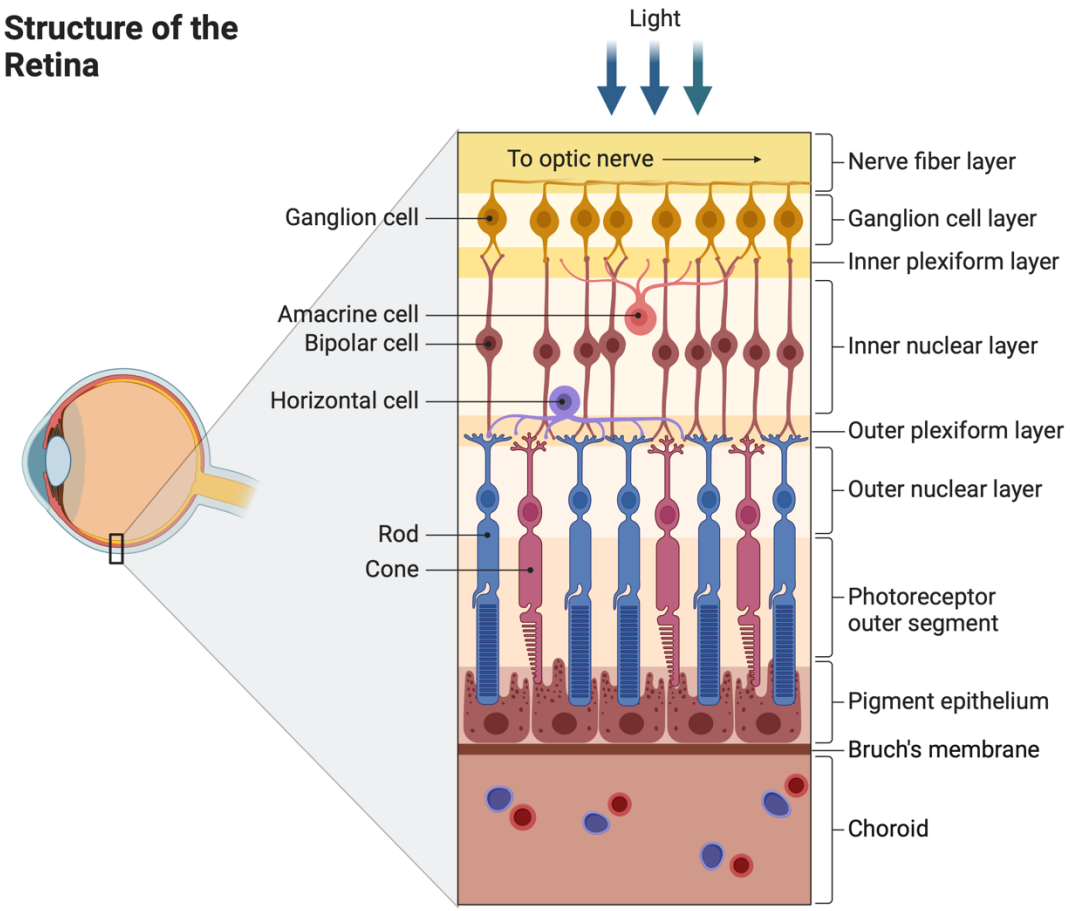

Figure 2.2 - Structure of the Human Retina.
Human retinal layers shown in relation to the macula of the eye. Adapted from "Structure of the retina", by BioRender.com (2024).

Photoreceptors are specialized cells within the layer of the retina which respond to light. There are three known photoreceptors rods, cones, and photosensitive retinal ganglion cells (pRGC). Rods are located on the outside of the macula and extend along the outer edge of the retina. These cells allow for peripheral vision as well as assist the other cells in motion detection and low light vision. Cones are concentrated at the center of the retina in the divot called the macula. Cone cells allow for vision in bright conditions as well as assist in detecting colour and fine details.

Posterior to the photoreceptors is a thin layer of cells called the RPE cells, which are part of the blood-ocular barrier. RPE cells have many functions, with the main ones being: (1) transport of nutrients, ions, vitamins, and water, (2) absorption of light and protection against harmful photooxidation, (3) conversion of trans-retinal into 11-cis-retinal, an essential component of the visual system, (4) phagocytosis of photoreceptor membrane, and (5) secretion of essential factors for structural integrity of the retina (Crabb et al. 2002; Jager 2008; Simó et al. 2010).

The choroid is made up of a layer of blood vessels that supply oxygen and nutrients to the retina. Bruch's membrane is found between the choroid and the retina, functioning as both a barrier and vital transport membrane of nutrients and metabolic waste from the RPE cells and photoreceptors (Strauss 2005). Oxygen, electrolytes, nutrients, and growth factors destined for RPE cells pass through the chorion and across Bruchs's membrane to the cells on the other side. Waste from the RPE cells and

photoreceptors travel across Bruch's membrane to be eliminated (Abdelsalam, Del Priore, and Zarbin 1999)

In mammals, the center of the visual field that contains the highest density of photoreceptors is called the macula. This high density of photoreceptors allows humans to perform everyday tasks such as make out fine details, read, and recognize faces (Jager 2008). The macula is the functional center of the eye and is the area where the light is focused by the anterior structures within the eye, as well as the location where all the cones, the receptors responsible for colour detection, reside. The high density of photoreceptors is responsible for the uptake of information communicated through light rays, conversion of this information into chemical impulses which are subsequently communicated to the brain via the optic nerve. Once the information has reached the brain, light rays are interpreted by the brain as images. The overall structure and condition of the macula is crucial for good vision; any small disturbances, such as an increase in cellular fluid, or bumps within the macula can cause a deterioration of vision clarity and quality (Jager 2008).

2.2 Age-Related Macular Degeneration

AMD is a progressive eye condition which affects the macula and results in a blurred central vision field making it hard for patients to perform everyday tasks such as reading and recognizing faces (Colijn et al. 2017; Moutray and Chakravarthy 2011). As AMD progresses the patients' vision may worsen or deteriorate at different rates depending on how their eye structure is being altered. There are presently two known forms of AMD, a slow progressing form referred to as dry or non-exudative AMD and a rapidly changing form called wet or exudative AMD (Bressler 1988; Hernández-Zimbrón et al. 2018). The first notable microscopic change that may occur in a patient who is developing AMD is the formation of small drusen deposits in between the RPE cells and Bruch's membrane. These drusen deposits are acellular polymorphous debris which accumulates and can be detected during a funduscopic eye examination as pale yellow regions in both the macula and retina (Crabb et al. 2002; Jager 2008; Abdelsalam, Del Priore, and Zarbin 1999). Drusen deposits can range from one deposit up to several hundred (Bressler 1988). As the number of drusen increase, a typical decrease in visual acuity is seen in patients (Bressler 1988). Although having some drusen deposits in the eye is not a complete indicator of AMD, many drusen deposits can be a common symptom of dry AMD and further monitoring of the patient's eyes is typically taken (Bressler 1988).

In contrast to the slower progressing non-invasive dry form, the wet or exudative AMD is a rapidly changing process. Wet AMD develops when there is the invasion of blood vessels from the choroid layer into the macula which is called choroid neovascularization (Bressler 1988; Noble and Chaudhary 2010). These abnormally grown blood vessels within the macula rupture easily, resulting in the accumulation of blood and fluid within the layers of the retina. (Bressler 1988; Noble and Chaudhary 2010). The increased fluid can cause the formation of a bump in the RPE cells, altering the shape of the macula leading to photoreceptor damage resulting in severe vision loss (Figure 2.3 and Figure 2.4**)**. Typically, an individual is diagnosed with dry AMD, which can then progress onto wet AMD. However, there are also known cases where wet AMD develops on its own.

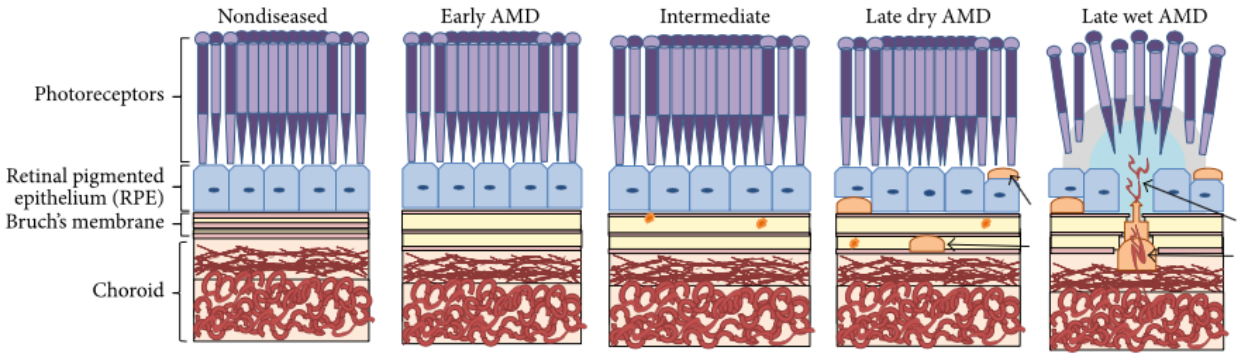

Figure 2.3 - Structural Changes During AMD Progression.
A normal retina can be seen on far left. Early AMD shows thickening of Bruch's membrane. Intermediate AMD shows small drusen deposits forming. Late Dry AMD shows small and large drusen deposits effecting Bruch's Membrane as well as the RPE cells. Late Wet AMD shows the choroidal neovascularization and subretinal fluid accumulation which leads to photoreceptor and RPE cells damage. Image adapted from Al Gwairi, O., et al. 2016.

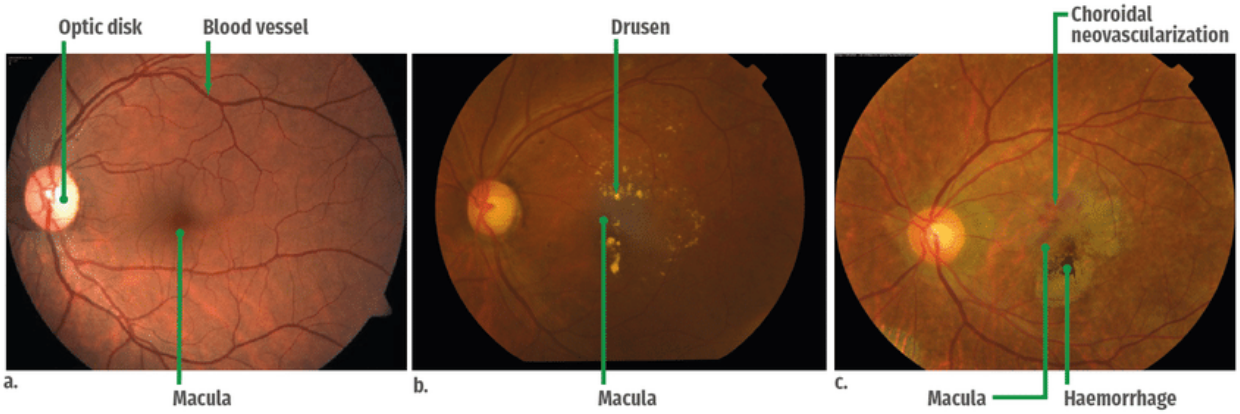

Figure 2.4 - Types of AMD
a. Normal image of eye b. Dry form of AMD showing drusen formation c. Wet form of AMD showing vascularization of the choroidal layer. Image from Acharya, U. R., et al. (2016)

The development of wet-AMD can be attributed to vascular endothelial growth factor A (VEGF-A) (Noble and Chaudhary 2010). Which is an important signalling molecule for cells that are deprived of oxygen. During this time, VEGF-A production is increased and induces vascular proliferation and migration of endothelial cells of pre-existing vessels. In the progression of wet-AMD, these blood vessels form and leak fluid into the retina which causes a change in retinal shape leading to retinal dysfunction ultimately resulting in the distortion of vision (Bressler 1988; Noble and Chaudhary 2010). VEGF-A is able to bind to either of the two VEGF receptors, VEGFR-1 and VEGFR-2, which are both closely related and share common ligands (Shibuya 2011). Receptor 1 is a kinase-impaired RTK whereas receptor 2 is a highly active kinase, both of which are required for the development of new vessels and angiogenesis.

2.3 Treatments for Dry AMD

Currently the treatments for AMD are stage dependent and are different for each individual. Typically, treatments for dry AMD are non-pharmacological – lifestyle changes such as improved nutrition and vitamin supplementation to support the health of the retina and the eye. If the disease continues to progress and develops into a more severe form of dry AMD, supplements containing increased concentrations of vitamins and minerals are often prescribed to support cell health and function (Bandello et al. 2017).

Two studies, AREDS and AREDS 2, illustrated that dietary supplements were able to slow down the progression of dry-AMD (Chew et al. 2014). AREDS was a randomized clinical trial with over 4000 patients. The participants were broken down into four groups – placebo, treatment with antioxidants, treatment with zinc oxide, or treatment with both antioxidants and zinc oxide. Patients who received the antioxidants or zinc oxide saw a 17% or 21% risk reduction as compared to the placebo group respectively. Patients who received the combination treatment of antioxidants and zinc oxide saw a risk reduction of 25% (Chew et al. 2014). AREDS2 looked at 4000 more patients and different formulations of supplements. Patients were divided into different groups and given either placebo, the original AREDS formulas, or the new modified one. Modifications included lutein and zeaxanthin, omega-3 fatty acids, or a combination of both. This study showed that lutein and zeaxanthin have a strong role in management of AMD and nutritional supplements correlated with a reduction in risk of developing AMD (Moutray and Chakravarthy 2011; Beatty et al. 2013).

2.4 Treatments for Wet AMD

Since its discovery in the early 1970's, wet AMD has yet to have a definitive cure. However, presently there are treatments which decrease the rate of disease progression with an overall goal of maintaining a patients' eyesight for as long as possible. Over the years, treatments for wet AMD have changed drastically and have improved as newer technologies became available. Originally, laser photocoagulation was thought to be a

powerful treatment for wet AMD (Burgess 1993). Laser photocoagulation used a powerful laser with hopes to prevent the growth of abnormal blood vessels in the back of the eye. The Macular Photocoagulation study looked at the efficacy of laser photocoagulation in the treatment of wet-AMD. Unfortunately, it was determined that laser photocoagulation was neither a safe nor effective way of managing wet-AMD; there were a large portion of the treatment group who experienced the unfortunate side effect of a loss of visual acuity (Burgess, 1993). Today, laser photocoagulation is rarely used as a management option due to the adverse side effect profiles (Al-Zamil and Yassin 2017).

In the 1990's, photodynamic therapy (PDT) was introduced as a possible treatment for wet-AMD and approved by the FDA (Bressler 1988). PDT technology used an intravenously dosed photosensitizer, Verteporfin, which was later activated through a red ocular laser at 689 nm. Once activated, the photosensitive dye formed reactive species which caused clots in blood vessels and prevented the progression of wet-AMD (Al-Zamil and Yassin 2017). PDT showed promising results in early testing and was adopted worldwide as a treatment for wet-AMD. However, due to some unexpected side effects as well as the cost of treatment, its use slowed down (Al-Zamil and Yassin 2017). Today, PDT is rarely used to treat wet-AMD due to the overall cost of treatments, the specialized equipment required, and the use of more favorable and effective treatments available today.

In 2004, a new era of treatments emerged for AMD, and anti-VEGF drugs grew in popularity due to the effective results and reduced cost of administration. Macugen (Pegaptanib) was the first anti-VEGF drug approved by the FDA and used for treatment of wet-AMD. Macugen was a revolutionary molecular approach used to directly combat the formation of new blood vessels within the eye. It works as a specific binding RNA aptamer to VEGF-165 isomer, preventing the VEGF from binding to a corresponding receptor. The VEGF Inhibition Study in Ocular neovascularization (VISION-1) treated patients with Macugen at 0.3, 1.0, or 3.0 mg of intravitreal injections every six weeks for 48 weeks. Results from this study showed that patients receiving the treatments showed improved visual acuity compared to the patients who received the aptamer control (Al-Zamil and Yassin 2017; Gragoudas, Cunningham, and Guyer 2004). Since the VISION-1 study, there have been newer and more effective anti-VEGF treatments that have emerged and replaced Macugen.

Avastin, an approved treatment for metastatic colorectal cancer was briefly used off label as a treatment for wet-AMD. Avastin showed stronger effectiveness and lower cost when compared to Macugen. Avastin is a mouse monoclonal antibody which was humanized through site-directed mutagenesis of a human antibody framework. Avastin binds to VEGF with the same affinity as the original human antibody. The success of Avastin lead to the development of Lucentis, which was specifically designed for treatment of wet-AMD.

Lucentis (Ranibizumab) was the second anti-VEGF drug approved by the FDA for treatment of wet AMD. Lucentis is a recombinant humanized IgG1 Monoclonal Fab fragment which has the specificity to bind to all biologically active isoforms of VEGF-A (Bakri et al. 2007). Lucentis was created using the same parent antibody as Bevacizumab (Avastin). Both Lucentis and Avastin work by blocking the binding of VEGF receptors which prevents the growth of new blood vessels in the eye.

Eylea (Aflibercept) is a VEGF-A decoy receptor which has a high binding affinity for both VEGF-A and VEGF-B isoforms as well as placental growth factors (Al-Zamil and Yassin 2017). Eylea is a recombinant fusion protein made up of the ligand-binding elements of the human VEGF receptor 1 and 2, fused to the human immunoglobulin G1 Fc fragment (Al-Zamil and Yassin 2017). A notable clinical study, VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet AMD (VIEW), compared treatments of Aflibercept to Ranibizumab (Heier et al. 2012). It showed that Aflibercept was superior to Ranibizumab (Al-Zamil and Yassin 2017; Heier et al. 2012). The increased effectiveness of Aflibercept is most likely accounted for by its higher binding affinity for VEGF when compared to Ranibizumab (Al-Zamil and Yassin 2017). Figure 2.5 summarizes the various treatment used historically to treat AMD along with its predecessor and successor.

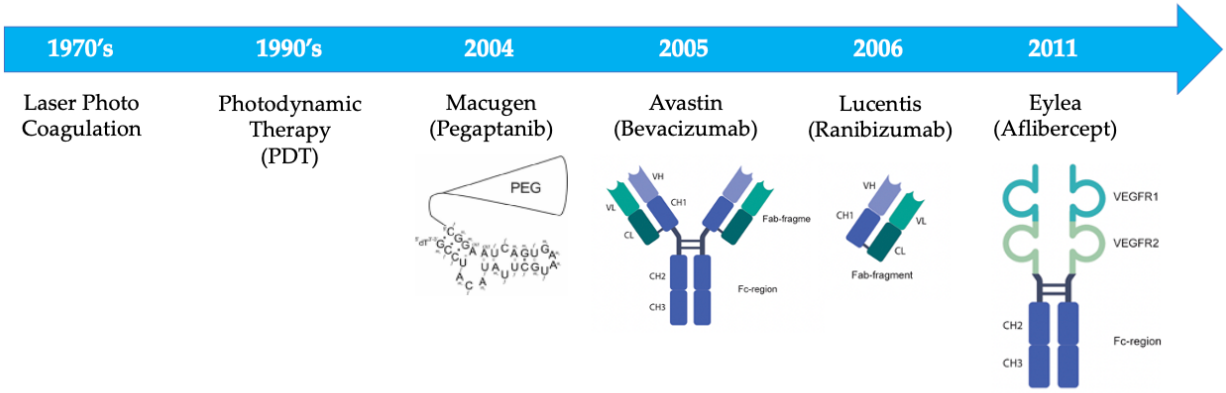

Figure 2.5 - A historical perspective on wet-AMD treatments.
Timeline of wet-AMD treatment development from early treatments to presently used treatments which are approved and commonly used in practice of treating AMD. Eylea and lucentis intravitreal injections cover over 95% of wet-AMD treatments in North America. Structures adapted from Lode et al 2019.

2.5 Risk Factors for AMD

AMD is the leading cause of blindness among people of European-descent over the age of 50 and accounts for approximately 10% of all blindness worldwide (Colijn et al. 2017) and is expected to double over the next 0 to 5 years (Colijn et al. 2017; Bressler 1988). Some major risk factors that can predispose an individual to AMD include; smoking, obesity, high dietary intake of fats, and low antioxidant or vitamin intake (Jager 2008). Dry-AMD diagnosis is typically accompanied with a medical recommendation to modify lifestyle and remove as many risk factors as possible. If the individual is a smoker, it is typically suggested that they try to quit smoking as it presents a 2.5 to 3 fold increase in development of both the dry and wet form of AMD (Colijn et al. 2017).

Smoking causes vasoconstriction to the blood vessels which reduces the supply of oxygen to the eyes (Frayser and Hickam 1964) resulting in cellular damage due to an impaired ability to transport nutrients to the cells and transport cellular waste product away. Smoking also introduces free-radicals into the body which can cause irreversible cellular damage as well as take part in depleting the available antioxidants available in the body to combat diseases such as AMD (Solberg, Rosner, and Belkin 1998). Oxidative damage to the eye and overall reduction of antioxidant enzymes in RPE cells can also lead to a depletion of macular pigment density, which serves as a crucial short-wavelength filter preventing photooxidation of the RPE cells (Hollyfield et al. 2008).

Cigarette smoking also leads to chronic ocular exposure to compounds which have been known to cause oxidative damage, vascular changes, and increases in inflammation which ultimately lead to progression in the pathogenic cascade of AMD (Solberg, Rosner, and Belkin 1998).

Genetic factors can also play a role in the development of AMD in individuals without high-risk lifestyles. Genetics can contribute to changes in the retina gene expression pattern and have been shown to be linked to oxidative stress and altered energy metabolism responses (Montezuma, Sobrin, and Seddon 2007). Certain ethnic groups may also display higher frequencies of the disease than others. For example, Caucasian populations have the highest prevalence of AMD (Kenney et al. 2013). Additionally, the presence of certain genetic impairments, such as in complement factor H function, may further accelerate the progression of AMD (Despriet et al. 2006). Although numerous research studies are ongoing to find a genetic marker which can be directly attributable to the development of AMD, it continues to be regarded as a multifactorial disease with a strong interaction between both environmental and genetic factors.

2.6 Oxidative stress

As discussed, AMD is likely a multifactorial disease which can be progressed by numerous factors one of which being oxidative stress. Oxidative stress can be defined as an excess of ROS within the cells causing damage to DNA, RNA, protein, and lipids.

ROS include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH-). Under normal conditions, ROS molecules are commonly used as a defense mechanism in the body against bacteria and other pathogens (Simon, Haj-Yehia, and Levi-Schaffer 2000). Enzymes such as Manganese-dependent Superoxide Dismutase (MnSOD) and catalase are responsible in the body for dissociation of ROS molecules of O_2^- and H_2O_2 respectively (Miriyala et al. 2012, Aebi 1974). Glutathione peroxidase 1 (GPx-1) is expressed in tissues and catalyzes the reduction of H_2O_2 to water and oxygen (Lubos, Loscalzo, and Handy 2011). Other protein markers such as caspase's are involved in the antioxidant response for the activation of apoptosis signalling under levels of chronic oxidative stress. Caspase-3 is understood as being the convergence point of mitochondrial dependant and independent pathways in cells undergoing apoptotic cell death (Balmer et al. 2015).

RPE cells are at high risk for oxidative stress build up as the microenvironment includes high oxygen consumption and metabolic demand mixed with unique chronic exposure to photooxidative stress (Jabbehdari and Handa 2021). RPE cells must maintain high metabolic activity to maintain the health of crucial photoreceptors, resulting in high proportions of mitochondria present in RPE cells. Mitochondria are known to produce approximately 90% of cellular ROS and that can increase exponentially in the presence of mitochondrial dysfunction (Jabbehdari and Handa 2021; Flohé 2016). High levels of ROS are also produced within the typical cell cycle

during phagocytosis of photoreceptors and can elevate ROS levels in the eye (Jabbehdari and Handa 2021).

2.7 Cellular Pathways

The development and progression of AMD involves a complex combination of various signaling pathways and cellular targets. Inflammation has been identified as a significant contributor to AMD pathophysiology (Ozaki et al. 2014). Additionally, genetic variants, modifiable factors such as smoking, dietary habits, and obesity, as well as environmental variables, have been associated with the progression from early to advanced stages of AMD (Montezuma, Sobrin, and Seddon 2007; Seddon et al. 2011). The role of genetics in AMD progression has been extensively studied, with polymorphisms in genes such as complement factor H (CFH) and age-related maculopathy susceptibility 2 (ARMS2) having a significant impact on the progression to advanced AMD (Vavvas et al. 2018). There are likely many more genetic factors that are currently unknown to be present in the development and progression of AMD; furthermore, the current understanding of the disease and pathways involved has not fully been uncovered. Vascular changes, including alterations in retinal and choroidal vasculatures, have been implicated in the progression of AMD (Koh et al. 2017; Lee et al. 2018; Trinh, Kalloniatis, and Nivison-Smith 2019).). Additionally, the impairment of RPE cell function and alterations in the angiogenic balance have been identified as crucial events in the molecular pathways leading to clinically relevant AMD changes

(Farnoodian et al. 2017). Overall, the development and progression of AMD involve a complex interplay of genetic, immunological, inflammatory, and environmental factors, as well as vascular and cellular pathways. Understanding these pathways and risk factors is crucial for the development of targeted interventions and personalized approaches to manage and potentially prevent the progression of AMD.

2.7.1 SIRT-1

SIRT-1 is a protein that has been the center of recent research due to its involvement in numerous biological pathways with a focus on longevity and cell survival. SIRT-1 has been found to be involved in diabetic nephropathy and regulation of lipid and glucose metabolism (Hase et al. 2021; W. Wang et al. 2019). Investigation of SIRT-1's involvement in skeletal muscle insulin sensitivity suggests a potential pathway in addressing metabolic disorders such as type 2 diabetes (Schenk et al. 2011). The role of SIRT-1 in improving function and health span has also been investigated, emphasizing SIRT-1's involvement in longevity pathways (Haigis and Sinclair 2010). The downstream effects of SIRT-1 include ROS detoxification and mitochondrial biogenesis and remodeling (Haigis and Sinclair 2010). SIRT-1's involvement in numerous metabolic pathways with critical functions make it a promising target for therapeutic intervention.

SIRT-1 has been implicated in ocular diseases such as AMD by means of reducing oxidative stress within the eyes (M. Zhou, Luo, and Zhang 2018). Furthermore, SIRT-1 is also known to be involved in the regulation of inflammation as it relates to neurodegenerative diseases (Sharma et al. 2012). With this understanding of SIRT-1's involvement in ocular disease, it is an attractive potential target in prevention of progression of AMD (Tan et al. 2020). Activation of the SIRT-1 pathway is illustrated in Figure 2.6.

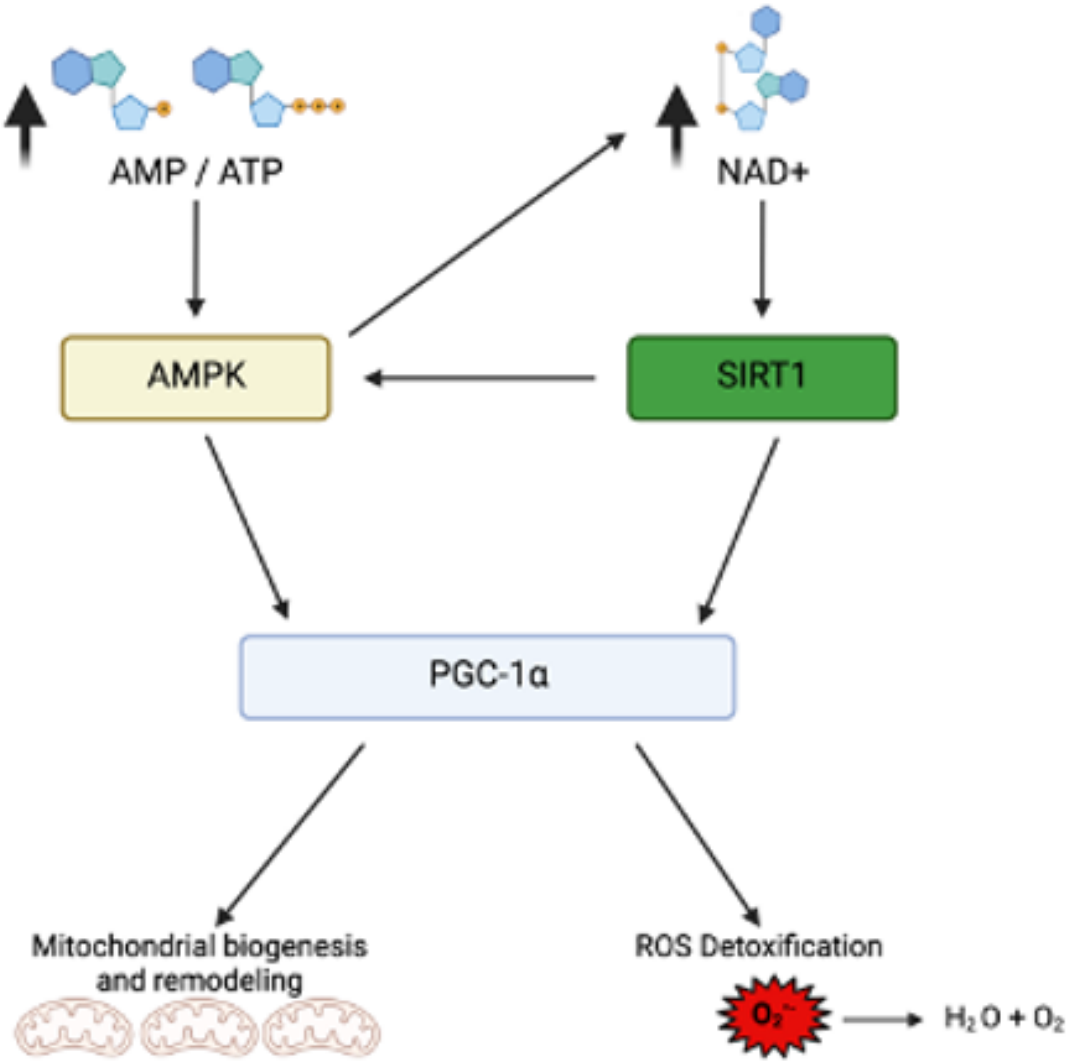

Figure 2.6 - SIRT-1 Activation.
Activation pathway of the SIRT-1 gene in mammalian cells. Key downstream roles involve mitochondrial biogenesis and remodeling as well as ROS detoxification. SIRT1 can deacetylate protein substrates in various signal transduction pathways to regulate gene expression, cell apoptosis and senescence, participate in the process of neuroprotection, energy metabolism, inflammation and the oxidative stress response in living organisms through the AMPK and PGC1alpha pathways. Figure created with BioRender.com (2024).

2.7.2 BIRC5

BIRC5 has been identified as a major target of cancer signaling pathways (Cao et al. 2019). It is primarily involved in cell proliferation and immune response related signaling pathways (Jiang et al. 2021; J. Wang et al. 2021). BIRC5 has been identified as a regulator of autophagy, mitosis, apoptosis, migration and invasion in many cancers (Adinew et al. 2022; T. Lin et al. 2019). BIRC5 has been noted as a possible novel cancer treatment target in ovarian cancer models (J. Xu et al. 2022). Although the BIRC5 pathway is not fully understood, its involvement in cell proliferation and immune response pathways may suggest its potential role in ocular diseases like AMD. Further investigation is warranted to determine a linkage of BIRC5 and the eye. The BIRC5 pathway is shown in Figure 2.7- Key molecules involved in the BIRC5 pathway are highlighted with focus being on the relationship between BIRC5 and inhibition of apoptosis.

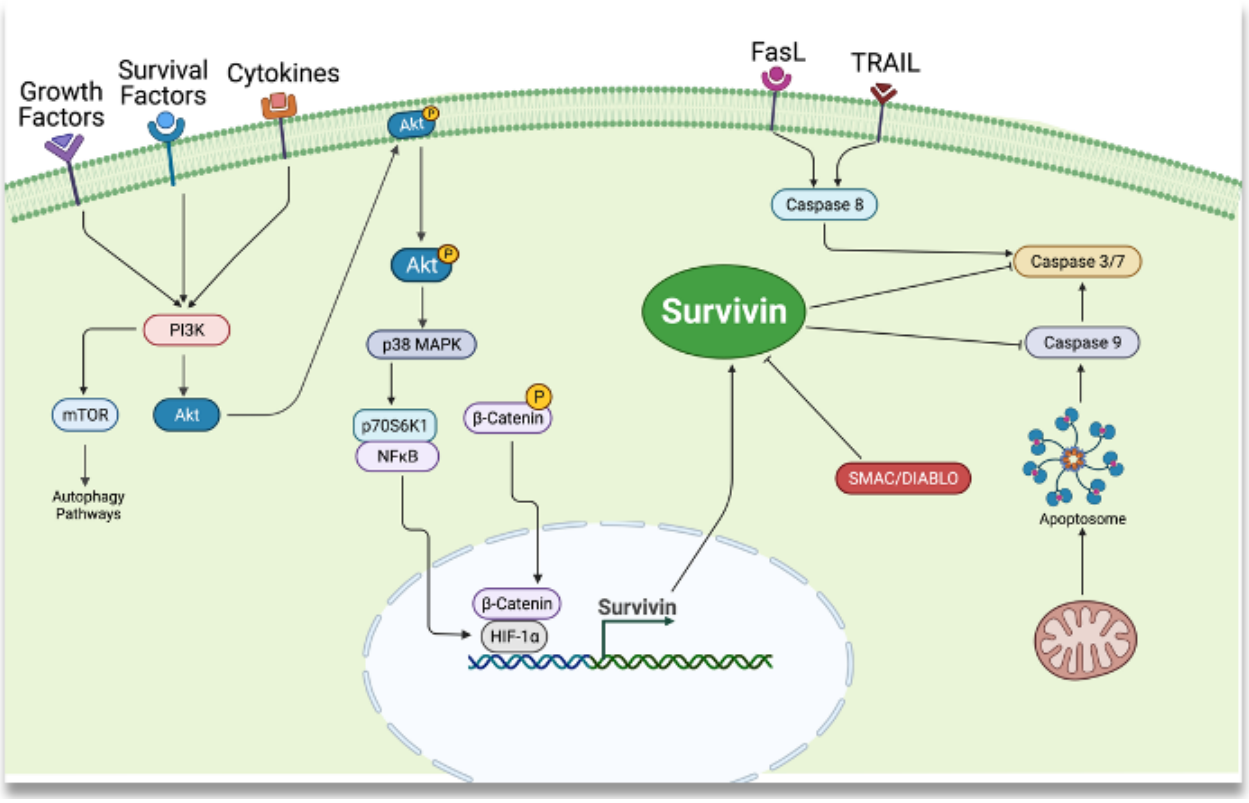

Figure 2.7 - Activation Pathway of BIRC5
Upstream and downstream signaling pathway of the BIRC5 (Survivin) gene in mammalian cells. Transcription of Survivin is modulated through the PI3K / AKT pathway as well as NfkB and beta-catenin dephosphorylation. Survivin interferes with the extrinsic apoptotic pathway through the indirect inhibition of caspase-8. Survivin also inhibits both the initiator caspase-9 and the effectors caspase-3 and caspase-7. Key roles involve caspase inhibition and autophagy pathways. Survivin has been shown to be inhibited through the SMAC/DIABLO pathways. Figure created with BioRender.com (2024).

2.8 ARPE-19 Cells

The ARPE-19 cell line is commonly used in modeling AMD due to its ability to mimic the behavior of retinal pigment epithelium cells, which are involved in the pathology of AMD (Kozlowski 2014). Studies have shown that ARPE-19 cells can be genetically modified to replicate specific mutations associated with AMD, such as the p.R345W mutation in the EFEMP1 gene, allowing researchers to study the mechanisms responsible for RPE pathology in inherited and age-related macular degenerations (Fernández-Godino, Bujakowska, and Pierce 2017; Fernández-Godino, Garland, and Pierce 2015). As such, ARPE-19 cells have become the standard cell line for investigating mechanisms as well as treatments relating to AMD – various compounds have been used to experiment on this line of cells to test its effect on cell survival, apoptosis, and inflammation (Xiao and Liu 2019; Yuanyuan Zhang et al. 2017). For example, ARPE-19 cells have been employed to examine the protective effects of mitochondrial-derived peptides in AMD and to study the effects of various naturally derived compounds on oxidative stress and mitochondrial dysfunction in RPE cells, which are relevant to AMD pathogenesis (C.-H. Chang et al. 2016; Z. Liu et al. 2007; Nashine et al. 2018). Overall, the use of ARPE-19 cells in modeling AMD has provided valuable insights into the pathophysiology of the disease and has facilitated the screening of potential therapeutic agents.

2.9 CRISPR Knock-Out Cells

CRISPR-Cas 9 is a method of genomic editing that can be pre-programmed to target a specific gene and make changes to the genomic sequence. CRISPR-Cas9 knockout cells are generated using the bacterial CRISPR/Cas9 system, which has revolutionized research involving mammalian genetics by enabling the rapid generation of isogenic cell lines and animal models with modified alleles (Wang et al. 2014). The process involves the use of the CRISPR-Cas9 system with a single guide-RNA (sgRNA) and protospacer adjacent motif (PAM) sequence to introduce double-strand breaks at specific genomic loci, followed by the repair of these breaks through non-homologous end joining or homology-directed repair mechanisms, resulting in the generation of knockout cells with targeted gene disruption (Alkanli et al. 2022; Karimian et al. 2019; C. L. Xu et al. 2019). The process in which CRISPR-Cas9 functions can be seen in Figure 2.8 CRISPR-Cas9 Overview. The technology of CRISPR-Cas9 based *in vitro* experiments allows for effective gene editing in different cell types and organisms, providing a powerful tool for investigating gene function and disease pathogenesis (Karimian et al. 2019).

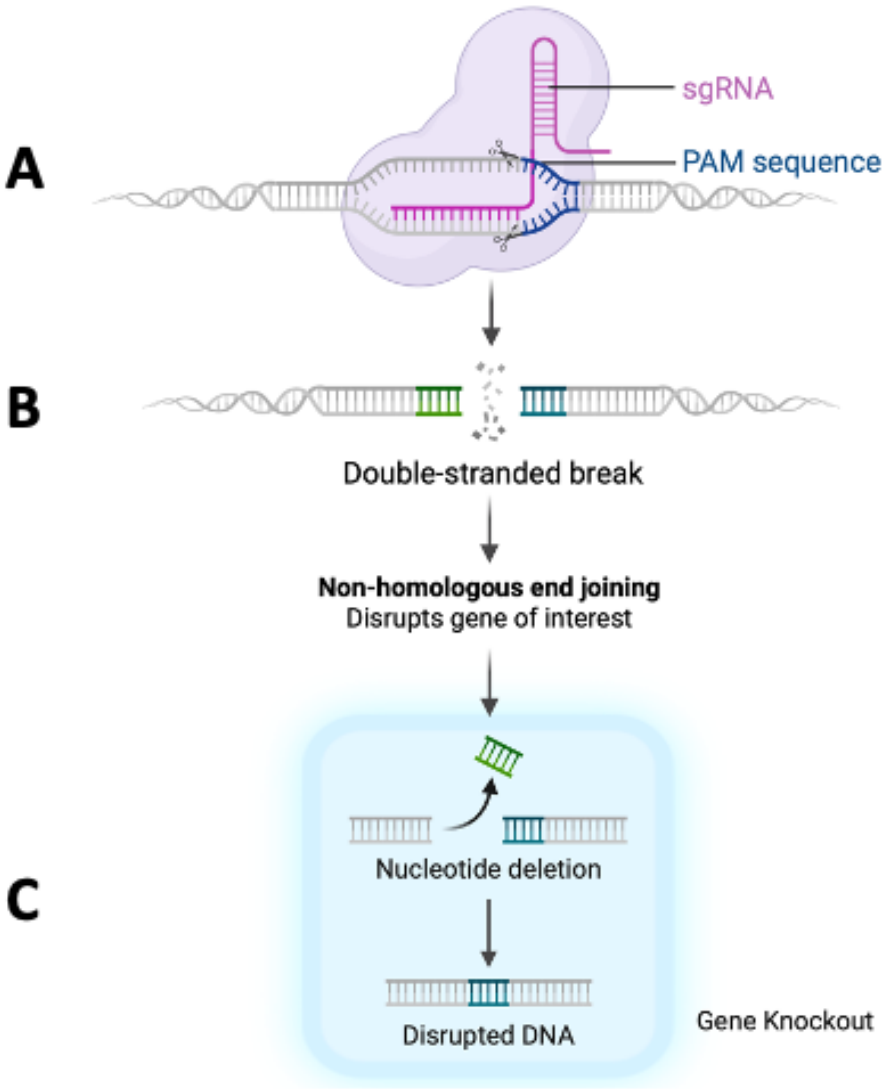

Figure 2.8 - CRISPR Cas-9 Overview.
A. CRISPR Cas-9 single guide RNA (sgRNA) and Protospacer-adjacent motif (PAM) sequence identifying the correct section of the DNA in which modification will be performed. B. A double stranded break is introduced by the cas protein causing repair pathways to take place. C. The result of disruption in the sequence creates a gene knockout. Figure created with BioRender.com (2024).

CRISPR-Cas9 knockout cells have significantly advanced the field of cell culture models for disease research. The CRISPR-Cas9 system has been widely used to efficiently and accurately generate knockout cells in various cell types, including cancer cell lines, human embryonic stem cells, induced pluripotent stem cells, and normal immune cells, allowing for the construction of disease models and the identification of potential therapeutic targets for these diseases (Chandrasekaran et al. 2022; Hart et al. 2015; Inoue et al. 2023; H. Wang et al. 2017; Yichen Wang et al. 2014; Yongping Zhang et al. 2017). CRISPR-Cas9 has demonstrated higher efficiency and versatility compared to previously used genome-editing techniques, such as zinc-finger nucleases and TALENs (Yichen Wang et al. 2014). In cancer research, CRISPR/Cas9 technology holds immense potential in cancer diagnosis and treatment and has been utilized to develop cancer disease models, such as medulloblastoma and mice models of glioblastoma (Akram et al. 2022).

The use of CRISPR-Cas9 knockout cells show promise in the study of ocular diseases, specifically in the study of AMD using ARPE-19 cells (Nguyen et al. 2023). Currently, Nguyen et al. (2023)'s group is the only one who has published in the field of AMD using ARPE-19 CRISPR-Cas9 knockout cells. The results of their work utilizing knockout models for AMD-associated genes such as POLDIP2 in human retinal pigment epithelial cells is encouraging (Nguyen et al. ,2023). As CRISPR-Cas9 becomes more accessible through ease of use and reduced costs, it will become even more

valuable as an *in vitro* model for studying the function of AMD-associated genes and their role in disease pathogenesis. Further studies of AMD and associated genes could unlock future therapeutic targets of the disease and provide a new approach to both screening for AMD as well as providing early treatment of the diagnosis to reduce the rate of vision loss in patients as they age.

2.10 Blue Light

Exposure to blue light can lead to the accumulation of oxidants and ultimately result in cell death and vision loss (Algvere, Marshall, and Seregard 2006; Taylor et al. 1990). Taylor, H.R., et al. (1990) showed that blue light can cause cellular damage within the eye similar to that seen in patients with AMD (Taylor et al. 1990). Blue light has become a more relevant and prevalent risk factor over the last several decades, with the increased use of cellphones, computers, and tablets. Blue light is defined as short high energy wavelengths of visual light between the wavelengths of 450 and 495 nm. This short wavelength has the ability to penetrate the back of the eye resulting in photooxidative damage to the layer of RPE cells on the back of the eye (Taylor et al. 1990). The retina is at high risk for oxidative stress and build-up of ROS due to the high proportion of polyunsaturated fatty acids, high oxygen consumption, and chronic exposure to visible and ultraviolet light spectrums.

In an animal study by Wielgus, A. R., et al. (2010) rats were exposed to 450 nm blue light for 6 hours. In the blue light-exposed rats, it was found that there was a much

higher concentration of N-retinyl-N-retinylidene ethanolamine (A2E) oxidization within the cells. This oxidized form of A2E is a metabolic waste product which is toxic to the retinas and was found in high concentrations in the rats that were exposed to blue light. This study suggests that there is a strong link between exposure to blue light and retinal injury (Wielgus et al. 2010).

Earlier work in our lab involved the development and testing of a blue light model for inducing oxidative damage in cell culture models with ARPE-19 cells. Through this testing of early models, a blue light panel was designed and assembled to illuminate cells from above and cause cellular damages (Figure 2.9 – Blue Light Apparatus).

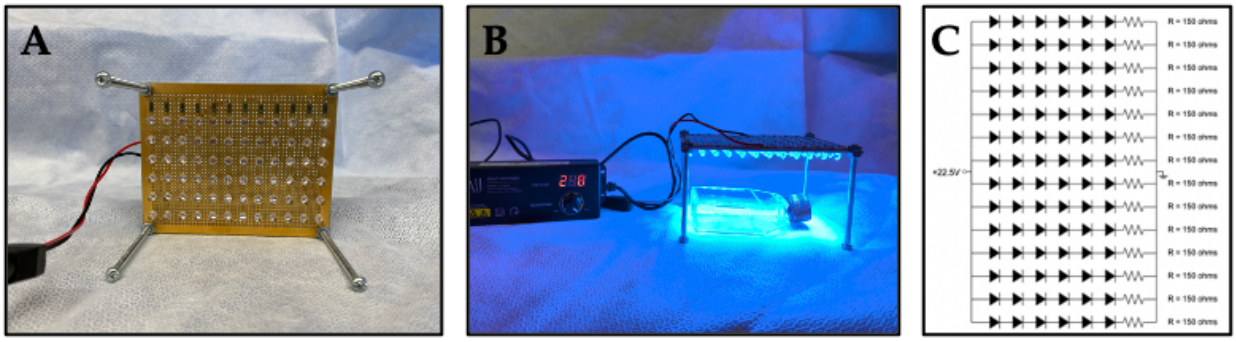

Figure 2.9 - Blue Light Apparatus.
A: 72 LED's connected on a computer board with power leads to supply 22.5 volts of power. B: LED panel supported on top of a tissue culture flask to show how the open sides allow for constant airflow around the tissue culture vessel in the tissue culture incubator. C: Wiring schematic that was designed to allow for 12 rows of 6 LED's to ensure full coverage of the tissue culture vessel below.

2.11 Sodium Iodate

SI – $NaIO_3$ – is an oxidizing agent used to create both *in vitro* and *in vivo* models of AMD (Soundara Pandi et al. 2021; Kannan and Hinton 2014). Use of SI as a method of retinal degeneration date back to 1941 where Sorsby describes the effect of injecting SI solution into rabbit retina (Sorsby 1941). SI has been used in both animal models (Kannan and Hinton, 2014) as well as in cell culture models. The most typical *in vivo* work however, was done in a murine model and *in vitro* in ARPE-19 cells (Kannan and Hinton 2014; Hanus et al. 2016).

SI has been shown to be a reliable model in studies of RPE damage and cellular protection within the eye (Kannan and Hinton 2014; Hanus et al. 2016) In both the study of dry and wet AMD. Accurate simulation of AMD in both animal and cell models is difficult due to the multiple factors that are involved in the complex pathophysiology of AMD. However, SI induced cell damage mimics the cellular changes and resultant physiology of AMD, as research has shown it to induce oxidative stress and apoptosis in RPE cells (Zhang et al., 2016). In both animal models and cell culture studies, it is hard to accurately simulate what occurs in the eye as AMD progresses due to the disease's multifactorial progression, therefore SI is considered an acceptable model to study cellular changes associated with AMD. SI have been utilized to study the protective effects of compounds against oxidative stress-induced senescence and photoreceptor cell loss in AMD models (S.-J. Chen et al. 2021; Du et al.

2018). In our studies, SI is used as a reproducible cellular stressor in which the degree of rescue or cellular protection by natural antioxidant compounds can be investigated through the use of various assays and imaging methodologies.

The oxidative pathway of SI induced RPE cell damage has yet to be fully understood. It is thought to have involvement in apoptosis, necroptosis, and ferroptosis cell death pathways with varying degrees of involvement (Balmer et al. 2015; Hanus et al. 2016; Kannan and Hinton 2014)

2.12 Antioxidants

Antioxidants are substances which can slow down the oxidative damages caused by free radicals (Halliwell, 1996). They can be found innately in the human body, are sold as supplements, but are also found abundantly in plants and the fruit of plants. These plant compounds can be broken down into groups of flavonoids, tannins, phenols, and lignans. Antioxidants can act in many different ways within the body. They can function to attenuate inflammation and inhibit cell proliferation. The chemical structure of each compound plays a large role in the metabolism and uptake for each antioxidant (Rice-Evans 2001).

Antioxidants and their potential role in disease treatment have been a subject of extensive research. The use of antioxidant supplements in reducing the morbidity and mortality of various diseases has been a topic of interest in the medical field. For example, several studies have investigated the effects of antioxidant supplementation in

cardiovascular conditions, cancer, neurodegenerative diseases, and AMD (Banerjee, Chawla, and Kumar 2021; Barbato et al. 2013; Bjelakovic et al. 2015; Bjelaković et al. 2007; Myung et al. 2013).

The role of oxidative stress in the progression of various diseases has been a major focus of recent research leading to investigations into the potential benefits of antioxidant-rich diets and supplements in mitigating adverse effects and slowing disease progression. In eye disease, antioxidant rich compounds have been tested in combination with other vitamins and minerals to show a reduction in risk of developing an advanced form of AMD ("The Age-Related Eye Disease Study" 2001)

While some studies have suggested potential benefits of antioxidant supplementation in mitigating the progression of certain diseases, the overall evidence regarding the efficacy of antioxidant supplements in disease management still needs to be further investigated. Further research is needed to elucidate the specific conditions in which antioxidant supplementation may be beneficial and to address the potential interactions and limitations. The key to such research would be a deeper and more complete understanding of the molecular pathways of the pathophysiology of AMD and other similar illnesses. This would allow for more targeted use of antioxidants.

In this study, the antioxidant compounds investigated are resveratrol, pterostilbene, lutein, zeaxanthin, beta-carotene, chebulagic acid, punicalagin, and

loganin. The chemical structures and molecular weights of the compounds is shown in Figure 2.10 - Antioxidant Compounds of Interest.

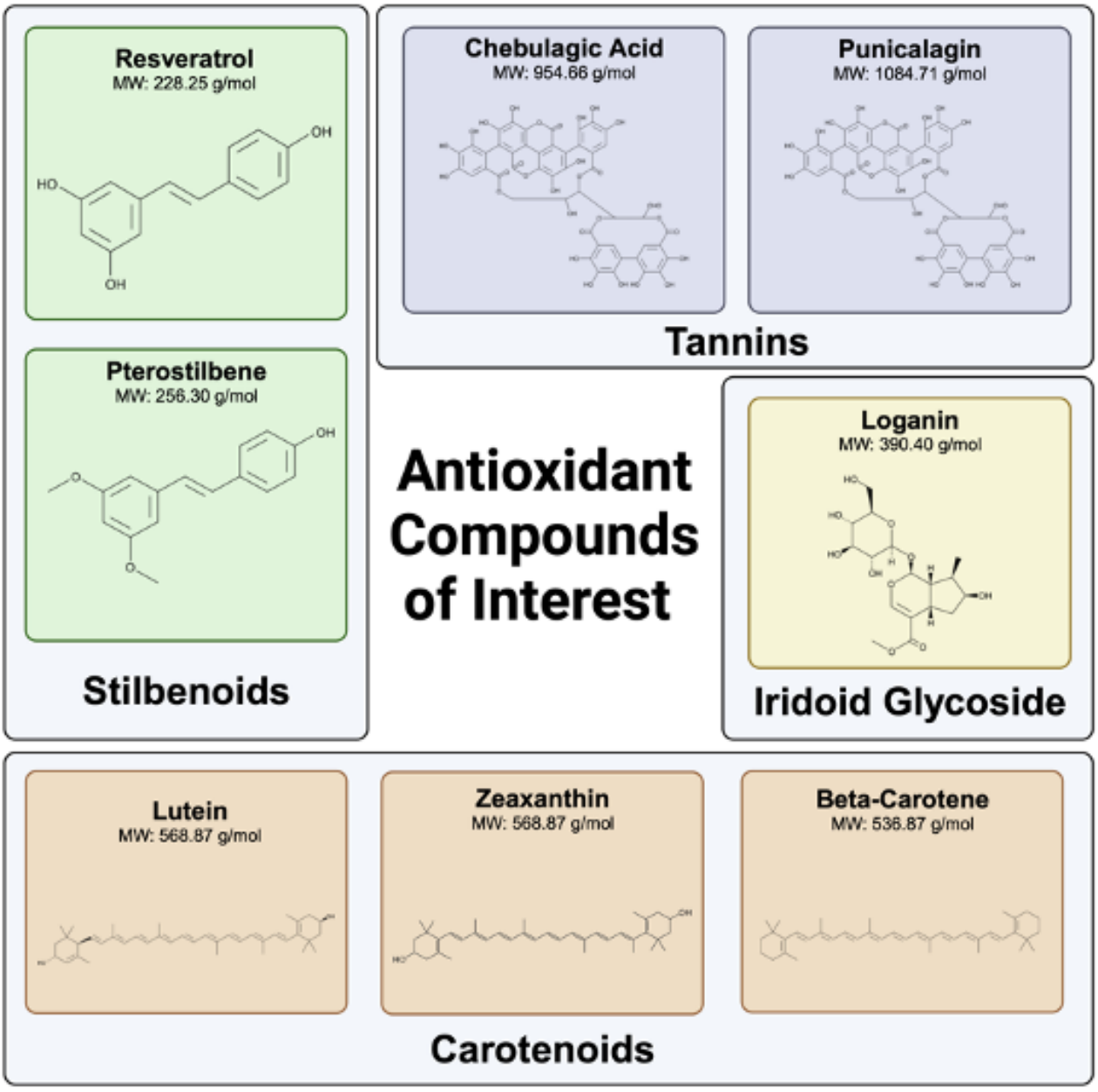

Figure 2.10 - Antioxidant Compounds of Interest
Antioxidants investigated in this study derive from 4 distinct classifications: Stilbenoids, Tannins, Carotenoids, and Iridoid Glycoside's. Molecular weight and chemical structure of each compound are summarized into their respective classification.

2.12.1 Resveratrol

Resveratrol (trans-3, 5, 4'trihydroxy- stilbene) is a polyphenol produced by a variety of plants in response to injury, stress, UV irradiation, or fungal attack (Frémont 2000; Burns et al. 2002; Shishodia and Aggarwal 2005). It was first identified in 1940 from the roots of the White Hellebore (Takaoka 1940; Silva et al. 2019) and shortly after was found in high concentration in Japanese knotweed(Silva et al. 2019). In traditional Chinese and Japanese medicine, resveratrol has been noted to treat conditions of the skin like inflammation and fungal infection (Shishodia and Aggarwal 2005). Resveratrol has a simple structure with two phenolic rings allowing it to exist in both a trans- and cis-isomer (Frémont 2000). The trans-isomer of resveratrol is typically studied due to its prevalence in nature and increased bioavailability (Burns et al. 2002; Frémont 2000). Resveratrol is found in the skins of some fruits such as grapes, blueberries, mulberries, and cranberries, as well as certain teas (Frémont 2000; Burns et al. 2002). It has been noted to be in highest concentration in the skins of grapes which are thought to be the highest source of resveratrol in the human diet (Silva et al. 2019).

Wine also can have high levels of resveratrol. Red wines are typically higher in resveratrol concentration than white wines; however, the overall concentration is dependent on many factors including type of grape, geographic origin, type of wine, growth practices and overall fermentation time (Frémont 2000).

Resveratrol has a variety of beneficial properties including antioxidative, anticarcinogenic, cardioprotective, anti-inflammatory, and anti-aging (Burns et al. 2002). Resveratrol has been studied for its antioxidant ability and has been noted to have a strong role in scavenging free radicals and mitigating oxidative stress (Frémont 2000; Bhat, KosmederII, and Pezzuto 2001). Tamaki, N., et al (2014) hypothesizes that resveratrol's mechanism of action to reduce oxidative stress functions through the SIRT1, AMP-activated protein kinase (AMPK) and nuclear factor E2-related (NRF2) defence pathways. Upon activation of these pathways, increased levels of resistance to oxidative stress and protection against inflammation take place (Tamaki et al. 2014).

Resveratrol has been investigated as a possible treatment for many different conditions within the body where oxidative stress plays a role. In cardiovascular diseases, resveratrol has been shown to reduce oxidative stress and potentially influence factors like inflammation and endothelial function (Samarjit Das and Dipak K. Das 2007). Resveratrol has shown promise in protecting the cells of the heart, brain, and kidneys. (Kalantari and Das 2010) and in slowing down the progression of aging by activating SIRT1 and PGC-1alpha pathways, and improving mitochondrial function (Kalantari and Das 2010).

Using both *in vitro* and *in vivo* models, resveratrol has shown promising results in mitigating the damaging effects of oxidative stress in eye disease. In ARPE-19 cell culture models, resveratrol has been shown to mitigate the damage induced by H_2O_2

and reduce oxidative stress levels within the cells (King, Bomser, and Min 2006). Results from *in vitro* studies indicate the possibility that resveratrol could be used as a treatment in diseases reliant upon oxidative stress, like AMD. Resveratrol has also been shown to have a regulatory effect on angiogenesis in many diseases. VEGF, a common growth factor in the wet form of AMD and also in tumor growth has been shown to be down regulated by treatments of resveratrol, reducing the overall number of blood vessels formed, and overall size of the tumor (Wu et al. 2018). Although the mechanism for inhibition of angiogenesis is not fully understood, it is thought resveratrol would be a possible means to treat diseases directly involved with VEGF, such as wet AMD with angiogenesis.

2.12.2 Pterostilbene

Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene) is a naturally occurring di-methylated derivative of resveratrol primarily found in blueberries (Estrela et al. 2013; McCormack and McFadden 2013). Pterostilbene has been studied and shows promise as a strong antioxidant (McCormack and McFadden 2013; Estrela et al. 2013), protective against cardiovascular disease (McCormack and McFadden 2013), and effective against cancer processes (Estrela et al. 2013), despite uncertainties regarding its mechanism of action. *In vivo* models using pterostilbene suggest a 60% greater bioavailability when compared to its analog resveratrol (McCormack and McFadden 2013). Pterostilbene's' higher bioavailability may come from the addition of two methyl

groups which would improve its lipophilic and oral absorption when compared to resveratrol (McCormack and McFadden 2013).

Pterostilbene has been shown to minimize the oxidative stress levels in a mouse neuronal cell model, indicating a very strong antioxidant capacity (B. Wang et al. 2016). Along with minimizing oxidative stress, pterostilbene showed significant results in reducing neuronal cellular apoptosis against glutamate-induced oxidative stress (B. Wang et al. 2016). Results from this study suggest that in a model of AMD, pterostilbene could also show promising protective effects in mitigating the oxidative stress induced damage of retinal cells *in vitro.*

2.12.3 Lutein

Lutein is a naturally occurring carotenoid which belongs to the xanthophylls group and is found primarily in various fruits, vegetables, and egg yolks (Marse-Perlman et al. 2001; Gong et al. 2017). Lutein can be found in highest concentrations in leafy green vegetables such as kale and spinach (S.-Y. Li et al. 2012). Lutein is an antioxidant which is not synthesized naturally in the human body, and only available through dietary means. Lutein is selectively taken up into eye tissues, with particularly high concentrations in the macula and lens (Johnson 2014; Norkus et al. 2010). Due to its unique properties, lutein has received increased attention with respect to its potential in promoting eye health (Shen and Lo 2018; Kelly et al. 2014).

Several studies have explored the antioxidant potential of lutein, especially in the context of eye diseases, particularly AMD and cataracts. Lutein's predisposition towards accumulating in the retina supports the possibility that it might be more effective in comparison to other compounds as an antioxidant in protecting retinal cells. A study by Marse-Perlman, A., et al. (2001) found an inverse association between the incidence of AMD and the level of serum lutein levels. Two notable clinical studies, AREDS and AREDS2, had lutein as a component of their nutritional supplementation intervention, which showed promising results in slowing down the progression of AMD (Chew et al. 2014; "The Age-Related Eye Disease Study" 2001).

The protective role of lutein in eye diseases is attributed to its multifaceted mechanisms, including the following: filtering harmful blue light, acting as an antioxidant, and stabilizing membrane integrity (Maci 2010). Lutein's antioxidant properties have been shown to attenuate apoptosis and autophagy induced by hypoxia in retinal cells, further underlining its potential in preserving retinal function and health (Fung, Law, and Lo 2016). Lutein's presence in the macula, in close proximity to lipid targets susceptible to oxidation, makes it well-suited to act as a biological antioxidant potentially reducing oxidative damage to the retina (Roberts, Green, and Lewis 2009; Miricescu et al. 2019). The protective effects of lutein within the eye extends beyond AMD, encompassing other inflammatory eye diseases such as uveitis, retinitis pigmentosa, and cataracts, highlighting its broad potential in safeguarding ocular

health (Sarialtin and Çoban 2018; Dinu et al. 2020). In addition to its role in eye health, lutein has been proposed to have broader health benefits, including potential preventive effects against cancer and cardiovascular diseases, which may also be linked to its antioxidant properties (Johnson 2000).

The specific interactions of lutein with proteins in the macula have been identified, shedding light on its unique role in human macular function and disease (B. Li et al. 2011). Furthermore, the potential of lutein as a health supplement for the prevention of cancer and retinal degenerative diseases has been recognized, further emphasizing its broader health implications (Álvarez et al. 2015). Incorporating lutein rich foods into the diet or considering supplementation of lutein may be a possible strategy in supporting eye health especially in those at high risk for development of AMD and other diseases where oxidative stress plays a key role.

2.12.4 Zeaxanthin

Zeaxanthin is a naturally occurring carotenoid which belongs to the xanthophylls group and is found primarily in various fruits, vegetables, and egg yolks (Marse-Perlman et al. 2001; Gong et al. 2017). Zeaxanthin is an analog of lutein and also has been shown to have strong antioxidative effects. Clinical trials have suggested that zeaxanthin, along with lutein, may play a significant role in the prevention and treatment of eye diseases such as AMD, cataract, and retinitis pigmentosa (L. Ma and Lin 2010). *In vitro* and *in vivo* studies have likewise demonstrated the potential of

zeaxanthin in protecting against chronic eye diseases, including AMD and cataracts (Murillo, Hu, and Fernandez 2019). Zeaxanthin has been found to bind to specific proteins in the macula of the human eye, providing protection against lipid membrane oxidation (Bernstein et al. 2016). Additionally, there is evidence that zeaxanthin may have some protective effect against inflammatory diseases of the eye, including AMD, uveitis, and retinal ischemia (Sarialtin and Çoban 2018).

The protective role of zeaxanthin in eye health is supported by its ability to absorb damaging blue light as it enters the eye, like lutein, to reduce the risk of eye disease (Johnson 2014). Zeaxanthin has also been shown to bind to antioxidant enzymes, which then stabilizes the proteins under chronic oxidative stress. This prevents the degradation of proteins under oxidative stress, a phenomenon seen recurrently in the retina, and an important step in the development of AMD (Tang et al. 2011). Dietary supplementation of zeaxanthin has been shown to provide a protective effect on the RPE cells, reducing the risk of progression to advanced stages of AMD, as shown in the AREDS2 study (Chew et al. 2014). In models of uveal melanoma, zeaxanthin has been shown to induce apoptosis of harmful cancer cells suggesting further use of zeaxanthin in treatment of ocular diseases (Bi et al. 2013).

Zeaxanthin and lutein act as vital compounds in maintaining the health of the macula through their strong antioxidant properties and potential protective effects against many different types of eye disease. Evidence from clinical trials and

experimental research support the promising effects of lutein and zeaxanthin as possible therapies for eye disease but also many different diseases of the body where oxidative stress plays a pivotal role.

2.12.5 Chebulagic Acid

Chebulagic acid is a tannin found in the chebulic myroblan, a fruit of *Terminalia chebula*, a tree native to parts of South Asia (H. Gao et al. 2008). Chebulagic acid is an active inhibitor of xanthine oxidase, and also has antibacterial and antifungal effects (Kongstad et al. 2014). Additionally, chebulagic acid shows strong potential as an antioxidant and also inhibits the VEGFR2 signaling pathways by way of its strong anti-angiogenic property (L. Lin et al. 2011; Athira et al. 2017). All these findings make chebulagic acid a promising avenue of therapy for many illnesses, including ones of an ocular nature.

Chebulagic acid has been reported to enhance insulin-mediated glucose uptake, indicating its potential in addressing metabolic aspects of eye diseases such as diabetic retinopathy (Shyni et al. 2014). Chebulagic acid has been found to exhibit neuroprotective effects, which could be beneficial in the context of neurodegenerative eye diseases such as AMD (Kim et al. 2014). Chebulagic acid has been shown to modulate NF-κB and MAPK signaling pathways, contributing to its anti-inflammatory action (Ekambaram et al. 2022). Given the role of inflammation and oxidative stress in

eye diseases, the anti-inflammatory and antioxidative potential of chebulagic acid may offer an additional management option.

2.12.6 Punicalagin

Punicalagin is a tannin commonly found in the seeds of pomegranates which has shown significant promise due to its antioxidant potential, and role in mitigating various diseases. Punicalagin has been noted to have antioxidant, anti-inflammatory, and anti-tumorigenic effects (Zhong, Reece, and Yang 2015). Punicalagin has been shown to protect against oxidative stress induced damage in various tissues including the liver, testes, and neural tubes (Rao et al. 2016; Yahui Zhang et al. 2022). Punicalagin has been found to promote cell autophagy, can protect cells from stress-induced cellular apoptosis, indicating its strong potential in cellular protection (Ying Wang et al. 2016).

Punicalagin has not been investigated in studies pertaining to eye health. However, its strong antioxidant potential offers promise to the future possibility of investigating this compound as a possible treatment for eye disease where oxidative stress is heavily involved.

2.12.7 Loganin

Loganin is an iridoid glycoside, originally found in *Cornus officinalis* (Japanese cornelian cherry tree) which has been the subject of extensive research due to its potential antioxidant properties and role in mitigating oxidative stress in various diseases (Park et al. 2021). Loganin has been shown to activate the Nrf2/HO-1 signaling

pathway, leading to the inhibition of inflammation and oxidative responses in macrophages (Park et al. 2021). Loganin has also been reported to exhibit potential anti-inflammatory effect; however the detailed mechanism is not yet understood (S. Liu et al. 2020). Loganin has shown promise in multiple biological activities including immunomodulation, antioxidation, and anti-inflammation (X. Chen et al. 2023).

Loganin has not been investigated in studies pertaining to eye health. Loganin's structure and antioxidant potential makes it a strong candidate for further investigation and treatment for eye diseases involving oxidative stress.

2.12.8 Beta-Carotene

Beta-carotene, a precursor to vitamin A, has been extensively studied for its role as a potential antioxidant in eye diseases. The AREDS studies demonstrated that daily oral supplementation with antioxidant vitamins and minerals, including beta-carotene, reduced the risk of developing advanced AMD by 25% at 5 years (Chew et al. 2014; "Lutein + Zeaxanthin and Omega-3 Fatty Acids for Age-Related Macular Degeneration" 2013). Beyond its antioxidant potential, Choo et al (2022) highlighted beta-carotene's potential in inhibiting free radical damage to DNA which is typically associated with diseases of the eye (Choo et al. 2022).

While beta-carotene has shown promise as an antioxidant in eye diseases, its effects on other health outcomes have been a subject of interest. High concentrations of beta-carotene has been associated with an increased risk of lung cancer and other

harmful outcomes in persons at high risk of lung cancer (O'Connor et al. 2022). Following the completion of the AREDS study, beta-carotene was replaced as a supplement in the therapeutic arm of the study by lutein and zeaxanthin due to the increased risk of lung cancer in smokers (Chew 2013). These findings suggest the need for cautious consideration of beta-carotene supplementation, especially in populations with specific health risks.

2.12.9 Antioxidant summary

Antioxidants provide a possible therapeutic benefit on AMD patients in the form of a supplement, as exemplified by the AREDS studies. Further research needs to be completed on the effect that each individual compound has as opposed to the effect they have in concert. This study aims to further understand the protective effects lutein, beta-carotene, zeaxanthin, loganin, chebulagic acid, punicalagin, resveratrol, and pterostilbene have on ARPE-19 cells. Following the results of the experiments of antioxidants alone, SI will be used as an oxidative compound and induce linear levels of oxidative stress. The ARPE-19 cells will be pre-treated with antioxidant compounds then exposed to the oxidative effects of SI. The later experiments will aim to understand the therapeutic effect of these antioxidant compounds on SI induced oxidative stress. Subsequently, an attempt will be made to delineate the mechanism of such protective mechanisms through the use of CRISPR-Cas9 ARPE-19 knockout cell lines. By knocking out pathways sequentially, the specific pathways for each antioxidant will hopefully be

elucidated. The overall goal of this study is to further understand the link between antioxidants and prevention of cell damage due to oxidative stress accumulation similar to that seen in AMD.

Chapter 3: Methods

3.1 Background

This study investigated the cytoprotective effects of pre-treatments with lutein, loganin, zeaxanthin, chebulagic acid, punicalagin, and beta-carotene on oxidative damages to RPE caused by SI. Measurements of cell viability, ROS production, cell death, and antioxidant levels were collected and analyzed. The hypothesis for this study was that the damaging oxidative effects of SI would be mitigated by a pre-treatment with antioxidant compounds. We hypothesized that the antioxidant compounds would result in improved cell viability and less overall oxidative stress within the RPE cells.

3.2 Specific Aims

The specific aims for this study are as follows.

1. Advance the previously optimized blue light model of AMD on ARPE-19 cells to study cell viability and protein expression of antioxidant enzymes

a. Measure cell viability of cells pre-treated with resveratrol and pterostilbene and exposed to blue light

b. Determine the protein expression of antioxidant enzymes, cell death markers, and lipid peroxidation with Western blotting techniques

2. Optimize a SI induced model of oxidative stress on ARPE-19 cells

a. Create a method of exposing cells to SI and inducing oxidative damage

b. Measure cell viability at various concentrations of SI on ARPE-19 cells

c. Determine levels of oxidative stress produced by SI exposure

d. Determine levels of cell death produced by SI exposure

3. Optimize the concentration and duration of bioactive compounds on ARPE-19 WT cells

a. Determine the dose response and optimal treatment concentrations of carotenoids (lutein, zeaxanthin, and beta-carotene) iridoid glycoside (loganin), tannins (punicalagin and chebulagic acid) and stilbenes (resveratrol and pterostilbene) on ARPE-19 WT cells.

b. Investigate cell viability of selected compounds alone and in the presence of SI on ARPE-19 WT cells

c. Investigate ROS levels of selected compounds alone and in the presence of SI on ARPE-19 WT cells

d. Determine the protein expression of antioxidant enzymes and cell death markers with protein blotting techniques on ARPE-19 WT cells

4. Investigate how a knockout of the BIRC5 (survivin) gene plays a role in cytoprotective effects of antioxidants on SI induced damage to ARPE-19 cells

a. Investigate cell viability of selected compounds alone and in the presence of SI on ARPE-19 BIRC5 KO cells

b. Investigate ROS levels of selected compounds alone and in the presence of SI on ARPE-19 BIRC5 KO cells

c. Determine the protein expression of antioxidant enzymes (MnSOD and catalase) and cell death markers (caspase-3) with protein blotting techniques on ARPE-19 BIRC5 KO cells.

5. Investigate how a knockout of the SIRT-1 (sirtuin-1) gene plays a role in cytoprotective effects of antioxidants on SI induced damage to ARPE-19 cells

a. Investigate cell viability of selected compounds alone and in the presence of SI on ARPE-19 SIRT-1 KO cells

b. Investigate ROS levels of selected compounds alone and in the presence of SI on ARPE-19 SIRT-1 KO cells

c. Determine the protein expression of antioxidant enzymes (MnSOD and catalase) and cell death markers (caspase-3) with protein blotting techniques on ARPE-19 SIRT-1 KO cells.

3.3 Cell Culture Methods

3.3.1 ARPE-19 Wild Type (WT) Cells

Human retinal pigment epithelial (RPE) cell line, ARPE-19, was obtained from the American Type Culture Collection (Manassas, VA, USA). ARPE-19 cells were cultured in T-75 or T-25 culture flasks (Sigma-Aldrich, St. Louis, MO, USA) with Dulbecco's Modified Eagle's Medium F12 supplemented with 15 mM HEPES and sodium bicarbonate (DMEM:F12) (Sigma-Aldrich, St. Louis, MO), 5% fetal bovine serum (FBS) (GE Health Care, Canada) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). Originally, ARPE-19 cells were propagated to 80% confluency and passaged. Cells were frozen under liquid nitrogen conditions for long term storage and to be used in all future experiments moving forward. All ARPE-19 cells were cultured at

37°C with 5% CO_2 and grown to approximately 90% to 95%confluency, prior to splitting or performing experiments.

3.3.2 Blue Light Exposure

ARPE-19 cells were exposed to blue light under standard incubation conditions for 12 hours. Blue light was delivered by a specifically designed LED manifold with 72 blue LED's calibrated to a wavelength of 470 nm (Figure 3.1). The LEDs were soldered to a PCB board in a series of 6 LEDs with a 150-ohm resistor. 12 rows of the LED resistor combinations were connected in parallel and connected to a power supply giving 24.8 volts per panel to ensure each LED received sufficient power at the correct wavelength. The LED panel was supported on top of the 96-well plate with a custom-designed support that held the panel 6 cm from the top of the 96-well plate. Exposure to blue light was carefully monitored using a timer in order to ensure all exposure times were identical.

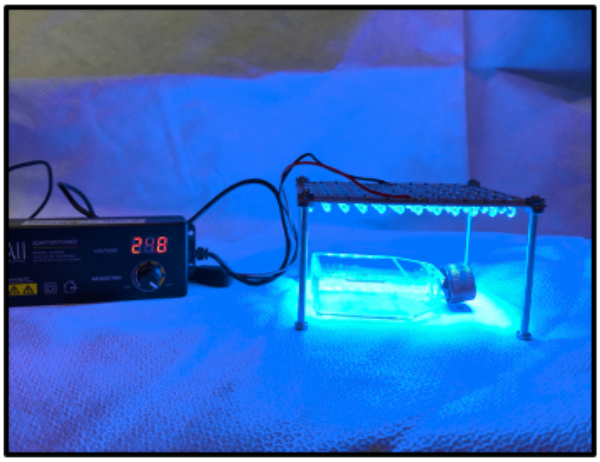

Figure 3.1 – Blue Light Pannel Exposing a T-25 Flask The blue light manifold supported on top of a T-25 flask to show how it is used in the tissue culture incubator. The LED panel is connected to a power supply and mechanical timer. These work together to ensure that the LED panel receives the required voltage of power for the proper duration of time.

3.3.3 ARPE-19 CRISPR Knock-out (KO) Cells

Two knock-out (KO) models of ARPE-19 cells, SIRT1 KO and BIRC5 KO, were designed by Synthego Biotech (Redwood, CA, USA). These cells were modified, cultured, and tested by Synthego for purity and knock-out success. Following these procedures, the cells were delivered to our lab where standard cell culture methods could begin. The KO cell lines were delivered at passage 4, cultured, and frozen back to create a stock supply of modified ARPE-19 cells. ARPE-19 cells were cultured at 37°C with 5% CO_2 and grown to approximately 95% confluency prior to splitting or performing experiments. DNA from KO cell lines was purified and analyzed through polymerase chain reaction (PCR) in order to ensure knockout remained present throughout the experiments.

3.3.4 DNA Extraction from Cells

DNA was extracted from WT, SIRT1 KO, and BIRC5 KO cells in order to perform in house verification of KO pools. The cells were grown up in 6 well plates and allowed to reach 90% confluency. Once reached, the cells were trypsinized and collected via centrifugation at 500xg for 5 minutes in a 2mL tube and then washed twice with phosphate buffered saline (PBS) prior to extracting cellular DNA. DNA was extracted through the use of a PureLink Genomic DNA Mini Kit from Invitrogen (Carlsbad, CA, USA). Cell lysate was prepared from the pelleted ARPE-19 cells using the provided

genomic lysis / binding buffer and ethanol. Once prepared, the cell lysate solution was then added to a spin column and centrifuged at 10000xg for 1 minute at room temperature. The supernatant was discarded, and the spin column was washed twice with provided wash buffer. Following washes, the column was allowed to dry before adding elution buffer and centrifuging for 90 seconds at maximum speed. The eluted product contains the genomic DNA, which was extracted from the ARPE-19 cells. Purified DNA was quantified before performing PCR analysis to ensure equal loading of sample to the reaction mixture. This DNA elution was kept at -20°C and used for identification PCR reactions.

3.3.5 PCR Analysis of Knockout Cell Lines

PCR was used as a means of ensuring successful KO of the selected genes was performed on ARPE-19 cells. This was performed in lab as a complement to the Interference of CRISPR Edits (ICE) analysis provided by Synthego on delivery of the ARPE-19 KO cells. Primers were designed specifically for WT, SIRT1 KO, and BIRC5 KO cells. These primers were synthesized by Eurofins Genomics (Toronto, ON, CA). The primers used for these reactions are listed in Table 3.1. PCR reactions were performed using FroggaBio (Toronto, ON, CA) 2x Taq Master mix, PCR grade water, primers, and DNA sample extracted from our three cell types. PCR reactions were performed in a BioRad Mini Thermocycler following regular PCR reaction templates with an annealing temperature of 65°C and an extension time of 30 seconds.

Table 3.1 - PCR primers for verification of SIRT1 KO and BIRC5 KO

	Sequence Name	Sequence (5' to 3')	% GC Content	Tm
1	**SIRT-1 FWD**	TTCAAGGGGCCAAGTTCACT	50	60.4
2	**SIRT-1 REV**	CTTCTCGATGGCAGTCAGCT	55	62.4
3	**SIRT1 ALO REV**	GCAACCTGTTCCAGGGAGTC	60	64.5
4	**SIRT1 BPII REV**	CCTGTTCCAGCGTGTCTGTCT	57.1	64.5
5	**SIRT1 ECORI REV**	CAACCTGTTCCAGCGTGTCTGAAT	50	64.6
6	**BIRC5 FWD**	GACCACCGCATCTCTACATTCAAGAAC	48.1	66.1
7	**BIRC5 REV**	GGCTGGCCAGAGAAGACTTA	55	62.4

Abbreviations: SIRT-1 – Sirtuin-1, BIRC5 – Survivin, KO – Knockout, Tm – Melting temperature of the primer, FWD – Forward, Rev – Reverse, ALO – ALOI Restriction Enzyme, BPII – BPII Restriction Enzyme, ECORI – ECORI Restriction Enzyme

3.3.6 Restriction Enzyme Digestion

PCR products were incubated at 37°C with appropriate restriction enzymes (summarized in PCR Primer Table**)** and reaction buffers (Thermofisher, ON, CA) in order to digest the PCR product for analysis. PCR primers were designed to amplify the segment of DNA that corresponds to the KO gene in question, and add a single cutter specific restriction enzyme site. Once created, this site allows for the quantitative analysis of PCR product as it corresponds with the knockout gene. Restriction enzyme digests were then loaded on an agarose gel and visualized.

3.3.7 Agarose Gel Electrophoresis

To visualize the products of PCR analysis reactions, samples were loaded onto a 1.2% agarose gel with sybrsafe DNA stain (Thermofisher, ON, CA) and subject to 150V in running buffer for 30 minutes using a Bio-Rad mini-gel apparatus. Following the running of the gel, it was visualized and imaged on a UV transilluminator to verify PCR product size.

3.4 Compound Preparation

3.4.1 Resveratrol Preparation

A resveratrol (Millipore Sigma, Oakville, ON, CA) 5 mM stock solution was prepared in dimethylsulfoxide (DMSO) and further diluted in distilled water. Varying concentrations of resveratrol were prepared by diluting 5 mM stock solutions into serum and antibiotic-free DMEM-F12 media where the final concentration of DMSO in cell culture would be less than 1%.

3.4.2 Pterostilbene Preparation

A pterostilbene (TCI Chemical) 5 mM stock solution was prepared in DMSO and further diluted in distilled water. Varying concentrations of pterostilbene were prepared by diluting 5 mM stock solutions into serum and antibiotic-free DMEM-F12 media where the final concentration of DMSO in cell culture would be less than 1%.

3.4.3 Lutein Preparation

A lutein (Cayman Chemicals, USA) 5 mM Stock solution was prepared in cell culture grade water, sterile filtered, and stored at -20°C until needed for experiments. Stock solutions were further diluted to 500 μM in serum and antibiotic free cell culture media, prior to being added to cells as treatments.

3.4.4 Chebulagic acid

Chebulagic acid (Cayman Chemicals, USA) 5 mM stock solution was prepared distilled cell culture grade water, sterile filtered, and stored at -20°C until needed for experiments. Stock solutions were further diluted to 500 μM in serum and antibiotic free cell culture media prior to being added to cells as treatments.

3.4.5 Loganin

Loganin (Cayman Chemicals, USA) 5 mM Stock solution was prepared in distilled cell culture grade water, sterile filtered, and stored at -20°C until needed for experiments. Stock solutions were further diluted to 500 μM in serum and antibiotic free cell culture media, prior to being added to cells as treatments.

3.4.6 Punicalagin

Punicalagin (Cayman Chemicals, USA) 5 mM Stock solution was prepared distilled cell culture grade water, sterile filtered, and stored at -20°C until needed for experiments. Stock solutions were further diluted to 500 μM in serum and antibiotic free cell culture media, prior to being added to cells as treatments.

3.4.7 Zeaxanthin

Zeaxanthin (Cayman Chemicals, USA) 1mM Stock solution was prepared distilled cell culture grade water, sterile filtered, and stored at -20°C until needed for experiments. Stock solutions were further diluted to 500 μM in serum and antibiotic free cell culture media, prior to being added to cells as treatments.

3.4.8 Beta-Carotene

Beta-carotene (Cayman Chemicals, USA) 5 mM Stock solution was prepared distilled cell culture grade water, sterile filtered, and stored at -20°C until needed for experiments. Stock solutions were further diluted to 500 μM in serum and antibiotic free cell culture media prior to being added to cells as treatments.

3.4.9 Sodium Iodate

Sodium Iodate ($NaIO_3$) (Sigma-Aldrich, St. Louis, MO, USA) was prepared in a 400 mM stock solution in cell culture grade distilled water, sterile filtered, aliquoted into 0.75 mL aliquots and stored at -20°C until needed for experiments. Prior to experiments, the stock aliquots were thawed and diluted in TDMEM to create a 50 mM working stock solution. This working stock was then added into the desired wells of a cell culture plate for treatment to ARPE-19 cells.

3.5 Compound Treatment

5 mM stock solutions of resveratrol, pterostilbene, lutein, chebulagic acid, loganin, punicalagin, zeaxanthin, and beta-carotene were diluted to 500 μM working stock solutions in serum and antibiotic free DMEM-F12 cell culture media. This working stock was then used to treat each desired well for 4 hours prior to exposing cells to 18 hours of SI in various concentrations. The final concentration of SI in each well was determined based on a final volume of 200 μL in a 96-well plate and 2 mL in a

6-well plate. These volumes were used to calculate the desired amount of working stock to add to each treatment well of ARPE-19 cells.

3.6 Sodium Iodate Treatment

ARPE-19 cells were treated with various concentrations of SI. SI was prepared as a 50 mM working solution from a 400 mM stock solution. Cells were seated in 96-well or 6-well plates, depending on the experiment being performed, and allowed to adhere for 24 hours. Following adherence, media was changed and a new media solution containing the SI was added to the well and the cells were incubated in the solution for 18 hours at 37°C with 5% CO_2. Following treatment, media was removed, and experiments were carried out as described by each experimental method.

3.7 Preparation of ARPE-19 cells for experiments

ARPE-19 cells were seeded with supplemented DMEM-F12 into 96-well plates with $5x10^5$ cells per well or 6-well plates with $7.5x10^6$ cells per well. Cells were then left undisturbed for 24 hours in order to allow them to adhere to the plate. Cells were grown in supplemented media along with the addition of various concentrations of antioxidant treatments and SI. Control wells not receiving any special treatment were given matching volumes of supplemented media to ensure identical volumes. The outer edge of the 96-well plate was not used due to uncontrolled evaporation from the wells. All experimental conditions were carried out in triplicate wells. Experiments that used blue light exposure were set up in two identical plates where one plate was kept in the

control condition (darkness) and the other was exposed to blue light for a pre-determined length of time.

3.7.1 Cell Viability Assay

Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc, USA) was used to determine cell viability. Measurements were made following the manufacturer's protocol. In brief, $5x10^5$ cells were plated into 96-well plates and allowed 24 hours to adhere and stabilize. After 24 hours, viability experiments were initiated as described in cell treatment method. Following treatment and exposure, treatment media was removed and10 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was added directly to each well and incubated for 3 hours. The absorbance was then read using a BioTek Cytation 5 96-well plate reader (BioTek, Winooski, VT, USA) at 450nm. Background absorbance was subtracted using a set of control wells that contained only media and MTS reagent. Values were expressed as a relative percentage of the controls for all three cell types.

3.7.2 Caspase 3/7 Fluorescence Assay

CellEvent Caspase 3/7 Green Detection Reagent (Invitrogen, Carlsbad, CA, USA) was used to investigate the activation of caspase-3 during apoptosis. Measurements for mean fluorescence values were made and images were collected following the manufacturer's protocol. Cells were treated as described in the cell culture treatment methodology section previously prior to adding the Casapse 3/7 Green Detection

Reagent as follows. The CellEvent Caspase 3/7 Green Detection Reagent was diluted to 2 μM in PBS with 5% FBS. Treatment media was aspirated from the wells and 100μL of the diluted reagent solution was added. The cells were incubated with the reagent for 30 minutes prior to imaging and reading fluorescence at 502nm/530nm excitation/emission maxima respectively using a BioTek Cytation 5 96-well plate reader with Gen5 version 3.06 (BioTek, Winooski, VT, USA). Imaging was conducted using the 465 nm LED cube for the Cytation 5 plate reader in imaging mode. Images were analyzed using the Gen5 Image analysis software and the mean value of fluorescence at 530nm was measured in each well.

3.7.3 Preparation of Total Cellular Protein Lysate

In order to collect sufficient quantities of total cellular protein, ARPE-19 cells were seeded in 6-well plates at $7.5x10^6$ cells and allowed to adhere to the plate and propagate for 24 hours. ARPE-19 cells were treated as described in the cell treatment methodology previously prior to stopping the treatment and collecting cells using trypsinization. Cells were subjected to centrifugation at 500xg for 5 minutes and the cell pellets were washed twice with PBS. Protein was extracted from the cell pellet using mechanical disruption with a lysis buffer containing 25 mM Tris (pH 7.5), 150 mM sodium chloride (NaCl), 1% Triton X-100, 2 mM Ethylenediaminetetraacetic acid (EDTA), 1% protease inhibitor cocktail (Sigma P8240), 1% sodium orthovanadate (ab120386), and 4% sodium fluoride (Thermo Scientific S299-100). Lysis was performed

using a sterile Qiagen 5 mm stainless-steel bead (Qiagen, Toronto, ON, CA) then homogenized using the Qiagen TissueLyser (Qiagen, Toronto, ON, CA) run at 20 Hz for 3 minutes. The bead was removed. The samples were then centrifuged at 20000xg for 15 minutes and supernatant transferred to a new tube for storage at -80°C. The concentration of protein in stored cellular lysates was quantified using a 660-assay kit (Thermofisher, ON, CA). The concentration of each protein was then used in preparation of sample with 4x SDS reducing buffer containing 125 mM Tris (pH 6.8), 2% SDS, 0.01% bromophenol blue and 10% glycerol, and 0.1 M beta mercaptol ethanol, resulting in a final concentration of 1X. Samples were then vortexed and heated at 100°C for 5 minutes prior to being loaded into SDS-Page gels. Cellular lysates were diluted to a final concentration of 0.5-1 μg/μL using dH_2O and 4x SDS reducing loading dye.

3.7.4 Western Blot Analysis

20 μg of each prepared protein sample was loaded into an SDS-PAGE gel (4-12% gradient gel, Genscript, PA, USA) and subjected to electrophoresis for 1 hour at 200 V. Following completion of the gel electrophoresis, the protein from the gel was transferred to a 0.45 μm nitrocellulose membrane (GE Healthcare, CA) electrophoretically using a Genscript E-Blot system (Genscript, PA, USA). Following transfer, the membrane was washed once with TBS with tween-20 (TBST) (20 mM Tris, 137 mM NaCl, 1% Tween-20) and stained with Ponceau S stain for 5 minutes. After

staining, the membranes were rinsed 5 times with dH_2O and excess stain was removed until lysate banding patterns were observed. The membranes were then images using the using Chemidoc imager (Bio-Rad Laboratories, Mississauga, ON, CA) in Ponceau S stain mode. The images were saved for later analysis and the membranes were distained using 0.1 M sodium hydroxide (NaOH) until all the red colour was removed from the membrane. The membrane was then washed twice with TBST and blocking solution was made. The membrane was blocked using 5% non-fat milk in TBST for 1 hour at room temperature on a Storvall Belly Dancer Shaker to allow for uniform coverage of blocking solution. Following blocking, membranes were incubated overnight at 4°C with the following antibodies: Caspase 3/7 (1:1000) (Cell Signaling, CA), catalase (1:1000) (Abcam, USA), and MnSOD (1:1000) (Millipore, CA). Membranes were washed using 1x TBST then incubated for 1 hour using an anti-rabbit secondary antibody (1:1000) (R&D Systems, USA) at room temperature.

Following secondary antibody incubation, membranes were again washed three times with TBST and proteins of interest were visualized by enhanced chemiluminescence (ECL). ECL was performed to detect protein banding patterns on immunoblot membranes by horseradish peroxidase-conjugated to secondary antibodies. ECL solution was made fresh every time with freezer stocks of luminol and coumaric acid. Membranes were incubated in ECL solution for 5 minutes with light agitation prior to detection. Chemiluminescent immunoblots were detected by using Chemidoc imager

(Bio-Rad Laboratories, Mississauga, ON, CA) in blot membrane mode and allowed to develop for 15 seconds of exposure time. Colorimetric images were also taken and merged with ECL images in order to visualize the protein marker bands to determine the size of protein bands present. Western blots were quantified using the ImageJ software to measure the area under the curve for each specific ECL band. Loading controls were used to normalize each blot and to eliminate any differences between experiments and membrane sets. All bands were normalized to their specific Ponceau S stain band intensity.

3.7.5 Oxidative Stress Assay

Intracellular ROS formation was detected using 2′,7′-dichlorodihydrofluorescein diacetate (CMH2-DCFDHA) probes according to the manufactures protocol (ThermoFisher Scientific). ARPE-19 WT and KO cells were seeded in a 6-well plate and treated with antioxidant supplement for 4 hours prior to exposure to SI for 18 hours. Following the incubation, treatment media was removed and fresh SDMEM-F12 media was added with 10uM CMH2-DCFDHA reagent at 37°C for 30 minutes. After washing the cells three times and adding 1x PBS cells were imaged and fluorescence intensity was measured using a multifunctional microplate reader (BioTek Cytation 5) with an excitation wavelength of 485 nm and emission wavelength of 528 nm. Images acquired were used as representative images for the overall fluorescence measurement read by

the plate reader. Images were compiled and fluorescence measurements were compared to control cells.

3.8 Statistical Analysis

The optimized data corresponds to 4-6 independent experiments or three (n=3) separate passages for Western blot experiments. All statistical analyses were performed using Graph Pad Prism 9 software (GraphPad Software, San Diego, CA, USA). Data was presented as mean ± standard error of the mean (SEM). All data used one-way ANOVAs with Tukey's post-hoc test with $p \leq 0.05$ indicating significance. Figures are presented with; * indicating $p<0.05$, ** indicating $p<0.01$, *** indicating $p<0.001$, and **** indicating $p<0.0001$.

Chapter 4: Results

4.1 Blue Light Study

Blue light irradiation has been shown to trigger apoptosis and oxidative stress in ARPE-19 cells. The aim of this study was to investigate the effects of two polyphenolic compounds in rescuing ARPE-19 WT cells from cell death, and the initial experiments were focused on a blue light induced model of oxidative stress and apoptosis. To this end, a blue-light manifold was created to induce oxidative damage to ARPE-19 cells through irradiation. This study further investigated antioxidants, oxidative stress, and cell death markers in ARPE-19 cells pre-treated with resveratrol or pterostilbene prior to exposure of blue light. Immunoblotting was used to measure protein expression of MnSOD, catalase, GPx-1, 4HNE, and caspase-3.

4.1.1 Effects of Resveratrol and Pterostilbene on MnSOD Protein Expression

MnSOD was investigated in this study due to its ability to mitigate oxidative stress. MnSOD, which is found in the mitochondria, plays a critical role in the dismutation of superoxide radical where it catalyzes the conversion of superoxide anion to H_2O_2 (Miriyala et al. 2012). Exposing ARPE-19 WT cells to blue light had no significant effect on MnSOD protein levels. Pre-treatment with resveratrol or pterostilbene also had no significant effect on MnSOD protein levels of the ARPE-19 WT cells (Figure 4.1).

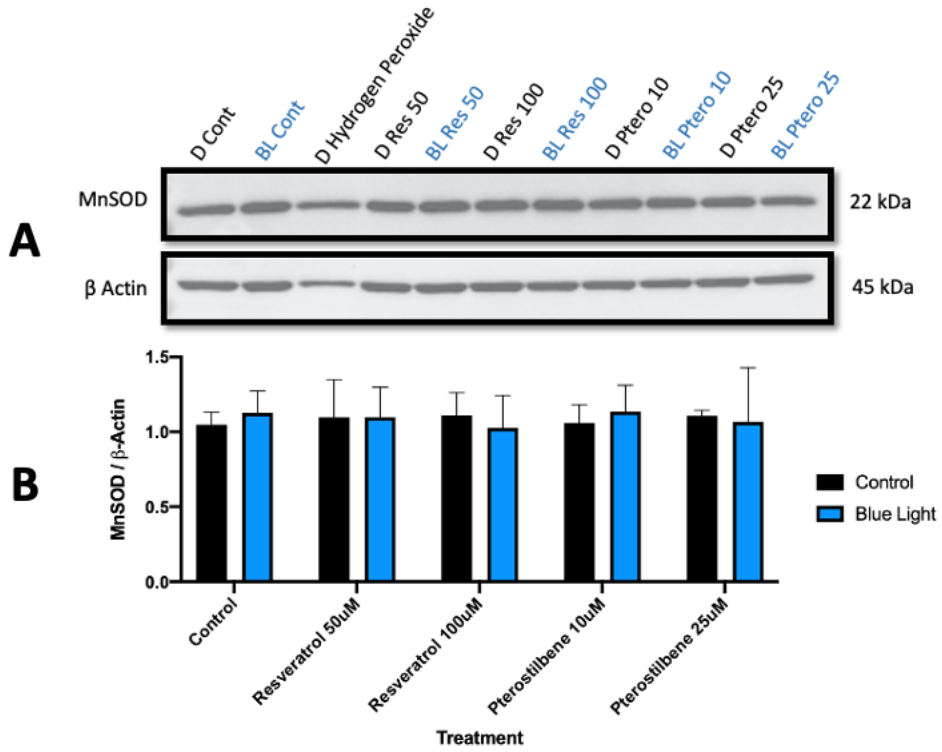

Figure 4.1 - MnSOD protein expression.
(A) Representative Western blot and (B) quantified band density of protein expression of MnSOD in ARPE-19 WT cells exposed to control or blue light with treatments of resveratrol (50 μM & 100 μM) and pterostilbene (10 μM & 25 μM). Cells were pre-treated for 4 hours with resveratrol and pterostilbene, followed by 12 hours of exposure to either blue light or kept in the dark. 300 μM of hydrogen peroxide was used as an oxidant to compare blue light exposure to. Protein expression was determined using Western blot and expressed as a ratio to β-actin. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test.

4.1.2 Effects of Resveratrol and Pterostilbene on Catalase Protein Expression

Catalase plays an important role in the reduction of hydrogen peroxide to water and oxygen (Aebi 1974). Exposing ARPE-19 WT cells to blue light showed no significant change on catalase protein levels. Pre-treatment with resveratrol or pterostilbene also had no significant effect on the protein expression of catalase in ARPE-19 WT cells (Figure 4.2).

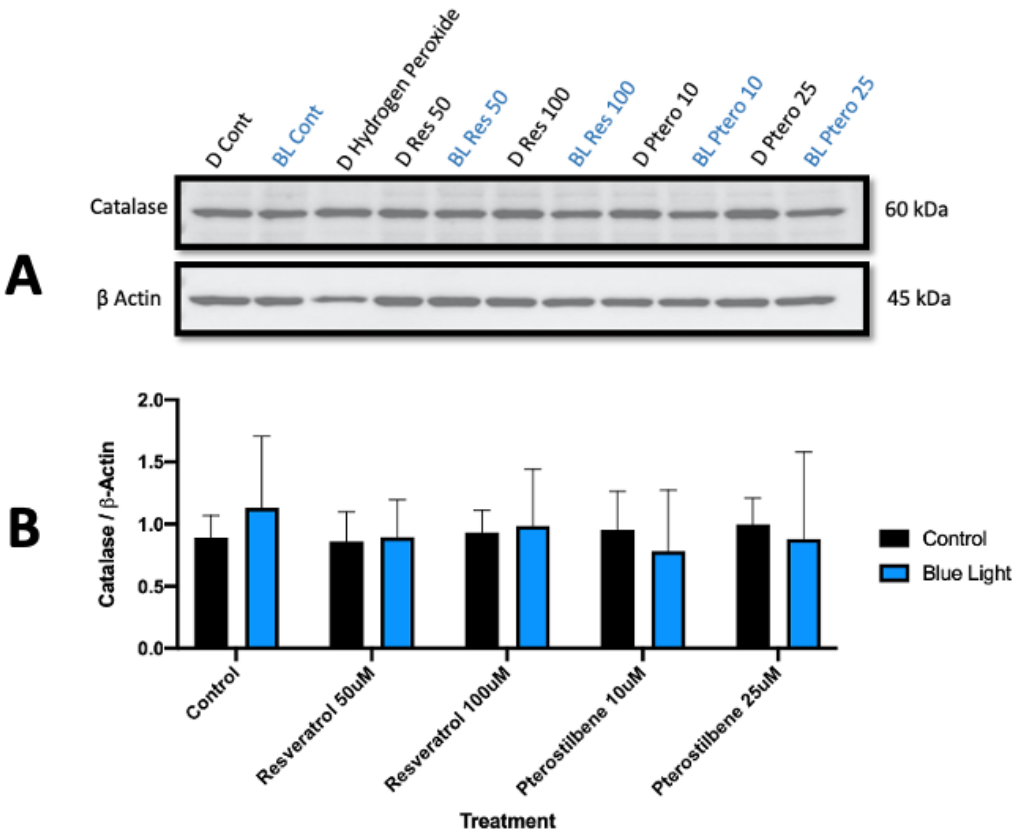

Figure 4.2 - Catalase protein expression.
(A) Representative Western blot and (B) and quantified band density of protein expression of catalase in ARPE-19 WT cells exposed to control or blue light with treatments of resveratrol (50 μM & 100 μM) and pterostilbene (10 μM & 25 μM). Cells were pre-treated for 4 hours with resveratrol and pterostilbene, followed by 12 hours of exposure to either blue light or kept in the dark. 300μM of hydrogen peroxide was used as an oxidant to compare blue light exposure to. Protein expression was determined using Western blot and expressed as a ratio to β-actin. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test.

4.1.3 Effects of Resveratrol and Pterostilbene on GPx-1 Protein Expression

GPx-1 plays a role in the detoxification of lipid hydroperoxides and hydrogen peroxide using glutathione as a substrate (Lubos, Loscalzo, and Handy 2011). Exposing ARPE-19 WT cells to blue light had no significant changes on GPx-1 protein levels. Pre-treatment with 50 μM and 100 μM of resveratrol or 10 μM of pterostilbene also had no significant effect on GPx-1 protein levels of the ARPE-19 WT cells. Pre-treatment of 25 μM pterostilbene showed a significant ($p<0.05$) reduction in GPx-1 protein levels as compared to control cells (Figure 4.3).

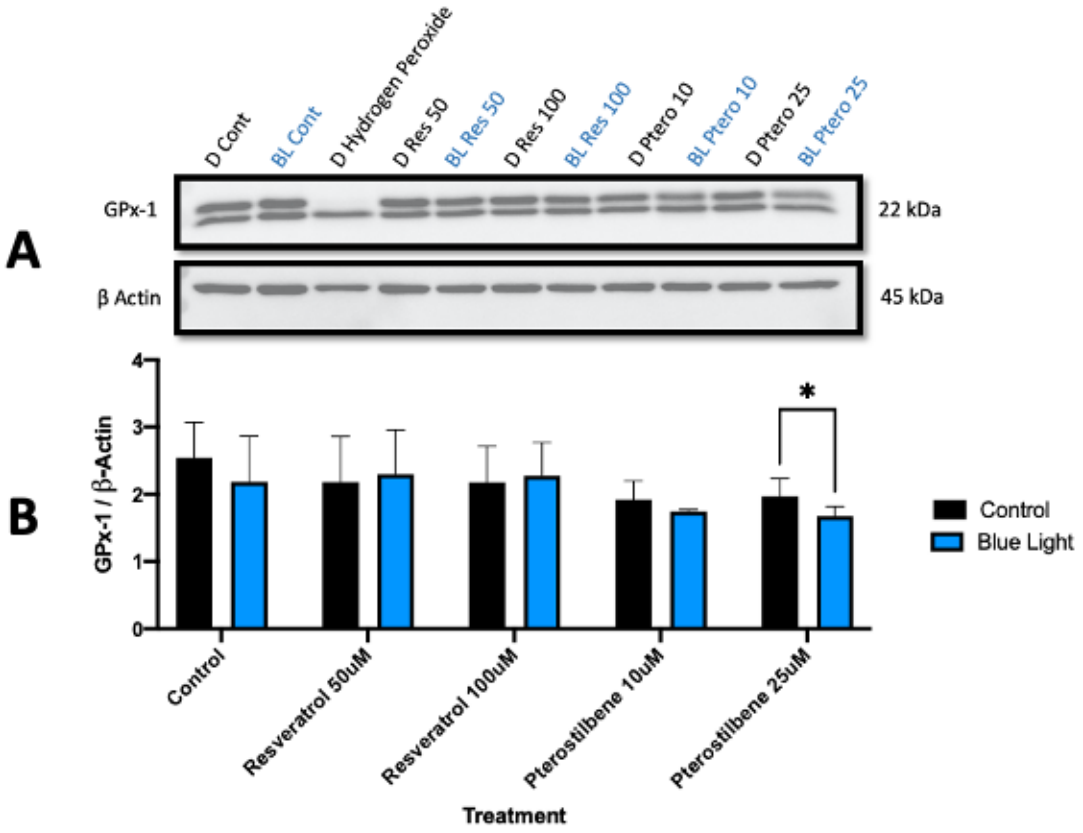

Figure 4.3 - GPx-1 protein expression.
(A) Representative Western blot and (B) and quantified band density of protein expression of catalase in ARPE-19 WT cells exposed to control or blue light with treatments of resveratrol (50 μM & 100 μM) and pterostilbene (10 μM & 25 μM). Cells were pre-treated for 4 hours with resveratrol and pterostilbene, followed by 12 hours of exposure to either blue light or kept in the dark. 300 μM of hydrogen peroxide was used as an oxidant to compare blue light exposure to. expression was determined using Western blot and expressed as a ratio to β-actin. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test (* indicates $p<0.05$).

4.1.4 Effects of Resveratrol and Pterostilbene on 4HNE Protein Adduct Formation

4HNE is a stable product of lipid peroxidation and is produced when free radicals remove electrons from lipids in the cell membrane increasing oxidative stress within cells(Łuczaj, Gęgotek, and Skrzydlewska 2017). Blue light was found to significantly increase ($p<0.05$) 4HNE protein adduct formation in ARPE-19 WT cells when compared to control cells kept in the dark. No significant changes were observed after treatments with resveratrol on 4HNE protein adduct formation. Pterostilbene (10 and 25 μM concentrations) was found to significantly increase ($p<0.05$) 4HNE protein adduct formation when comparing control cells to cells that were exposed to blue light after pre-treatment with pterostilbene (Figure 4.4).

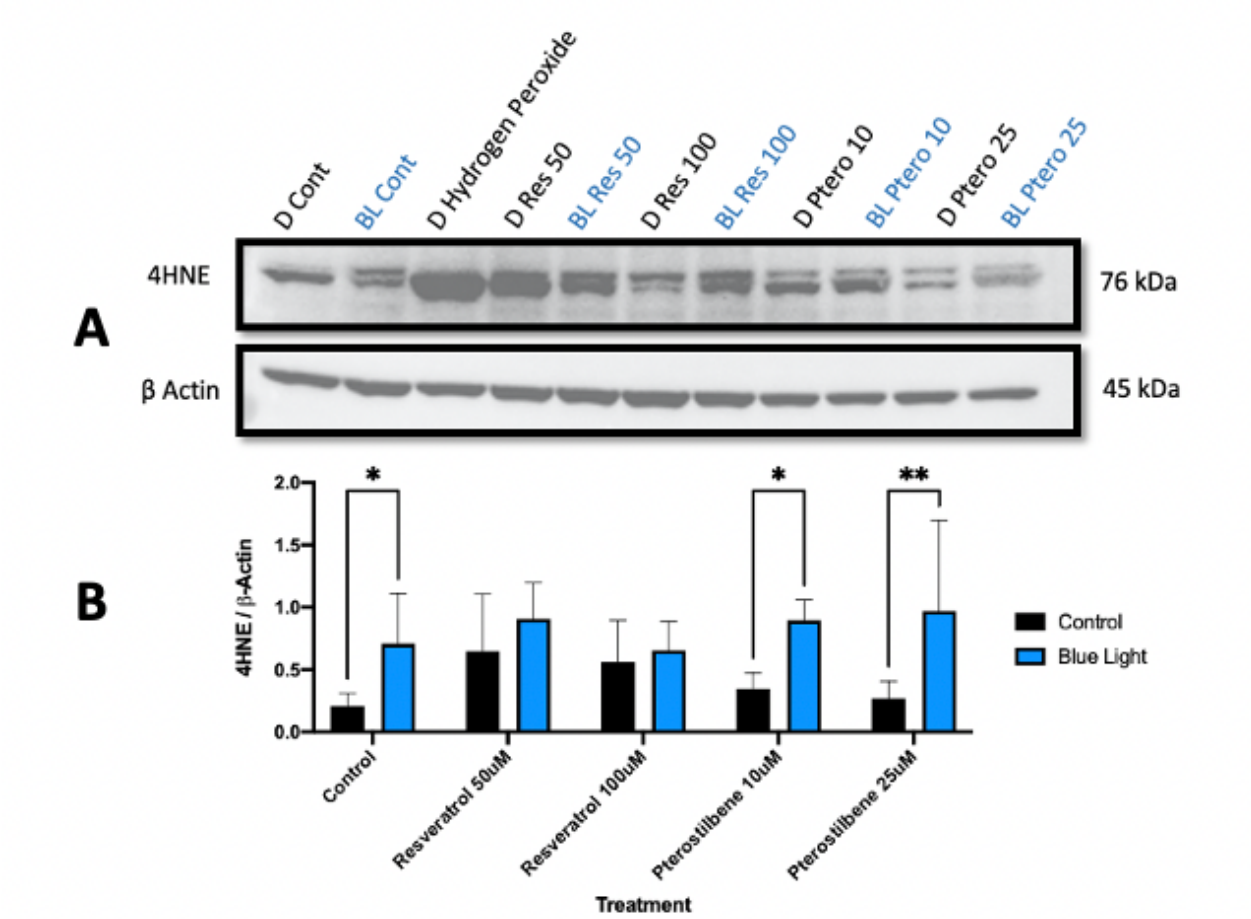

Figure 4.4 - 4-HNE protein adduct formation.
(A) Representative Western blot and (B) and quantified band density of protein adduct formation of 4HNE in ARPE-19 WT cells exposed to control or blue light with treatments of resveratrol (50 μM & 100 μM) and pterostilbene (10 μM & 25 μM). Cells were pre-treated for 4 hours with resveratrol and pterostilbene, followed by 12 hours of exposure to either blue light or kept in the dark. 300 μM of hydrogen peroxide was used as an oxidant to compare blue light exposure to. Protein expression was determined using Western blot and expressed as a ratio to β-actin. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (* indicates $p<0.05$, ** indicates $p<0.01$).

4.1.5 Effects of Resveratrol and Pterostilbene on Caspase-3 Protein Expression

Caspase-3 is a prominent signaling molecule in the apoptosis pathway. Exposing ARPE-19 WT cells to blue light had no significant effect on caspase-3 protein expression levels. Pre-treatment with resveratrol or pterostilbene also showed no significant effects in caspase-3 levels of the ARPE-19 WT cells (Figure 4.5).

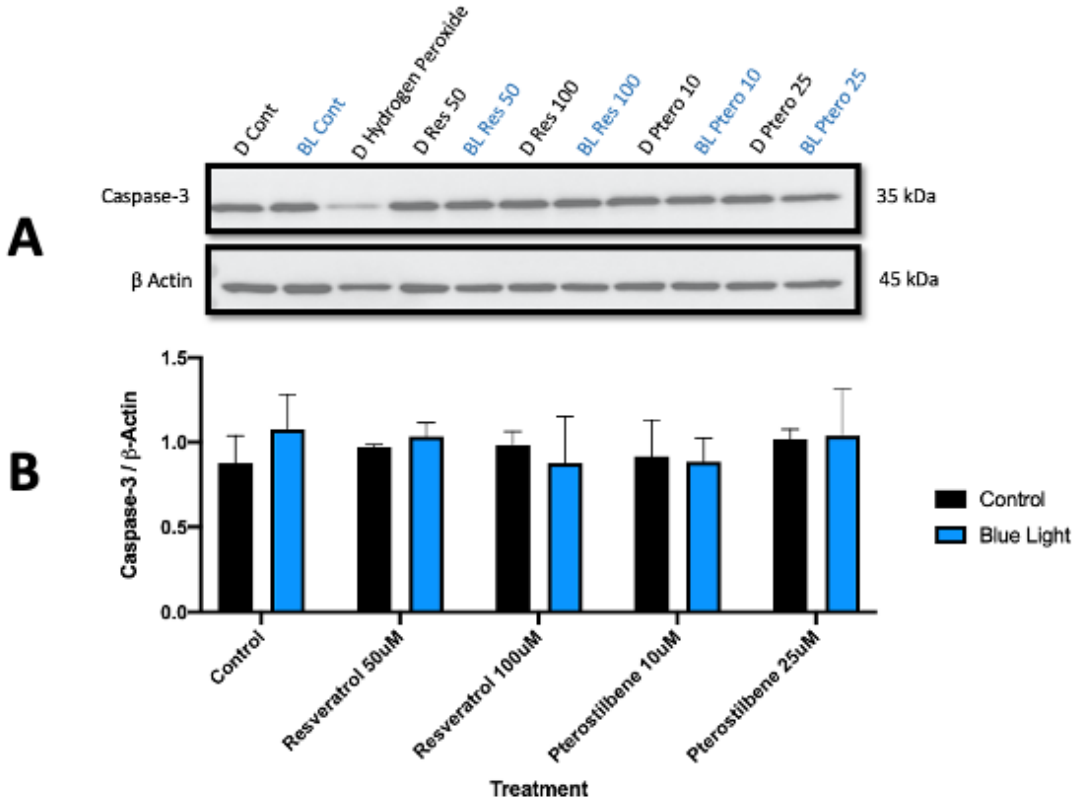

Figure 4.5 - Caspase-3 protein expression.
(A) Representative Western blot and (B) and quantified band density of protein expression of caspase-3 in ARPE-19 WT cells exposed to control or blue light with treatments of resveratrol (50 μM & 100 μM) and pterostilbene (10 μM & 25 μM). Cells were pre-treated for 4 hours with resveratrol and pterostilbene, followed by 12 hours of exposure to either blue light or kept in the dark. 300 μM of hydrogen peroxide was used as an oxidant to compare blue light exposure to. Protein expression was determined using Western blot and expressed as a ratio to β-actin. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test.

4.1.6 Blue Light Study Summary

One major limitation of the blue light irradiation was the fluctuation in intensity of the light and the increase in overall temperature of the incubator. It was discovered that the LEDs would become fatigued after 20-30 exposures, resulting in an overall reduction in blue light intensity. The fatigue can be observed in the LED panels through a darkening of the internal workings of the LED. Originally, they have a bright silver colour, but as they cycle and age, the silver turns to a bronze colour. After noticing the change in colour, the panel was tested with a lux meter. The findings from the lux meter indicated that the intensity of blue light from an old panel and a new panel was roughly a 30% reduction in intensity. This reduction of intensity resulted in a 30% increase in cell viability when comparing blue light treated cells to control cells. Following this finding, the blue light exposure experiments were halted in exchange for a more stable chemical compound for inducing blue-light-like damage to ARPE-19 cells was to be investigated.

Multiple stressors (hydrogen peroxide, blue light, and SI) were being simultaneously investigated as oxidative stressors used in cell culture models of oxidative stress in ARPE-19 cells. From our investigation and the limitations of the blue light exposure, SI was determined to be the most suitable treatment for this study's goals. SI was acquired and initial testing on ARPE-19 cells was initiated, and treatment conditions were optimized.

4.2 Compound Optimization

4.2.1 SI Optimization

SI was selected based on its ability to produce linear increase in oxidative stress and cause damages to the retinal cells that are typically seen in progression of AMD. To investigate the oxidant potential of SI on ARPE-19 cells, an MTS assay was used as a measure of cellular viability. Dilutions of SI were added so the final concentration in the well was calculated to be 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, and 30 mM. SI was left incubating with the cells for 18 hours prior to being removed and new media with MTS reagent being added and incubated for 3 hours. At lower concentrations (up to 10 mM), it was found that SI resulted in an increase in cell viability, whereas, at higher concentrations the cellular viability of the ARPE-19 WT cells was found to decrease. Significant ($p<0.05$) decrease in cell viability was found at 15, 17.5, 20, and 30 mM concentrations of SI (Figure 4.6).

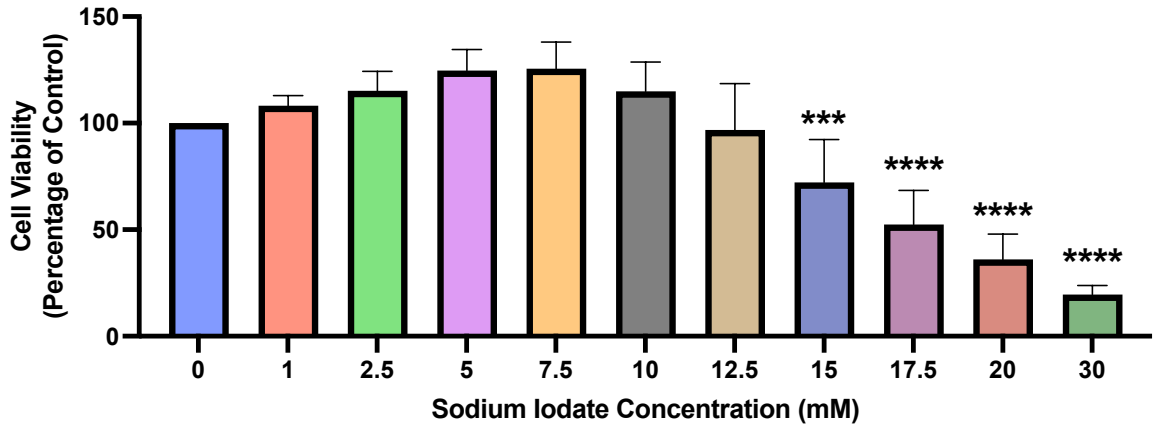

Figure 4.6 - SI dose response on ARPE-19 WT cells using MTS assay.
ARPE-19 WT cells were treated with SI in supplemented media for 18 hours prior to performing MTS assay as a measure of cellular viability. Data presented as mean ± SEM of n=6 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test (*** indicates $p<0.001$, **** indicates $p<0.0001$).

To investigate the ability of SI to induce cell death of the ARPE-19 WT cells, Caspase 3/7 fluorescence imaging was performed. Results from the Caspase 3/7 imaging illustrate that as concentration of SI increased, significant ($p<0.05$) increases of caspase 3/7 mean fluorescence intensity was observed (Figure 4.7). Based on the cell viability and cell death data, SI was chosen as the inducer of cell damage for experiments moving forward.

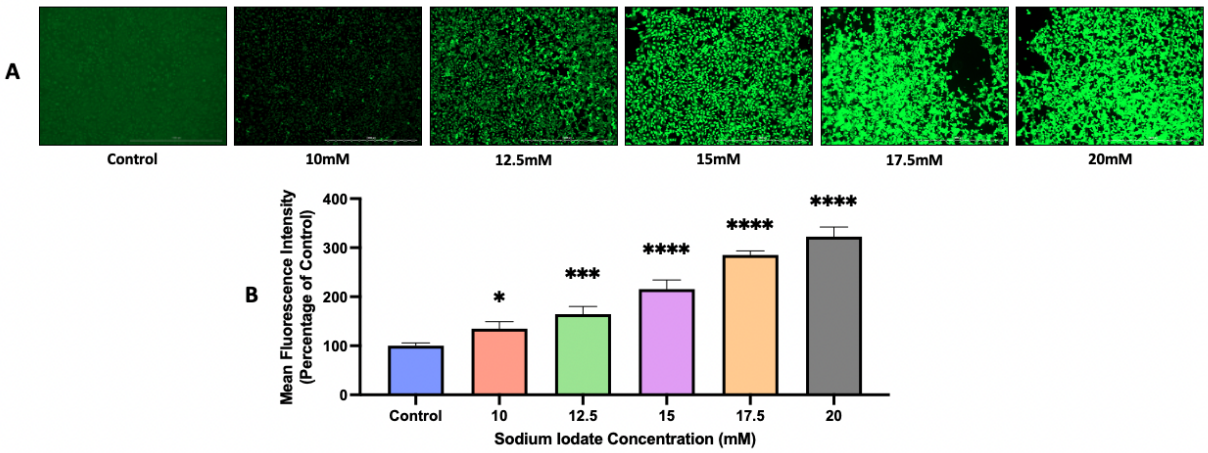

Figure 4.7 - SI dose response on ARPE-19 WT cells using Caspase 3/7 Fluorescence Imaging Technique.

A. Representative Fluorescence Imaging of ARPE-19 WT cells were treated with SI in supplemented media for 18 hours prior to performing Caspase 3/7 fluorescence imaging as a detector of apoptosis. **B.** Mean fluorescence intensity measurements of images from A of increasing SI concentrations compared to control (0 mM SI). Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (* indicates $p<0.05$, *** indicates $p<0.001$, **** indicates $p<0.0001$).

4.2.2 Antioxidant Compound Optimization Overview

Prior to investigating the beneficial effects of antioxidants in the presence of SI as the oxidant, treatment conditions and treatment durations needed to be optimized. A search of primary literature was performed for each of the compounds on various human cell lines (as many have not been investigated in ARPE-19 cells) and a starting range of concentrations were collected and organized. Upon determination of the expected concentrations, initial experiments used these as a starting point, and depending on the results of the cell viability assay (MTS assay), concentrations were either increased or decreased based on the goal of the treatment. The MTS assay and ARPE-19 WT cells were used in optimizing the treatment protocols for the future experiments.

4.2.3 Compound Optimization Results

Results from the optimization of the bioactive compounds of resveratrol, pterostilbene, lutein, punicalagin, loganin, chebulagic acid, beta-carotene, and zeaxanthin can be found in Table 4.1 –Bioactive compound optimization as well as in each subsequent figure with the full results of the experiments (referenced in the table).

Table 4.1 – Bioactive Compound Optimization using MTS assay.
Summary of all concentrations tested in the optimization of each compound. Statistical significance as compared to the control is shown with respect to each concentration.

Compound Name	Concentrations Tested (μM)	Figure
Resveratrol	25, 50, 75, 100, 150, 200, 250	Figure 4.8
Pterostilbene	5[d], 10[d], 20[d], 30[d], 40[d]	Figure 4.9
Lutein	10[b], 25[c]	Figure 4.10
Punicalagin	5, 10, 20	Figure 4.11
Loganin	20, 40	Figure 4.12
Chebulagic Acid	5[b], 20[c], 40[a]	Figure 4.13
Beta-Carotene	2.5, 5, 10, 20, 40	Figure 4.14
Zeaxanthin	1, 2.5, 5, 10[b], 20[d], 30[d]	Figure 4.15

([a] indicates $p<0.05$, [b] indicates $p<0.01$, [c] indicates $p<0.001$, [d] indicates $p<0.0001$.)

Resveratrol (0-250 μM) showed no significant change in cell viability when compared to the control across all concentrations tested, indicating it is likely not cytotoxic to the cell (Figure 4.8). Pterostilbene showed a damaging effect on ARPE-19 cells as there was a significant decrease ($p<0.0001$) in cell viability. Cell viability was reduced by over 50% with 5 μM treatments and over 90% with treatments greater than 10 μM of pterostilbene (Figure 4.9).

Lutein (0-25 μM) showed a significant ($p<0.01$) decrease in cell viability when compared to the control cells. The decrease in viability for lutein was 22% with the 10 μM treatment and 31% for the 25 μM treatment (Figure 4.10). Punicalagin (0-20 μM) showed no significant change when compared to the control cells across all concentrations tested (Figure 4.11). Loganin (0-40 μM) showed no significant change when compared to the control cells across all concentrations tested (Figure 4.12).

Chebulagic acid (0-40 μM) showed a significant decrease ($p<0.05$) in cell viability when compared to the control cells. The cell viability was decreased most significantly ($p<0.001$) by 25% at 20 μM (Figure 4.13). Beta-carotene (0-40 μM) showed no significant change in cell viability when compared to the control cells across all concentrations tested, indicating it is likely not cytotoxic to the cell and may garner cellular protection when exposed to oxidative stress (Figure 4.14).

Zeaxanthin (0-30 μM) showed a significant increase ($p<0.01$) in cell viability at concentrations 10, 20 and 30 μM when compared to the control cells. This increase in viability indicates zeaxanthin is not cytotoxic to the cells (Figure 4.15).

From the results on the bioactive compound optimization on ARPE-19 cells, 7 compounds were tested as a pre-treatment to SI exposure. Concentrations for further testing were selected for each compound based on results from these optimization experiments. Resveratrol was tested at 50 and 100 μM, lutein at 10 and 25 μM, punicalagin at 10 and 25 μM, loganin at 10 and 40 μM, chebulagic acid at 5, 20, and 40 μM, beta-carotene at 2.5, 5, 10, 20, and 40 μM, and finally zeaxanthin at 1, 2.5, 5, 10, 20, and 30 μM as shown in Table 4.1.

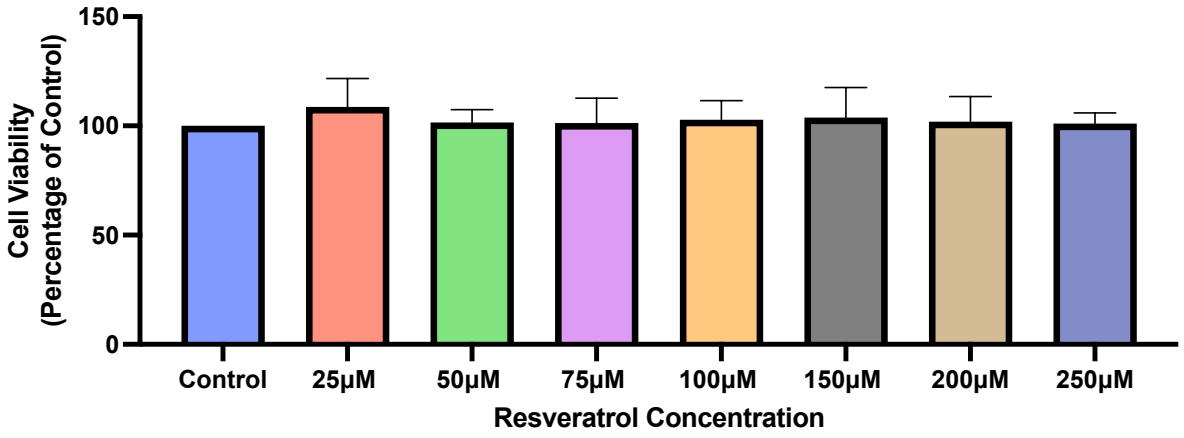

Figure 4.8 - Resveratrol dose response on ARPE-19 WT cells using MTS assay. ARPE-19 WT cells were treated with resveratrol in supplemented media for 22 hours prior to performing MTS assay as a measure of cellular viability. Treatments were compared to control cells and cell viability was expressed as a percentage of control. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test.

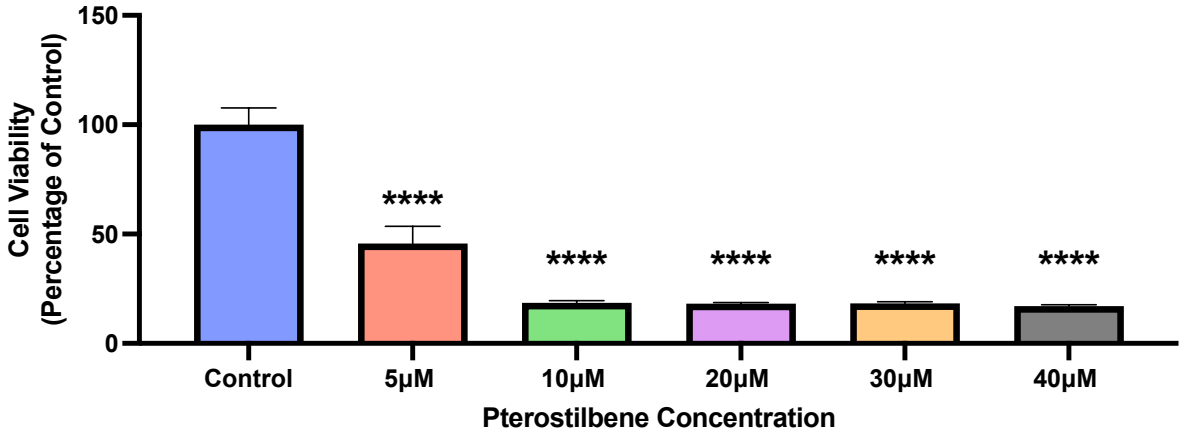

Figure 4.9 - Pterostilbene dose response on ARPE-19 WT cells using MTS assay. ARPE-19 WT cells were treated with pterostilbene in supplemented media for 22 hours prior to performing MTS assay as a measure of cellular viability. Treatments were compared to control cells and cell viability was expressed as a percentage of control. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test (**** indicates $p<0.0001$).

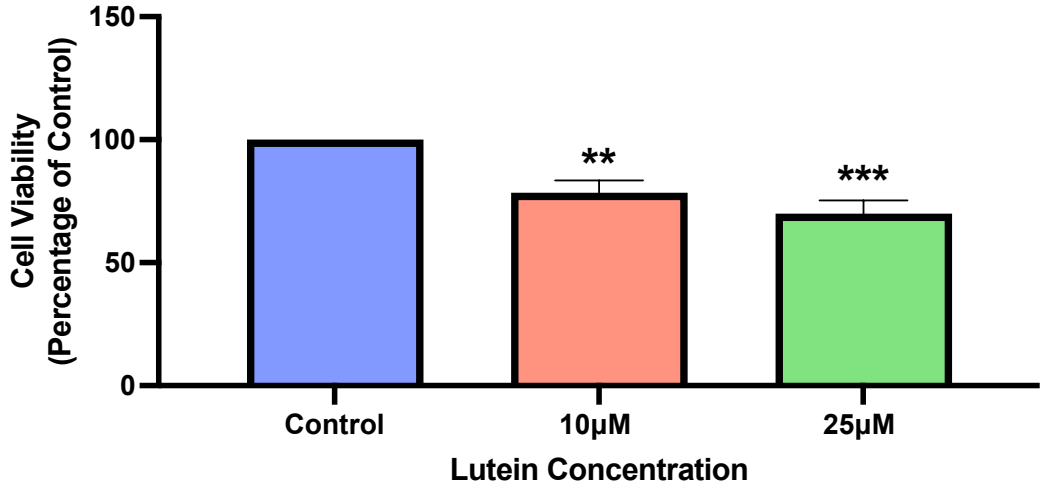

Figure 4.10 - Lutein dose response on ARPE-19 WT cells using MTS assay.
ARPE-19 WT cells were treated with lutein in supplemented media for 22 hours prior to performing MTS assay as a measure of cellular viability. Treatments were compared to control cells and cell viability was expressed as a percentage of control. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test (** indicates $p<0.01$, *** indicates $p<0.001$).

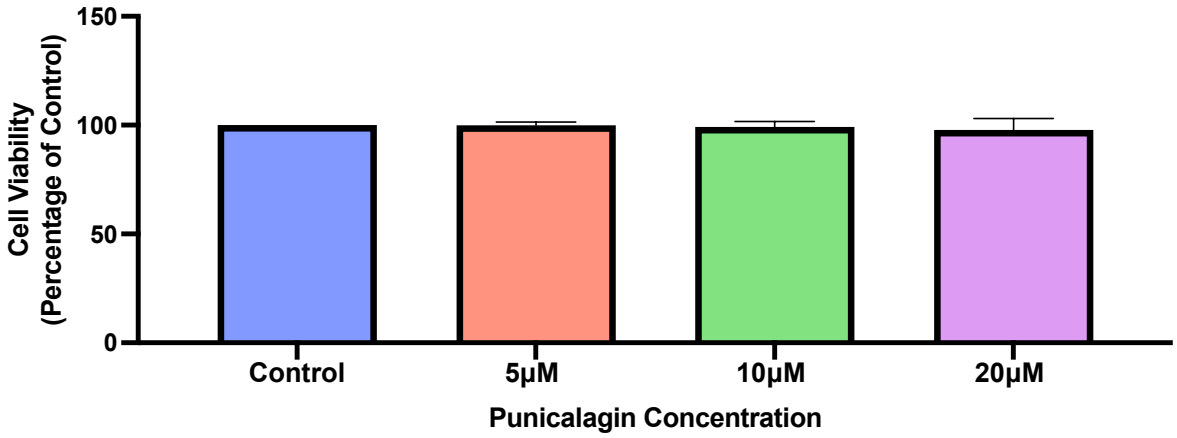

Figure 4.11 - Punicalagin dose response on ARPE-19 WT cells using MTS assay. ARPE-19 WT cells were treated with punicalagin in supplemented media for 22 hours prior to performing MTS assay as a measure of cellular viability. Treatments were compared to control cells and cell viability was expressed as a percentage of control. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test.

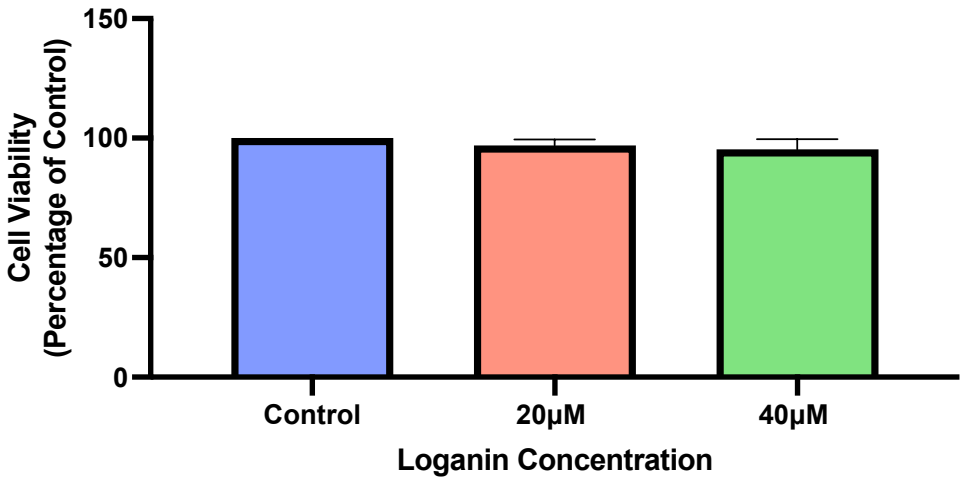

Figure 4.12 - Loganin dose response on ARPE-19 WT cells using MTS assay.
ARPE-19 WT cells were treated with loganin in supplemented media for 22 hours prior to performing MTS assay as a measure of cellular viability. Treatments were compared to control cells and cell viability was expressed as a percentage of control. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test.

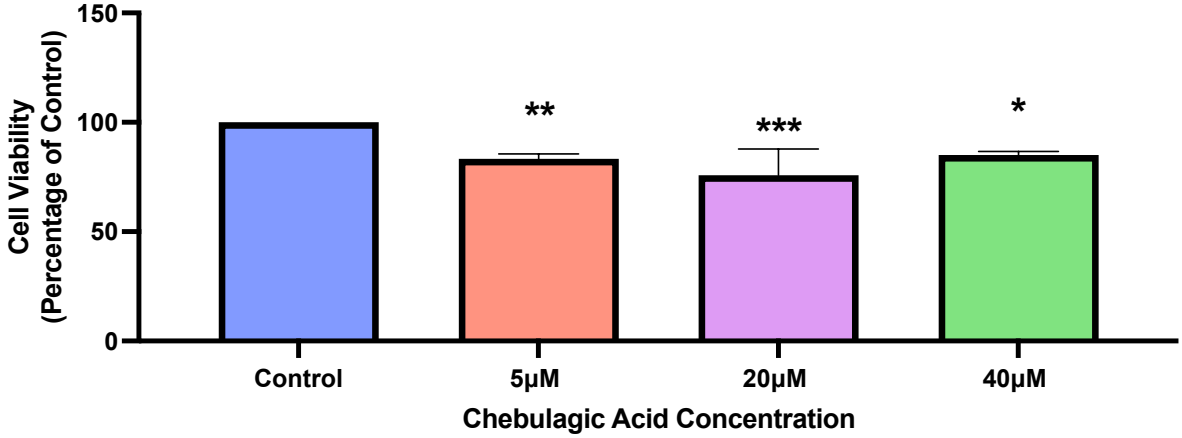

Figure 4.13 - Chebulagic acid dose response on ARPE-19 WT cells using MTS assay. ARPE-19 WT cells were treated with chebulagic acid in supplemented media for 22 hours prior to performing MTS assay as a measure of cellular viability. Treatments were compared to control cells and cell viability was expressed as a percentage of control. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.)

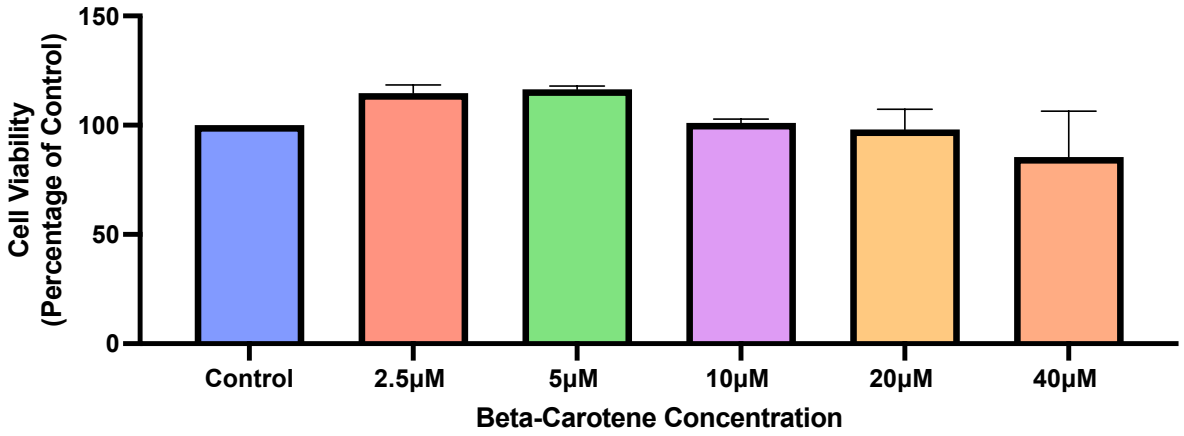

Figure 4.14 - Beta-Carotene dose response on ARPE-19 WT cells using MTS assay. ARPE-19 WT cells were treated with beta-carotene in supplemented media for 22 hours prior to performing MTS assay as a measure of cellular viability. Treatments were compared to control cells and cell viability was expressed as a percentage of control. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test.

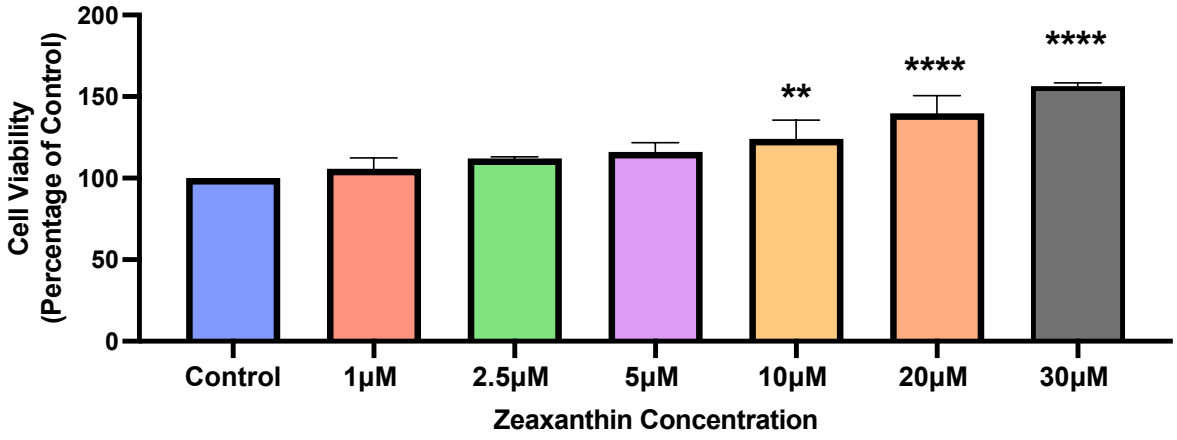

Figure 4.15 - Zeaxanthin dose response on ARPE-19 WT cells using MTS assay. ARPE-19 WT cells were treated with zeaxanthin in supplemented media for 22 hours prior to performing MTS assay as a measure of cellular viability. Treatments were compared to control cells and cell viability was expressed as a percentage of control. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test (** indicates $p<0.01$, **** indicates $p<0.0001$).

4.3 Compound Optimization with SI Treatments

Following the optimization of the bioactive compounds and SI exposure, in order to test the hypothesis that these compounds can protect ARPE-19 WT cells from the oxidative effects of SI, combination treatments were performed. MTS assay was used as a method of quantifying cellular viability of the ARPE-19 WT cells. ARPE-19 WT cells were pre-treated for 4 hours with the appropriate concentrations of bioactive compound, prior to exposing the cells to SI for 18 hours. Results from the optimization of resveratrol, lutein, punicalagin, loganin, chebulagic acid, beta-carotene, and zeaxanthin with SI can be found summarized in Table 4.2 – MTS Assay – Compound and SI Optimization.

Table 4.2 – MTS Assay – Compound and SI Optimization.
Summary of all concentrations tested in the optimization of each compound with exposure to SI. Significance from the appropriate control is shown with respect to each concentration.

SI Concentration (mM)	Compound Name	Concentrations Tested (μM)
12.5		50, 100
15	**Resveratrol**	0, 50, 100
17.5		0, 50, 100
12.5		10, 25[c]
15	**Lutein**	10, 25
17.5		10, 25[c]
12.5		10[d], 25[d]
15	**Punicalagin**	10[d], 25[d]
17.5		10[d], 25[d]
12.5		20, 40
15	**Loganin**	20, 40
17.5		20, 40[c]
12.5		5[d], 20[d], 40[d]
15	**Chebulagic Acid**	5[d], 20[d], 40[d]
17.5		5[c], 20[c], 40[b]
12.5		2.5[b], 5[d], 10, 20[a], 40[c]
15	**Beta-Carotene**	2.5, 5[c], 10, 20[a], 40[b]
17.5		2.5, 5, 10, 20, 40
12.5		1, 2.5, 5, 10[b], 20[b], 30[d]
15	**Zeaxanthin**	1[a], 2.5[b], 5[b], 10[d], 20, 30
17.5		1, 2.5[a], 5[b], 10[c], 20, 30

([a] indicates $p<0.05$, [b] indicates $p<0.01$, [c] indicates $p<0.001$, [d] indicates $p<0.0001$.)

Following the combination pre-treatment of bioactive compound prior to exposure to SI, the results suggest that resveratrol had no significant effect on ARPE-19 WT cells exposed to various concentrations of SI. Lutein (25 μM) showed significant ability to maintain viability with 12.5 and 17.5 mM exposure to SI. Loganin showed no effect on cell viability at 12.5 and 15 mM of SI, however, at 17.5 mM of SI with a 40 μM pre-treatment, cell viability was significantly increased when compared to the control.

Punicalagin and chebulagic acid decreased cell viability when used as a pre-treatment prior to exposure to SI. Comparing the cell viability data between the compound alone and in combination with SI, it was evident that there is a possible interaction between the bioactive compound and SI becoming more cytotoxic to the cells. Punicalagin resulted in greater than 80% reduction in cell viability when compared to the respective control ($p<0.0001$). Chebulagic acid at higher concentrations of SI (15 and 17.5 mM) resulted in upwards of 70% reduction in cell viability when compared to its respective controls ($p<0.01$).

Beta-carotene showed significant ($p<0.01$) ability to maintain cell viability at its lower testing range (2.5 and 5 μM) with up to 15 mM of SI exposure. At higher concentrations of beta-carotene (20 and 40 μM) cell viability was significantly reduced ($p<0.05$) when compared to its controls. Zeaxanthin showed the most promising results of offering cytoprotection from the exposure to SI. Maintenance of cell viability was seen with pre-treatments of 2.5 to 10 μM zeaxanthin prior to exposure to 15 and 17.5

mM of SI. 10 μM zeaxanthin offered the highest ability to maintain cell viability following exposure to 15 mM of SI with 145% ($p<0.0001$) increase in cell viability when compared to the control exposure of 15 mM of SI.

From the results of the optimization of bioactive compound with SI exposure, it was decided that the 15 and 17.5 mM SI exposures would be used for further experiments. 15 and 17.5 mM of SI consistently showed significant decreases in cell viability of ARPE-19 WT cells where the 12.5 mM exposure occasionally was not significantly different than control cells. Of the 7 compounds that were tested with SI, resveratrol and loganin showed no significant deviation from control cells. Punicalagin and chebulagic acid showed significant reductions in cell viability indicating a possible increased cytotoxicity of the compounds when exposed to SI. Lutein, zeaxanthin, and beta-carotene showed increased ability to maintain cell viability at various concentrations when exposed to SI and were the three compounds used for further experimentation to study cell viability, oxidative stress and antioxidant status. Lutein was tested at 10 and 25 μM, zeaxanthin at 2.5 and 10 μM, and beta-carotene at 5 and 20 μM.

4.4 ARPE-19 WT MTS Assay Results

Results from the MTS assay with lutein, zeaxanthin and beta-carotene can be found in Table 4.3 – ARPE-19 WT - MTS Assay – Compound and SI as well as in each subsequent figure with the full results of the experiments (referenced in the table).

Table 4.3 – ARPE-19 WT - MTS Assay – Compound and SI.
Summary of results from the MTS assay using ARPE-19 WT cells pre-treated with bioactive compounds then exposed to 15 and 17.5 mM of SI. Significance from the appropriate control is shown with respect to each concentration.

SI Concentration (mM)	Compound Name	Concentrations Tested (μM)	Figure
15	**Lutein**	10[c], 25[d]	Figure 4.16
17.5		10[c], 25[c]	
15	**Zeaxanthin**	2.5[a], 10[b]	Figure 4.17
17.5		2.5, 10[c]	
15	**Beta-Carotene**	5[d], 20[b]	Figure 4.18
17.5		5, 20	

([a] indicates $p<0.05$, [b] indicates $p<0.01$, [c] indicates $p<0.001$, [d] indicates $p<0.0001$.)

Lutein treatment alone caused a significant ($p<0.05$) reduction in cell viability when compared to control cells for both 10 and 25 μM concentrations. When exposed to 15 and 17.5 mM of SI alone, ARPE-19 cells had a significant ($p<0.05$) reduction in cell viability when compared to the control cells. Pre-treatment with 10 and 25 μM lutein prior to SI exposure resulted in a significant ($p<0.05$) maintenance of cell viability. Both the 10 and 25 μM pre-treatments of lutein in the presence of 15 and 17.5 mM of SI exposure illustrated a significant ($p<0.001$) protection in cellular viability on the ARPE-19 WT cells when compared to the SI treated alone WT cells. At the 17.5 mM exposure to SI, both 10 and 25 μM of lutein resulted in approximately the same protective effect on cellular viability when compared to the control (Figure 4.16).

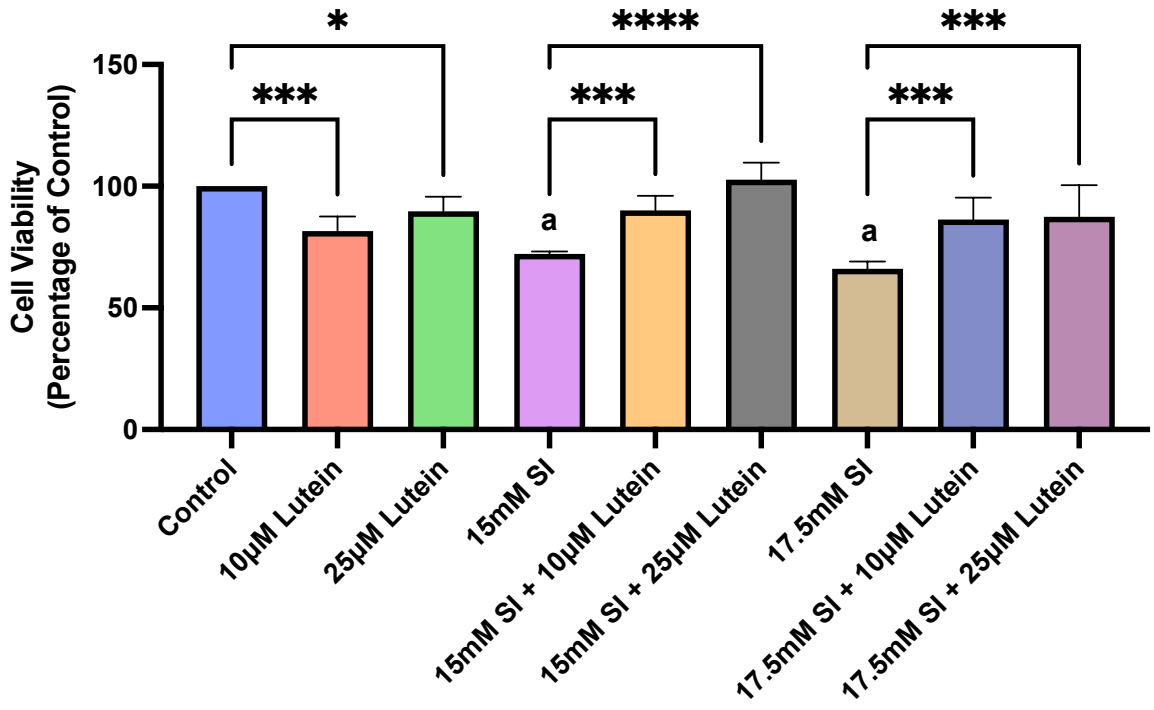

Figure 4.16 - Effect of lutein on ARPE-19 WT cell viability using MTS assay. ARPE-19 WT cells were pre-treated with lutein in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing MTS assay as a measure of cellular viability. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$.)

Zeaxanthin treatments alone showed no significant change from ARPE-19 WT control cells. Cell viability was significantly ($p<0.05$) reduced in ARPE-19 cells when exposed to 15 and 17.5 mM of SI alone. Zeaxanthin pre-treatments showed an increased maintenance of cell viability when compared to 15 and 17.5 mM SI exposure alone cells. Pre-treatment with 2.5 and 10 μM of zeaxanthin at 15 mM of SI resulted in a significant ($p<0.05$) protection in cellular viability over the control. At 17.5 mM exposure to SI, 10 μM of zeaxanthin resulted in a significant ($p<0.001$) protection in cellular viability when compared to the control. The 2.5 μM concentration of zeaxanthin resulted in no significant change in cellular viability when compared to the control (Figure 4.17).

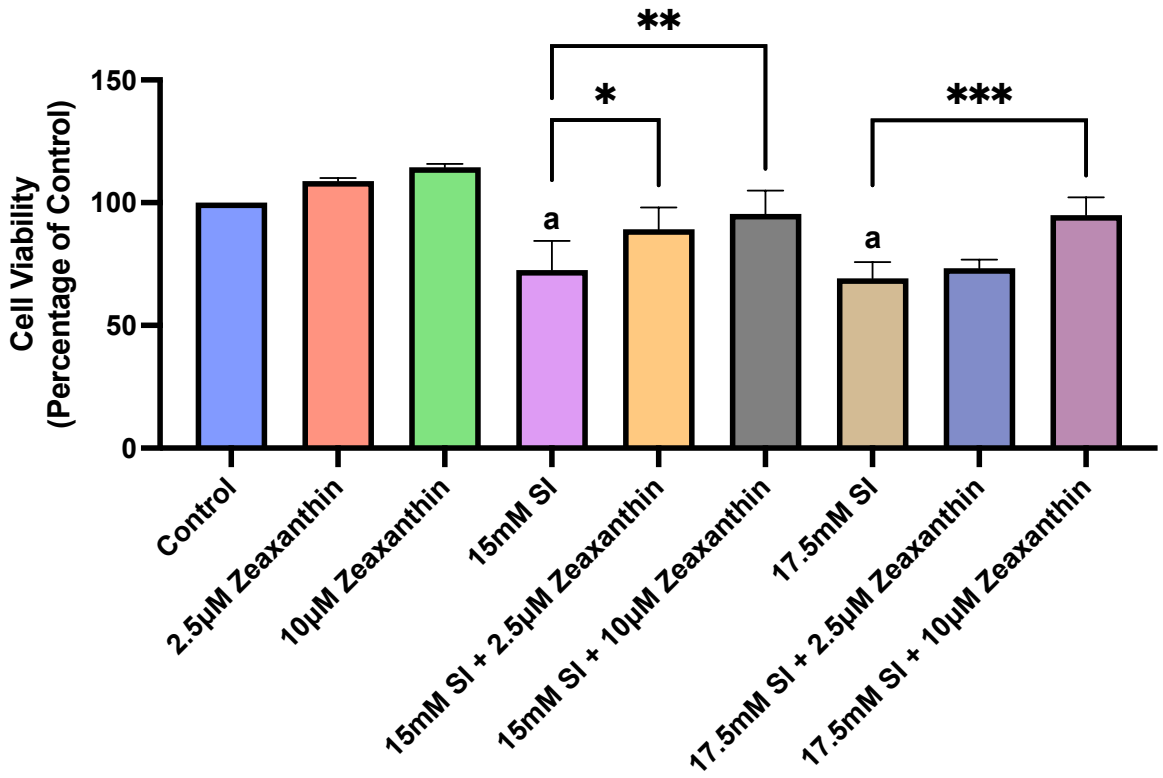

Figure 4.17 - Effect of zeaxanthin on ARPE-19 WT cell viability using MTS assay. ARPE-19 WT cells were pre-treated with Zeaxanthin in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing MTS assay as a measure of cellular viability. Data presented as mean ± SEM of n=4 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.)

Beta-carotene showed the least effect on cell viability of all three compounds tested. Pre-treatment with both 2.5 and 20 μM of beta-carotene at 17.5 mM of SI resulted in no significant change in viability when compared to the control. Exposure to 15 and 17.5 mM of SI alone resulted in a significant ($p<0.05$) reduction in cell viability when compared to control cells. Pre-treatment with 5 μM of beta-carotene resulted in a significant ($p<0.0001$) protective maintenance of cellular viability, whereas the higher 20 μM pre-treatment resulted in an overall significant decrease ($p<0.01$) in cellular viability at the same exposure to 15 mM of SI. Beta-carotene pre-treatment combined with 17.5 mM SI exposure resulted in no significant change in cell viability when compared to 17.5 mM SI alone exposed cells (Figure 4.18).

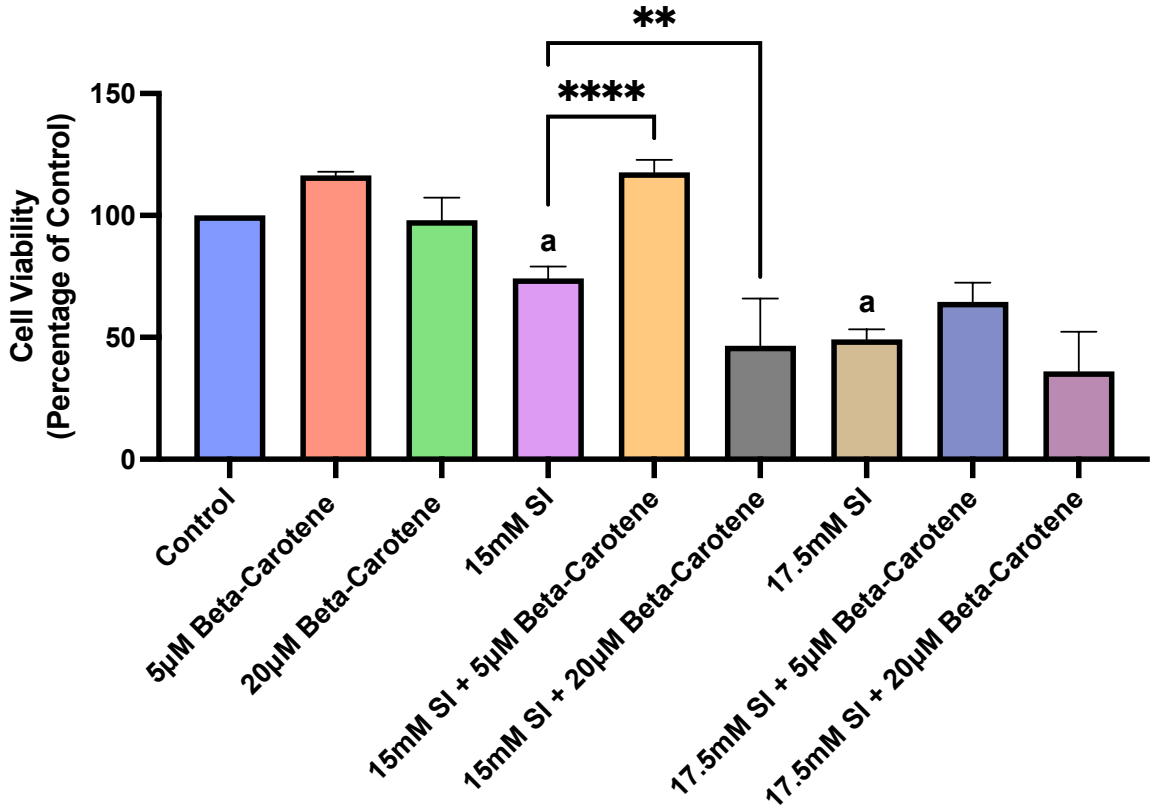

Figure 4.18 - Effect of beta-carotene on ARPE-19 WT cell viability using MTS assay. ARPE-19 WT cells were pre-treated with beta-carotene in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing MTS assay as a measure of cellular viability. Data presented as mean ± SEM of n=4 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, ** indicates $p<0.01$, **** indicates $p<0.0001$.)

4.4.1 Combined effect of the antioxidant compounds and SI – Summary

Based on the results of MTS cell viability assay, it was determined that lutein, zeaxanthin, and beta-carotene had the most promising protective effect on ARPE-19 WT cells. A summary of results from all concentrations of antioxidant studied is summarized in Table 4.3. Lutein, zeaxanthin, and beta-carotene were selected to proceed forward with further experimentation and determine the effects of each compound on genetically modified ARPE-19 knockout cell models. By studying these compounds with a KO model, the mechanistic pathways in which each compound is playing a role in protection of the ARPE-19 cells can be investigated.

4.5 ARPE-19 KO Cell Lines

To investigate the biological effect and role each compound has on ARPE-19 cells, two KO cell lines were created. The first KO cell line was a KO of BIRC5, or the survivin gene. These cells were constructed by Synthego Biotech (Redwood, CA, USA). Upon receiving the cell line, a KO analysis was also conducted illustrating the success of the KO and the deletions or insertions that were created through the knockout construction. The results of the KO analysis can be seen in Figure 4.19**.** From the analysis, the pooled KO line has a KO score of 37%, indicating that the cell line itself has increased difficulty surviving and proliferating with the BIRC5 gene missing. KO was successfully created, and the cell line was ready to be used in our cell culture experiments.

Figure 4.19 - BIRC5 KO ICE Analysis
Interference of CRISPR Edits (ICE) analysis provided by Synthego Biotechnology (Redwood, CA). Summary figure shows the guide target used for the knockout, the knockout score, as well as the contribution percentage of each DNA type found in the KO cell pool.

The second KO cell line was a KO of SIRT-1or the Sirtuin 1 gene. These cells were constructed by Synthego Biotech (Redwood, CA, USA). Upon receiving the cell line, a KO analysis was also conducted showing the success of the KO and the deletions or insertions that were created through the KO construction. The results of the KO analysis can be found in Figure 4.20. From the analysis, the pooled KO line has a KO score of 94%, indicating that the cell line is functioning as it should and there were minimal effects on cell proliferation with the SIRT-1 gene missing. The KO was successfully created, and the cell line was ready to be used in our cell culture experiments.

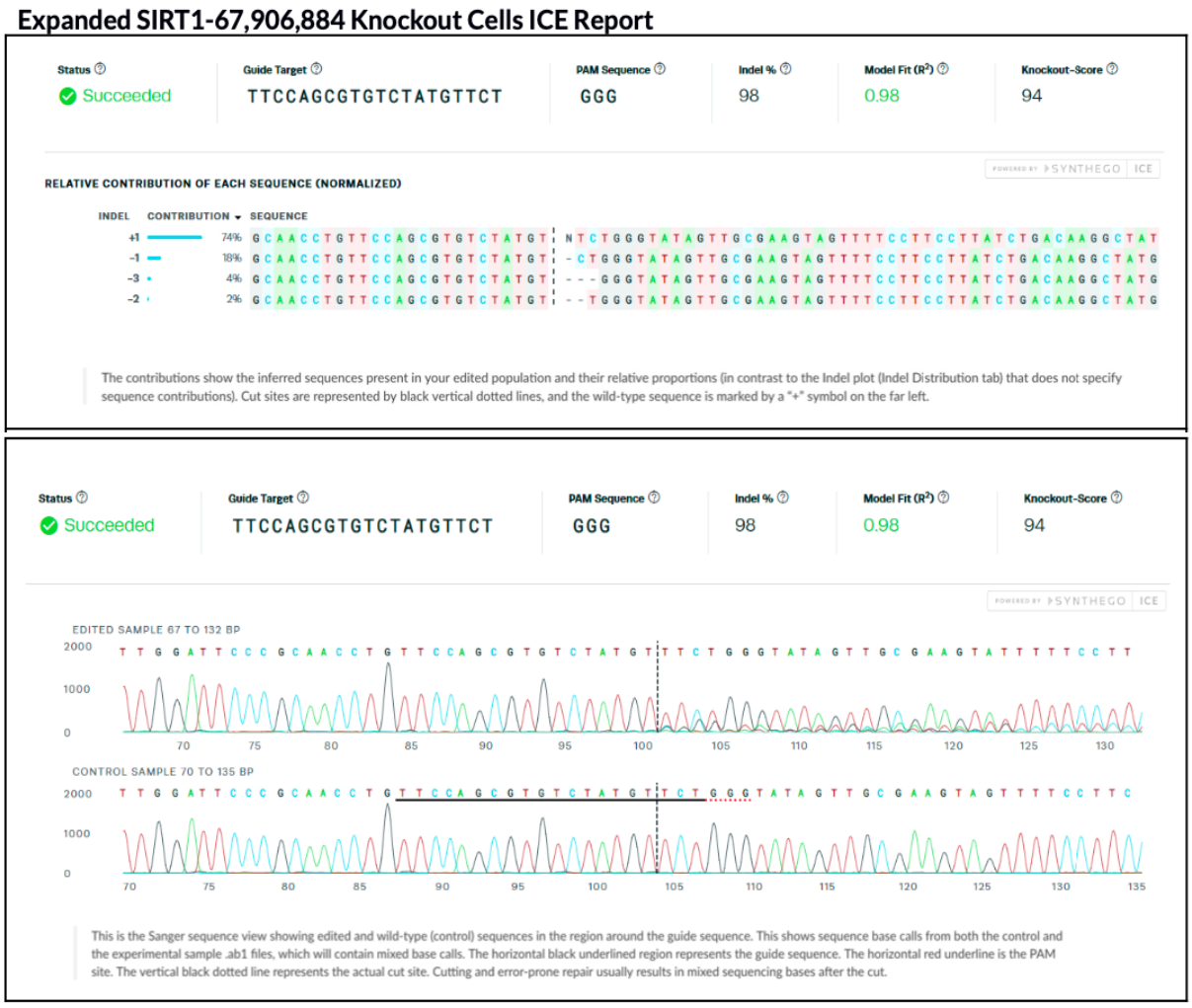

Figure 4.20 - SIRT-1 KO ICE Analysis
Interference of CRISPR Edits (ICE) analysis provided by Synthego Biotechnology (Redwood, CA). Summary figure shows the guide target used for the knockout, the knockout score, as well as the contribution percentage of each DNA type found in the KO cell pool.

To further analyze and confirm the success of the acquired KO cells prior to experiments, PCR primers were specifically designed for each KO and PCR analysis was performed. Results from the PCR analysis can be found in Figure 4.21**.** From the results, both knockout pools were confirmed to contain knockouts as expected. The PCR primers used were designed to amplify the segment of DNA that corresponds to the KO gene in question and add a single cutter specific restriction enzyme site. With the addition of the single cutter restriction enzyme site, digests can be performed and if the knockout sequence is present in the cell, when the DNA is digested with the specific cutter, a secondary band will be produced. Restriction enzyme digests were then loaded on an agarose gel and visualized. For the BIRC5 KO digestion, comparison between the WT and KO samples indicate the presence of a second band indicating the KO is present. The SIRT-1 KO digestion also confirms the presence of a second band indicating the KO is present.

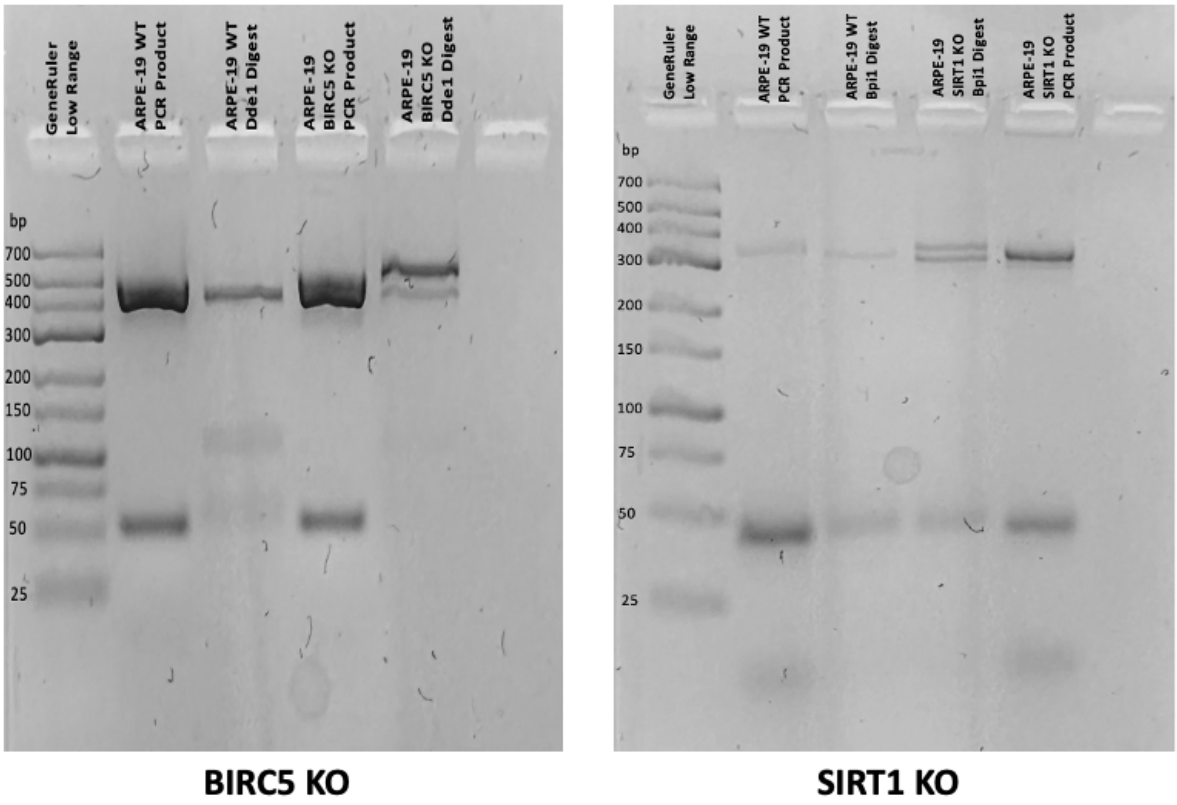

Figure 4.21 – Restriction Enzyme Digests of Knockout Cells.
BIRC5 and SIRT-1 KO cell DNA was purified and PCR was run using specifically designed primers to create restriction enzyme sites (Table 3.1 PCR Primers). Digests of PCR product were performed and yielded a secondary band when compared to WT control, indicating the presence of KO cells.

4.5.1 ARPE-19 BIRC5 KO MTS Assay

In order to investigate the mechanistic pathway in which our carotenoids, lutein, zeaxanthin, and beta-carotene function, KO cell lines of ARPE-19 cells were created and were tested with the same experiments as the ARPE-19 WT cell lines. For each experiment, untreated KO control cells were used as a reference and the results were expressed as a percentage of control. ARPE-19 BIRC5 KO cells were pre-treated for 4 hours with the appropriate concentrations of bioactive compound, prior to exposing the cells to SI for 18 hours. Following the 22 hours or pre-treatment and exposure, an MTS assay was performed to quantify cell viability. Results from the MTS assay with lutein, zeaxanthin and beta-carotene can be found in Table 4.4 – ARPE-19 BKO - MTS Assay – Compound and SI as well as in each subsequent figure with the full results of the experiments (referenced in the table).

Table 4.4 – ARPE-19 BKO - MTS Assay – Compound and SI.
Summary of results from the MTS assay using ARPE-19 BIRC5 KO cells pre-treated with bioactive compounds then exposed to 15 and 17.5 mM of SI. Significance from the appropriate control is shown with respect to each concentration.

SI Concentration (mM)	Compound Name	Concentrations Tested (μM)	Figure
15	**Lutein**	10^{c}, 25^{d}	Figure BKO Lutein
17.5		10^{d}, 25^{d}	
15	**Zeaxanthin**	2.5, 10^{b}	Figure BKO Zeaxanthin
17.5		2.5, 10^{d}	
15	**Beta-Carotene**	5, 20	Figure BKO Beta-carotene
17.5		5^{d}, 20^{b}	

(^{a} indicates $p<0.05$, ^{b} indicates $p<0.01$, ^{c} indicates $p<0.001$, ^{d} indicates $p<0.0001$.)

Lutein (10 and 25 μM) treatments of ARPE-19 BIRC5 KO cells caused a significant ($p<0.001$) reduction in cell viability compared to untreated KO cells. When exposed to 15 and 17.5 mM of SI alone, ARPE-19 BIRC5 KO cells saw significant ($p<0.05$) reduction in cell viability when compared to the control cells. Pre-treatment with 10 and 25 μM of lutein prior to exposure to SI resulted in a significant protective maintenance ($p<0.01$) in cellular viability with both 15 and 17.5 mM SI concentrations. When treated with 10 μM of lutein and exposed to SI, cell viability was protectively maintained by 14% at 15 mM and 28% at 17.5 mM when compared to SI exposure alone. When pre-treated with 25 μM of lutein and exposed to SI, cell viability was protectively maintained by 33% at 15 mM and 46% at 17.5 mM when compared to SI exposure alone (Figure 4.22).

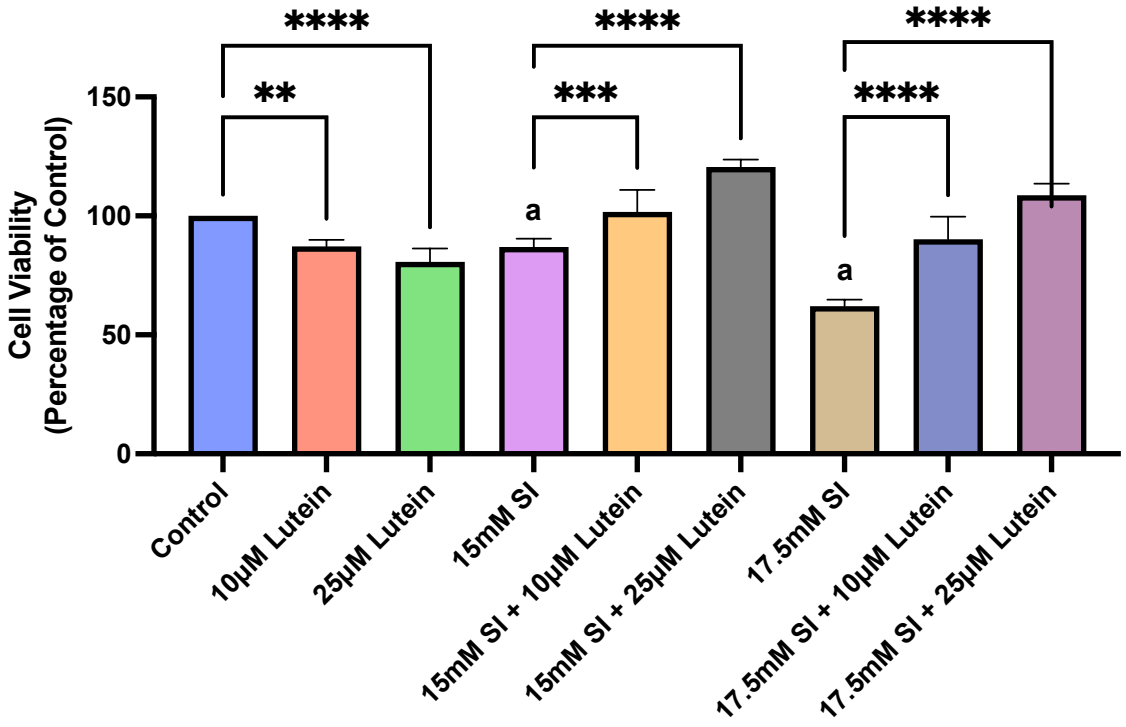

Figure 4.22 - Effect of lutein on ARPE-19 BIRC5 KO cell viability using MTS assay. ARPE-19 BIRC5 KO cells were pre-treated with lutein in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing MTS assay as a measure of cellular viability. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$, **** indicates $p<0.0001$.)

Zeaxanthin (10 μM) treatment of ARPE-19 BIRC5 KO cells caused a significant ($p<0.01$) increase in cell viability compared to untreated control cells. When exposed to 15 and 17.5 mM of SI alone, ARPE-19 BIRC5 KO cells showed a significant ($p<0.05$) reduction in cell viability when compared to the control cells. Pre-treatment with 2.5 μM of zeaxanthin prior to exposure to SI caused a non-significant change in cellular viability. When pre-treated with 2.5 μM of zeaxanthin and exposed to SI, cell viability was maintained by 6% at 15 mM and 12% at 17. 5 mM when compared to SI exposure alone. When treated with 10 μM of zeaxanthin and exposed to SI, cell viability was significantly decreased ($p<0.01$) by 20% at 15 mM and 30% at 17.5 mM when compared to SI exposure alone (Figure 4.23).

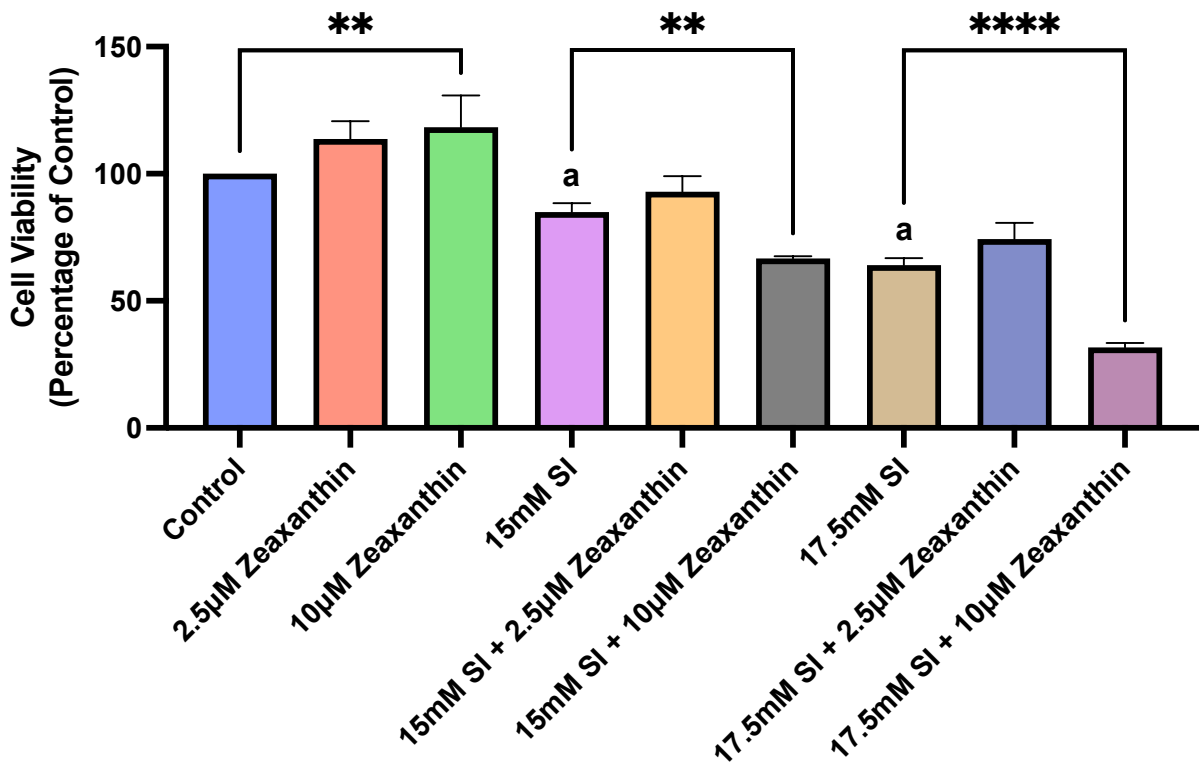

Figure 4.23 - Effect of zeaxanthin on ARPE-19 BIRC5 KO cell viability using MTS assay.

ARPE-19 BIRC5 KO cells were pre-treated with Zeaxanthin in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing MTS assay as a measure of cellular viability. Data presented as mean ± SEM of n=4 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, ** indicates $p<0.01$, **** indicates $p<0.0001$.)

Beta-carotene (5 and 20 μM) treatments to ARPE-19 BIRC5 KO cells caused a significant ($p<0.0001$) increase in cell viability compared to untreated cells. When exposed to 15 and 17.5 mM of SI alone, ARPE-19 BIRC5 KO cells saw significant ($p<0.05$) reduction in cell viability when compared to the control cells. Pre-treatment with 5 μM of beta-carotene prior to exposure to SI was found to cause an increase in ability to maintain cellular viability at 15 mM but a significant ($p<0.01$) maintenance of cell viability at 17.5 mM of SI exposure when compared to SI exposed cells alone. When pre-treated with 5 μM of beta-carotene and exposed to SI, cell viability was maintained by 22% ($p<0.0001$) at 17.5 mM when compared to SI exposed cells. When treated with 20 μM of beta-carotene and exposed to SI, cell viability was maintained by 6% at 15 mM and 13% ($p<0.01$) at 17.5 mM when compared to SI exposed alone cells (Figure 4.24).

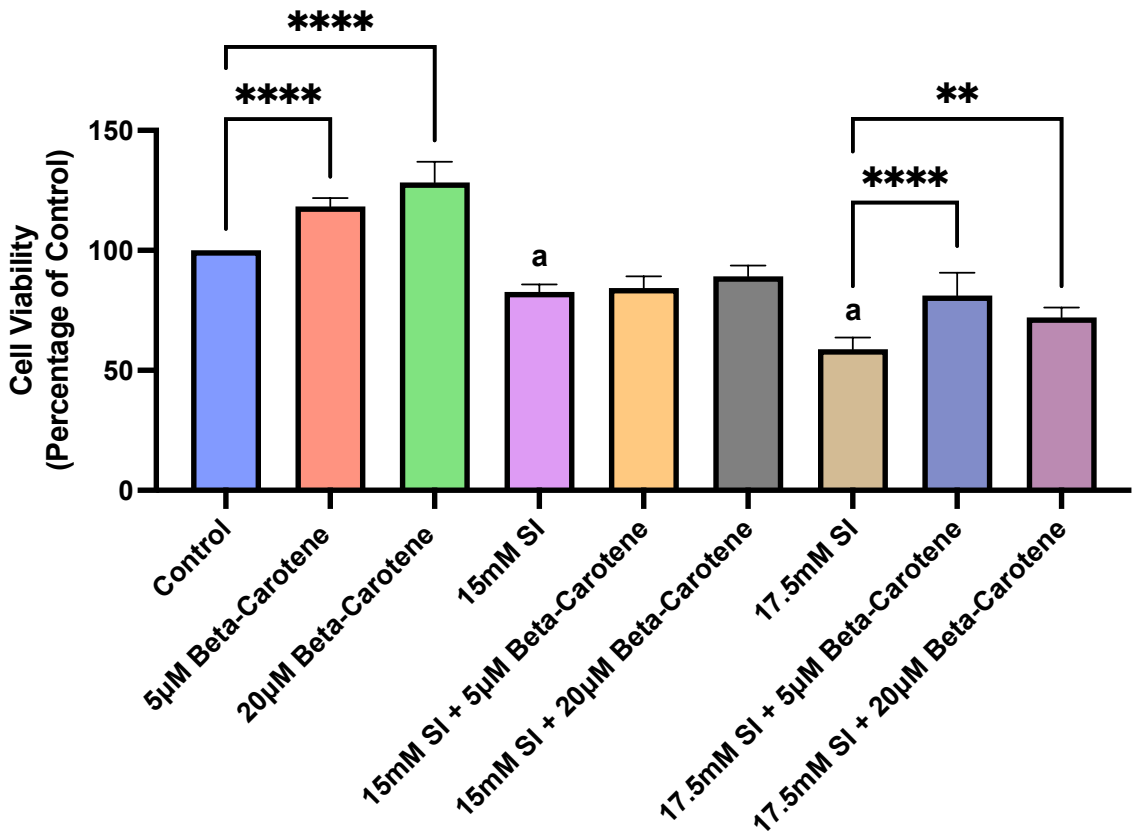

Figure 4.24 - Effect of beta-carotene on ARPE-19 BIRC5 KO cell viability using MTS assay.

ARPE-19 BIRC5 KO cells were pre-treated with beta-carotene in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing MTS assay as a measure of cellular viability. Data presented as mean ± SEM of n=4 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, ** indicates $p<0.01$, **** indicates $p<0.0001$.)

4.5.2 ARPE-19 BIRC5 KO MTS Assay – Summary

From the results of the MTS assay, it can be concluded that lutein, zeaxanthin and beta-carotene all have protective effects in terms of maintaining cell viability in BIRC5 KO ARPE-19 cells. The most significant finding is that the protective effect of zeaxanthin and beta-carotene is hindered by the KO when comparing results to the WT cells. This indicates there is possible linkages to the BIRC5 pathway and antioxidant intervention with SI damages. Lutein pre-treatment on the BIRC5 KO cells illustrated no significant changes from the WT cells and major trends remained consistent.

4.5.3 ARPE-19 SIRT-1 KO MTS Assay

To investigate the mechanistic pathway in which our test compounds of lutein, zeaxanthin, and beta-carotene function, KO cell lines of ARPE-19 cells were created and underwent matching experiments to the ARPE-19 WT cell lines. An MTS assay was used as a quantifiable measurement of cellular viability. For each experiment, untreated KO control cells were used as a reference and the results were expressed as a percentage of control. ARPE-19 SIRT-1 KO cells were pre-treated for 4 hours with the appropriate concentrations of bioactive compound, prior to exposing the cells to SI for 18 hours. Following the 22 hours or pre-treatment and exposure, the MTS assay was performed to quantify cell viability. Results from the MTS assay with lutein, zeaxanthin and beta-carotene can be found in Table 4.5 – ARPE-19 SKO - MTS Assay – Compound and SI as well as in each subsequent figure with the full results of the experiments (referenced in the table).

Table 4.5 – ARPE-19 SKO - MTS Assay – Compound and SI.
Summary of results from the MTS assay using ARPE-19 SIRT-1 KO cells pre-treated with bioactive compounds then exposed to 15 and 17.5 mM of SI. Significance from the appropriate control is shown with respect to each concentration.

SI Concentration (mM)	Compound Name	Concentrations Tested (μM)	Figure
15	**Lutein**	10[a], 25[d]	Figure 4.25
17.5		10[a], 25[d]	
15	**Zeaxanthin**	2.5[c], 10[d]	Figure 4.26
17.5		2.5[d], 10[d]	
15	**Beta-Carotene**	5, 20	Figure 4.26
17.5		5[b], 20	

([a] indicates $p<0.05$, [b] indicates $p<0.01$, [c] indicates $p<0.001$, [d] indicates $p<0.0001$.)

Lutein (10 μM) treatment on ARPE-19 SIRT-1 KO cells caused a significant ($p<0.05$) reduction in cell viability compared to untreated cells. Exposure to 15 and 17.5 mM resulted in significant ($p<0.05$) reductions in cell viability when compared to the untreated control cells. Cell viability was significantly ($p<0.05$) maintained when treated with 10 μM of lutein and exposed to both concentrations of SI when compared to SI exposed cells alone. When pre-treated with 25 μM of lutein and exposed to SI, cell viability was maintained by 30% at 15 mM and 35% at 17.5 mM (Figure 4.24).

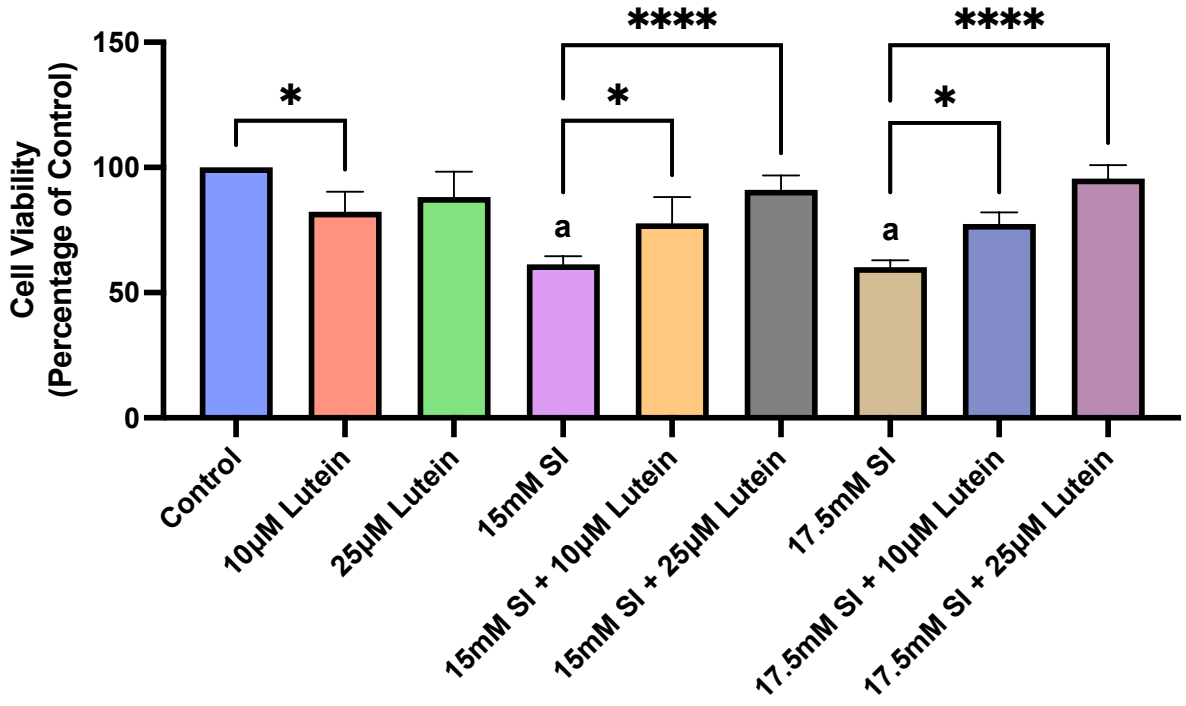

Figure 4.24 - Effect of lutein on ARPE-19 SIRT-1 KO cell viability using MTS assay. ARPE-19 SIRT-1 KO cells were pre-treated with lutein in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing MTS assay as a measure of cellular viability. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, **** indicates $p<0.0001$.)

Zeaxanthin (2.5 and 10 μM) treatments on ARPE-19 SIRT-1 KO cells caused a significant ($p<0.01$) increase in cell viability when compared to untreated cells. Exposure to 15 and 17.5 mM resulted in significant ($p<0.05$) reductions in cell viability when compared to the untreated control cells. Pre-treatments with 2.5 and 10 μM concentrations of zeaxanthin in the presence of SI was found to cause significant ($p<0.0001$) protective maintenance of cellular viability in ARPE-19 SIRT-1 KO cells when compared to SI exposed cells alone. When pre-treated with 2.5 μM of zeaxanthin and exposed to both concentrations of SI, cell viability was significantly maintained ($p<0.001$). When treated with 10 μM of zeaxanthin and exposed to SI, cell viability was maintained by 47% at 15 mM and 42% at 17.5 mM when compared to SI exposed alone cells (Figure 4.25).

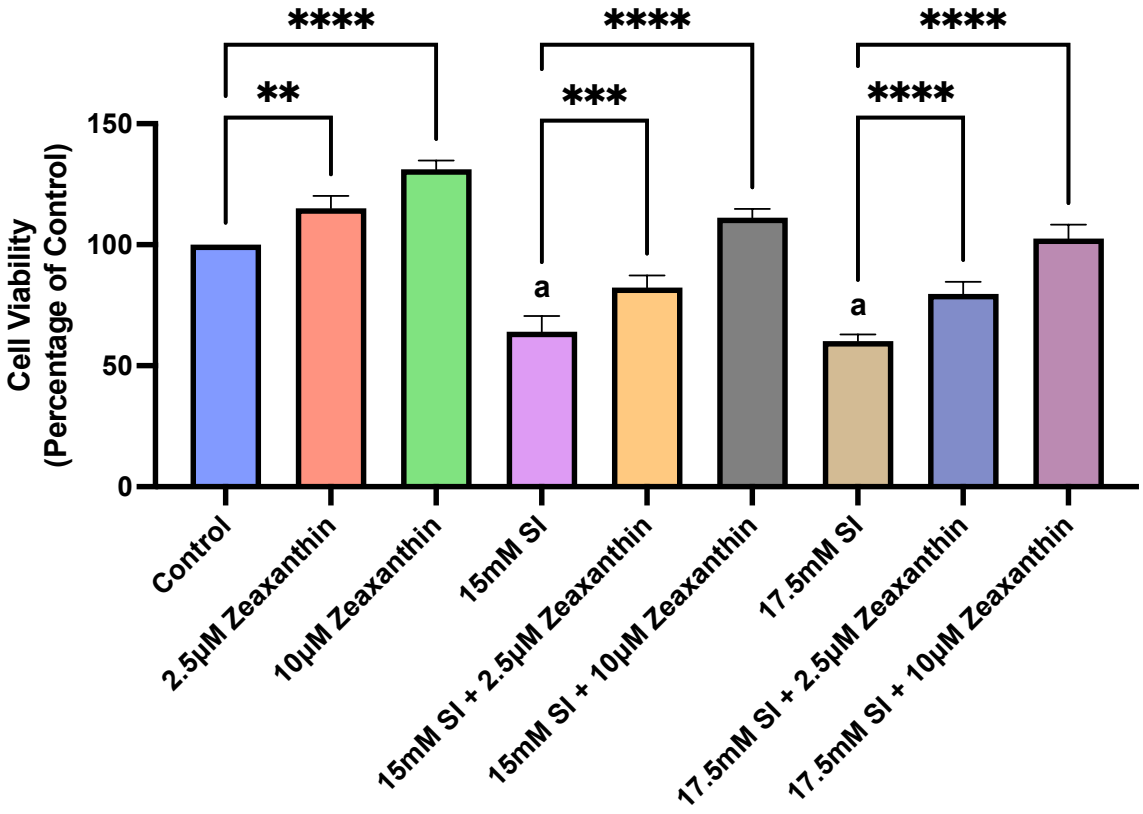

Figure 4.25 - Effect of zeaxanthin on ARPE-19 SIRT-1 KO cell viability using MTS assay.

ARPE-19 SIRT-1 KO cells were pre-treated with Zeaxanthin in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing MTS assay as a measure of cellular viability. Data presented as mean ± SEM of n=4 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$.)

Beta-carotene (2.5 and 10 μM) treatments on ARPE-19 SIRT-1 KO cells caused a significant ($p<0.01$) increase in cell viability when compared to untreated cells. Exposure to 15 and 17.5 mM resulted in significant ($p<0.05$) reductions in cell viability when compared to the untreated control cells. Pre-treatment with 5 μM concentrations of beta-carotene was found to cause no significant change in cellular viability at 15 mM of SI but a significant ($p<0.01$) increase in protective maintenance in cell viability at 17.5 mM of SI exposure. When pre-treated with 5 μM of beta-carotene and exposed to SI, cell viability was maintained by 6% at 15 mM and 29% ($p<0.05$) at 17.5 mM when compared to cells exposed to SI alone. When pre-treated with 20 μM of beta-carotene and exposed to SI, cell viability was maintained by 12% at 15 mM and 18% at 17.5 mM when compared to cells exposed to SI alone (Figure 4.26).

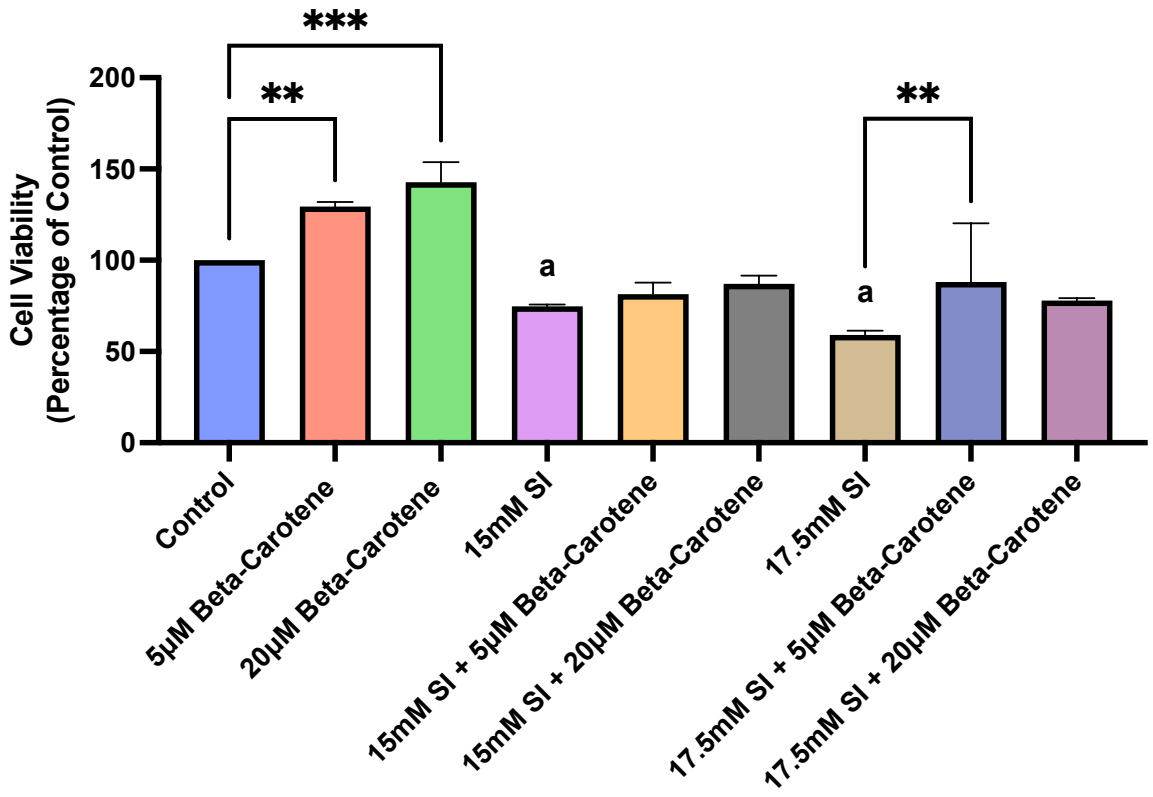

Figure 4.26 - Effect of beta-carotene on ARPE-19 SIRT-1 KO cell viability using MTS assay.

ARPE-19 SIRT-1 KO cells were pre-treated with beta-carotene in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing MTS assay as a measure of cellular viability. Data presented as mean ± SEM of n=4 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, ** indicates $p<0.01$, *** indicates $p<0.001$.)

4.5.4 ARPE-19 SIRT-1 KO MTS Assay – Summary

From the results of the MTS assays, lutein, zeaxanthin, and beta-carotene all exhibited protective effects on the SIRT-1 knockout model of ARPE-19 cells. Furthermore, no major deviation from the WT model suggests that protective effects of lutein, zeaxanthin, and beta-carotene are not solely modulated through the SIRT-1 pathway. More studies need to be conducted to investigate the complete mechanism of protection; however, the main trends and significance remains consistent with the wildtype results.

4.6 CMH2-DCFDHA Assay

CMH2-DCFDHA assay was used to assess oxidative stress within the WT and KO cells. Following incubation of the compounds for 4 hours, SI was added to the wells to result in a final concentration of 15 and 17.5 mM and allowed to incubate for 18 hours. Following the 22 hours total incubation time, the treatment media was removed, and CMH2-DCFDHA assay was performed. The mean fluorescence intensity (MFI) of each well was measured using a Biotek Cytation 5 multiplate reader and expressed as a percentage of untreated control.

4.6.1 ARPE-19 WT

4.6.1.1 ARPE-19 WT CMH2-DCFDHA Assay – SI & Lutein

Lutein (10 and 25 μM) caused a significant ($p<0.0001$) increase in intracellular oxidative stress when compared to untreated control cells. SI caused a significant ($p<0.05$) increase in oxidative stress by 125% at 15 mM and 185% and 17.5 mM. Pre-treatment with lutein illustrated a significant reduction ($p<0.001$) in intracellular ROS when compared to the SI only control cells. When pre-treated with 10 μM of lutein and exposed to SI, intracellular ROS was reduced by 18% at 15 mM and 70% at 17.5 mM. When pre-treated with 25 μM of lutein and exposed to SI, intracellular ROS was reduced by 49% at 15 mM and 110% at 17.5 mM (Figure 4.27).

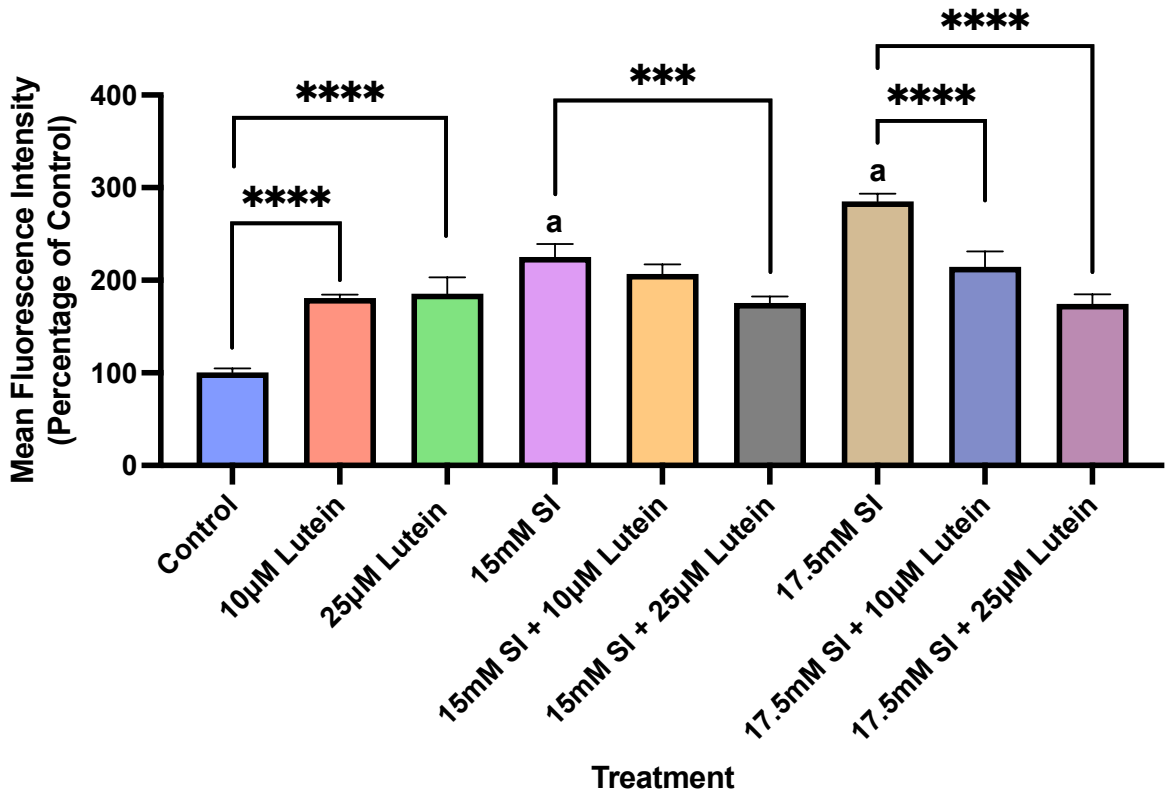

Figure 4.27 - Lutein and SI treatments on ARPE-19 WT cells using CMH2-DCFDHA Assay.

ARPE-19 WT cells were pre-treated with Lutein in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing the CMH2-DCFDHA assay to quantify reactive oxygen species. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, *** indicates $p<0.001$, **** indicates $p<0.0001$.)

4.6.1.2 ARPE-19 WT CMH2-DCFDHA Assay – SI & Zeaxanthin

Zeaxanthin (2.5 and 10 μM) caused a significant ($p<0.001$) increase in intracellular oxidative stress when compared to untreated control cells. Exposure to 15 and 17.5 mM of SI resulted in significant ($p<0.05$) increases in intracellular oxidative stress when compared to untreated control cells. SI resulted in an increase of oxidative stress by 151% at 15 mM and 172% and 17.5 mM. Pre-treatment with zeaxanthin showed a significant reduction ($p<0.001$) in intracellular ROS when compared to the non-treated SI controls. When pre-treated with zeaxanthin at 2.5 μM and exposed to SI, intracellular ROS was reduced by 78% at 15 mM and 89% at 17.5 mM. When pre-treated with 10 μM of zeaxanthin and exposed to SI, intracellular ROS was decreased by 91% at 15 mM and 88% at 17.5 mM (Figure 4.28).

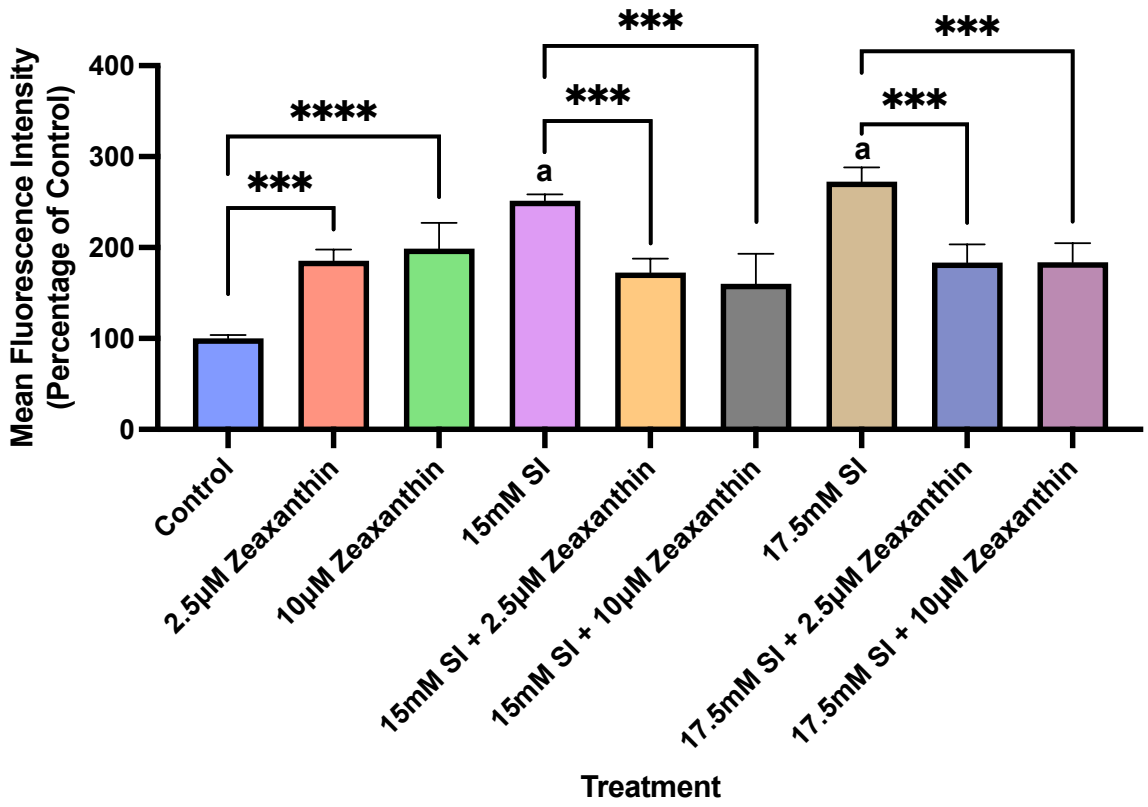

Figure 4.28 - Zeaxanthin and SI treatments on ARPE-19 WT cells using CMH2-DCFDHA Assay.

ARPE-19 WT cells were pre-treated with zeaxanthin in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing the CMH2-DCFDHA assay to quantify reactive oxygen species. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, *** indicates $p<0.001$, **** indicates $p<0.0001$.)

4.6.1.3 ARPE-19 WT CMH2-DCFDHA Assay – SI & Beta-carotene

Beta-carotene (5 and 20 μM) caused a significant ($p<0.05$) increase in intracellular oxidative stress when compared to untreated control cells. Exposure to 15 and 17.5 mM of SI resulted in significant ($p<0.05$) increases in intracellular oxidative stress when compared to untreated control cells. SI caused an increase of oxidative stress by 115% at 15 mM and 163% and 17.5 mM. Pre-treatment with beta-carotene illustrated a significant reduction ($p<0.05$) in intracellular ROS when compared to the non-treated SI controls at 17.5 mM of SI and 20 μM of beta-carotene. When pre-treated with beta-carotene at 5 μM and exposed to SI, intracellular ROS was reduced by 6% at 15 mM and 34% at 17.5 mM. When pre-treated with 20 μM of beta-carotene and exposed to SI, intracellular ROS was decreased by 13% at 15 mM and 73% ($p<0.01$) at 17.5 mM (Figure 4.29).

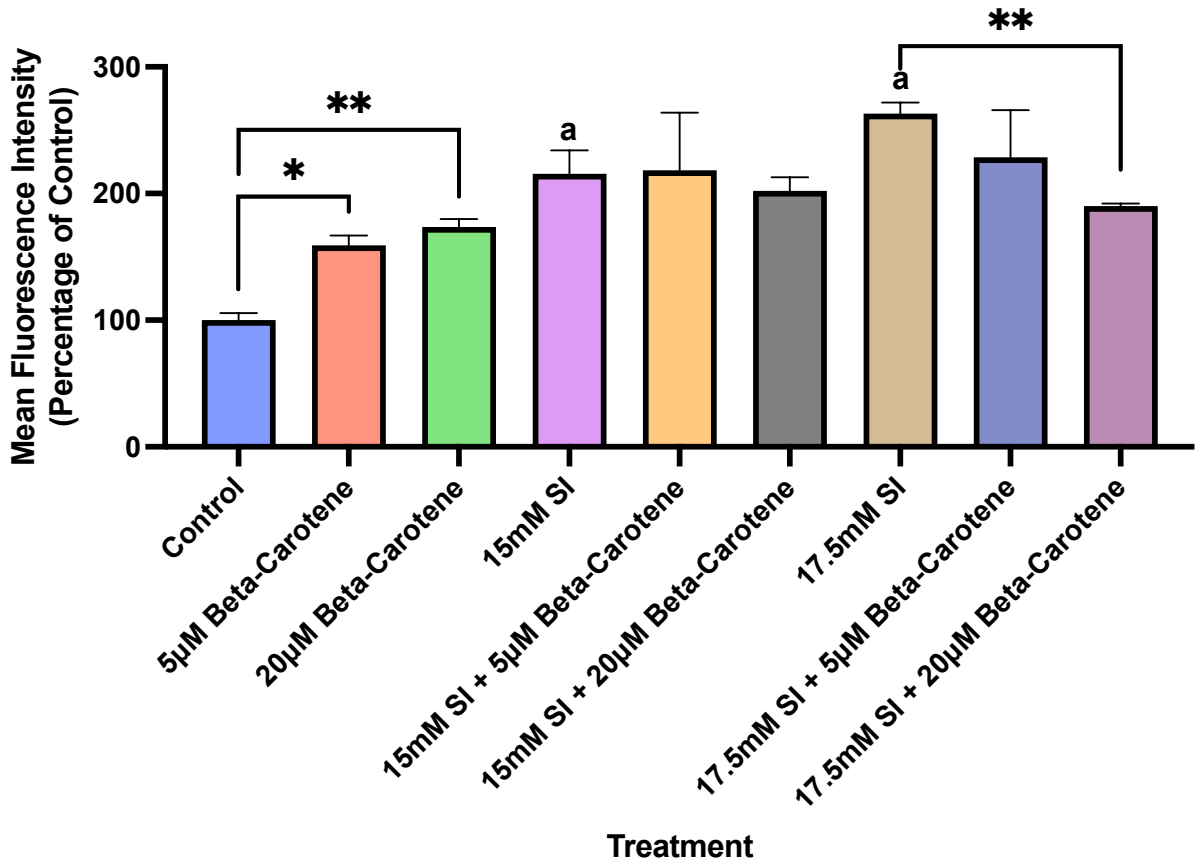

Figure 4.29 - Beta-Carotene and SI treatments on ARPE-19 WT cells using CMH2-DCFDHA Assay.

ARPE-19 WT cells were pre-treated with beta-carotene in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing the CMH2-DCFDHA assay to quantify reactive oxygen species. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$.)

4.6.2 ARPE-19 BIRC5 KO

4.6.2.1 ARPE-19 BIRC5 KO CMH2-DCFDHA Assay – SI & Lutein

Lutein (10 and 25 μM) caused a significant ($p<0.05$) increase in intracellular oxidative stress when compared to untreated control cells. SI caused an increase of oxidative stress by 45% at 15 mM and 40% and 17.5 mM as compared to untreated control cells. Pre-treatment with lutein illustrated a significant reduction ($p<0.01$) in intracellular ROS when compared to the non-treated SI controls. When pre-treated with 10 μM of lutein exposed to SI, intracellular ROS was reduced by 32% at 15 mM and 28% at 17.5 mM. When pre-treated with 25 μM of lutein and exposed to SI, intracellular ROS was reduced by 33% at 15 mM and 29% at 17.5 mM (Figure 4.30).

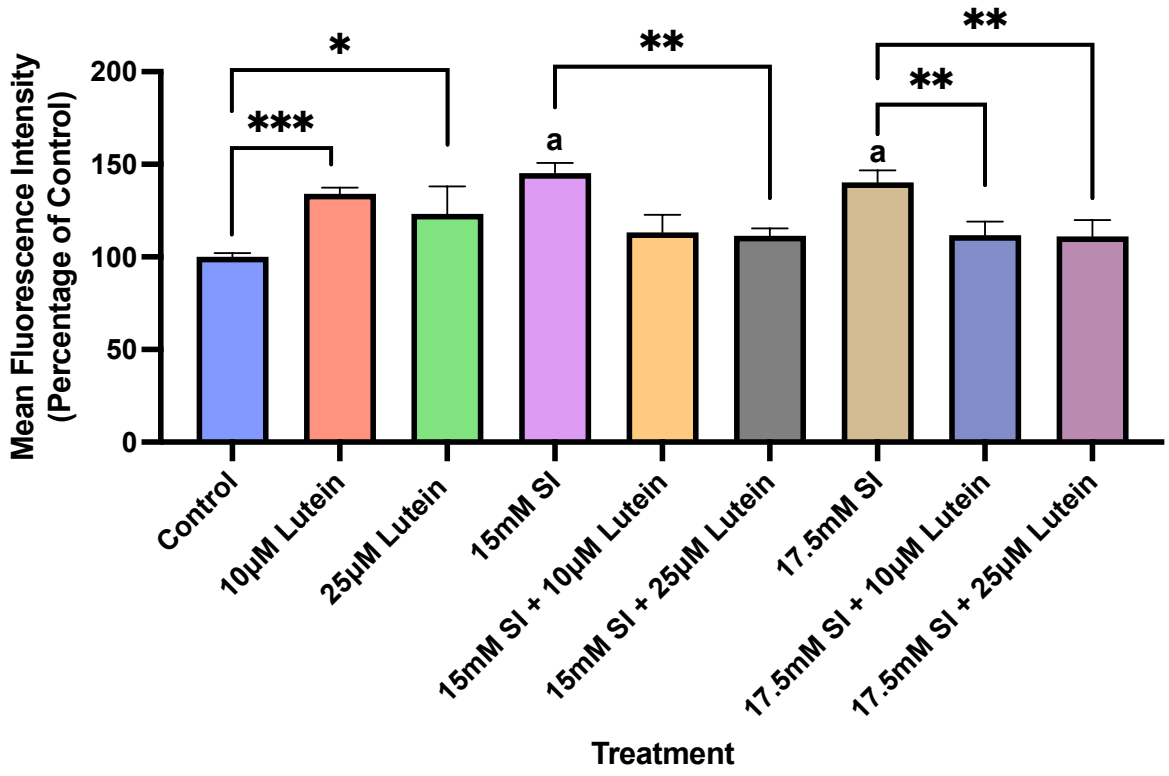

Figure 4.30 - Lutein and SI treatments on ARPE-19 BIRC5 KO cells using CMH2-DCFDHA Assay.

ARPE-19 BIRC5 KO cells were pre-treated with Lutein in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing the CMH2-DCFDHA assay to quantify reactive oxygen species. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $P<0.001$.)

4.6.2.2 ARPE-19 BIRC5 KO CMH2-DCFDHA Assay – SI & Zeaxanthin

Zeaxanthin (2.5 and 10 μM) caused a significant ($p<0.001$) increase in intracellular oxidative stress when compared to untreated control cells. SI caused an increase of oxidative stress by 151% at 15 mM and 191% and 17.5 mM when compared to untreated control cells. Pre-treatment with zeaxanthin illustrated a significant increase ($p<0.0001$) in intracellular ROS when compared to the non-treated SI controls. When pre-treated with zeaxanthin at 2.5 μM and exposed to SI, intracellular ROS was increased by 68% at 15 mM and 86% at 17.5 mM. When pre-treated with 10 μM of zeaxanthin and exposed to SI, intracellular ROS was increased by 66% at 15 mM and 110% at 17.5 mM (Figure 4.31).

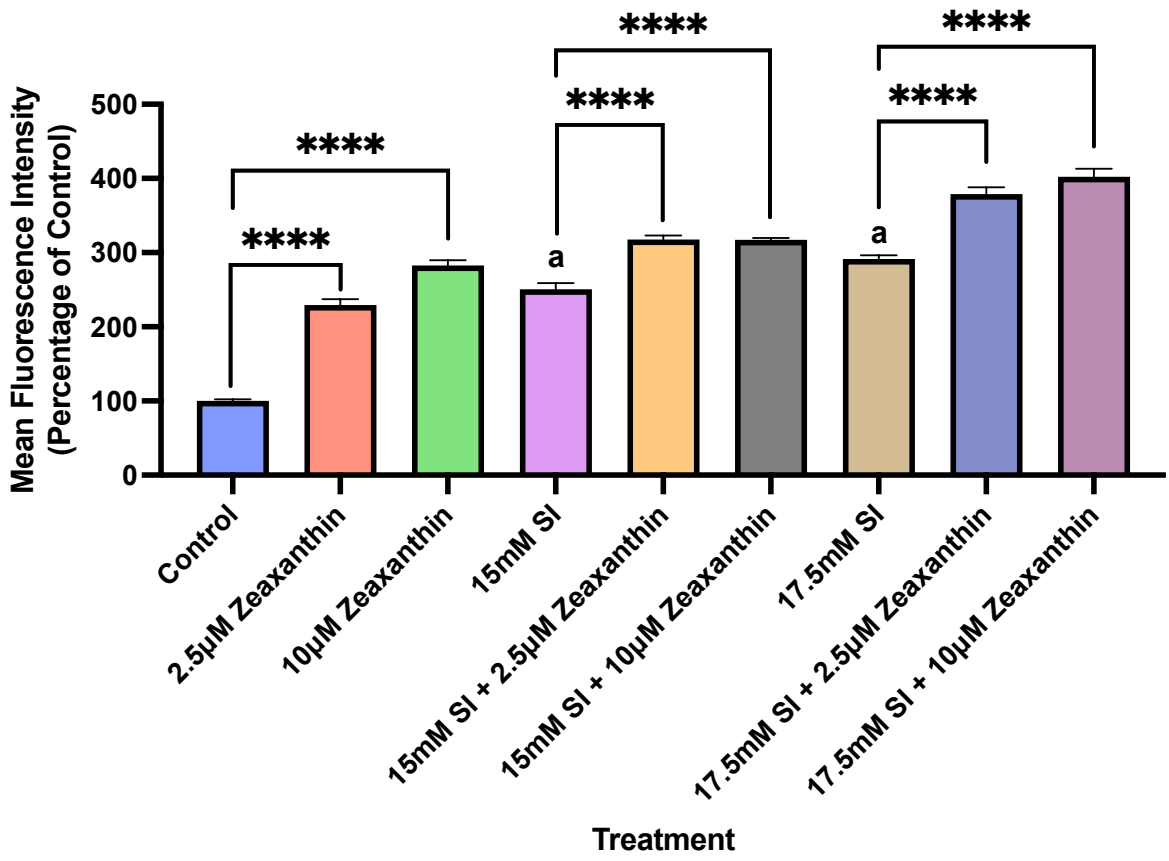

Figure 4.31 - Zeaxanthin and SI treatments on ARPE-19 BIRC5 KO cells using CMH2-DCFDHA Assay.

ARPE-19 BIRC5 KO cells were pre-treated with zeaxanthin in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing the CMH2-DCFDHA assay to quantify reactive oxygen species. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, **** indicates $p<0.0001$.)

4.6.2.3 ARPE-19 BIRC5 KO CMH2-DCFDHA Assay – SI & Beta-carotene

Beta-carotene (5 and 20 μM) caused a significant ($p<0.0001$) increase in intracellular oxidative stress when compared to untreated control cells. SI caused an increase of oxidative stress by 133% at 15 mM and 166% and 17.5 mM when compared to untreated control cells. Pre-treatment with beta-carotene illustrated a significant reduction ($p<0.001$) in intracellular ROS when compared to the non-treated SI controls. When pre-treated with beta-carotene at 5 μM and exposed to SI, intracellular ROS was reduced by 36% at 15 mM and 37% at 17.5 mM ($p<0.05$). When pre-treated with 20 μM of beta-carotene and exposed to SI, intracellular ROS was decreased by 16% at 15 mM and 54% at 17.5 mM ($p<0.0001$) (Figure 4.32).

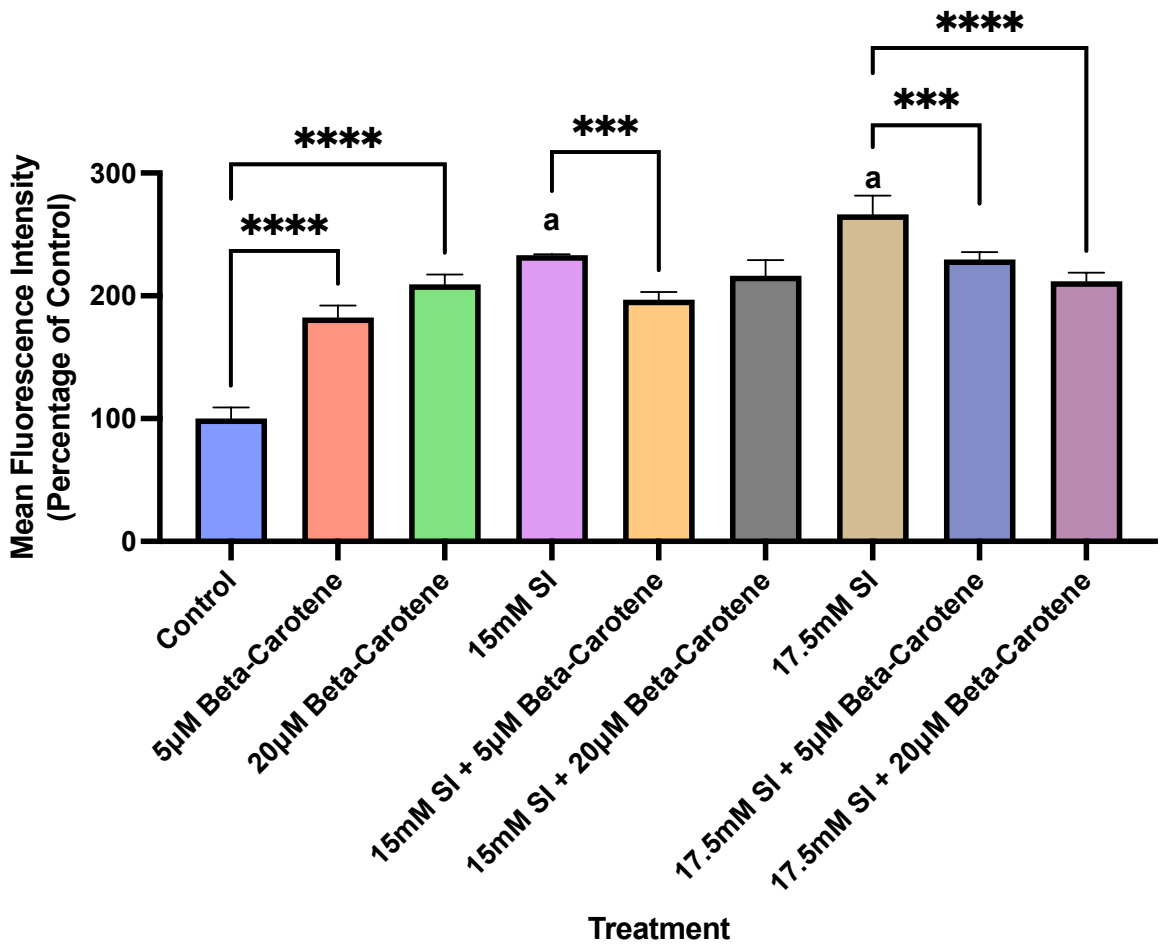

Figure 4.32 - Beta-Carotene and SI treatments on ARPE-19 BIRC5 KO cells using CMH2-DCFDHA Assay.

ARPE-19 BIRC5 KO cells were pre-treated with beta-carotene in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing the CMH2-DCFDHA assay to quantify reactive oxygen species. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, *** indicates $p<0.001$, **** indicates $p<0.0001$.)

4.6.3 ARPE-19 SIRT-1 KO

4.6.3.1 ARPE-19 SIRT-1 KO CMH2-DCFDHA Assay – SI & Lutein

Lutein (10 and 25 μM) caused a significant ($p<0.0001$) increase in intracellular oxidative stress when compared to untreated control cells. SI caused an increase of oxidative stress by 122% at 15 mM and 157% and 17.5 mM. Results from the pre-treatment with lutein illustrated a significant reduction ($p<0.05$) in intracellular ROS when compared to the non-treated SI controls. When pre-treated with 10 μM of lutein and exposed SI, intracellular ROS was reduced by 32% at 17.5 mM. When pre-treated with 25 μM of lutein and exposed to SI, intracellular ROS was reduced by 38% at 15 mM and 74% at 17.5 mM (Figure 4.33).

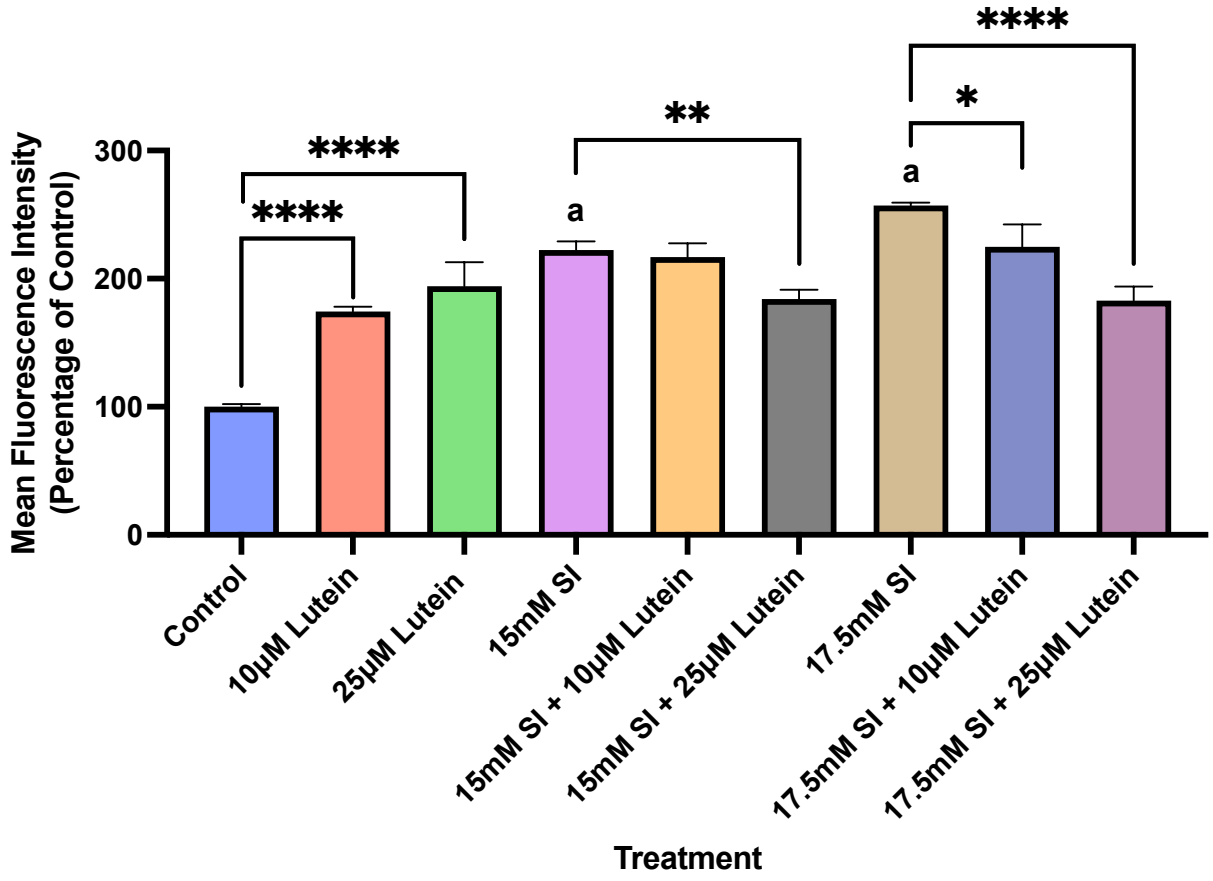

Figure 4.33 - Lutein and SI treatments on ARPE-19 SIRT-1 KO cells using CMH2-DCFDHA Assay.

ARPE-19 SIRT-1 KO cells were pre-treated with Lutein in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing the CMH2-DCFDHA assay to quantify reactive oxygen species. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$, **** indicates $p<0.0001$.)

4.6.3.2 ARPE-19 SIRT-1 KO CMH2-DCFDHA Assay – SI & Zeaxanthin

Zeaxanthin (2.5 and 10 μM) caused a significant ($p<0.0001$) increase in intracellular oxidative stress when compared to untreated control cells. Exposure to 15 and 17.5 mM of SI resulted in significant ($p<0.05$) increases in intracellular oxidative stress when compared to untreated control cells. SI caused an increase of oxidative stress by 156% at 15 mM and 191% and 17.5 mM. Pre-treatment with zeaxanthin illustrated a significant reduction ($p<0.0001$) in intracellular ROS when compared to the non-treated SI controls at both concentrations of zeaxanthin with 17.5 mM of SI exposure (Figure 4.34).

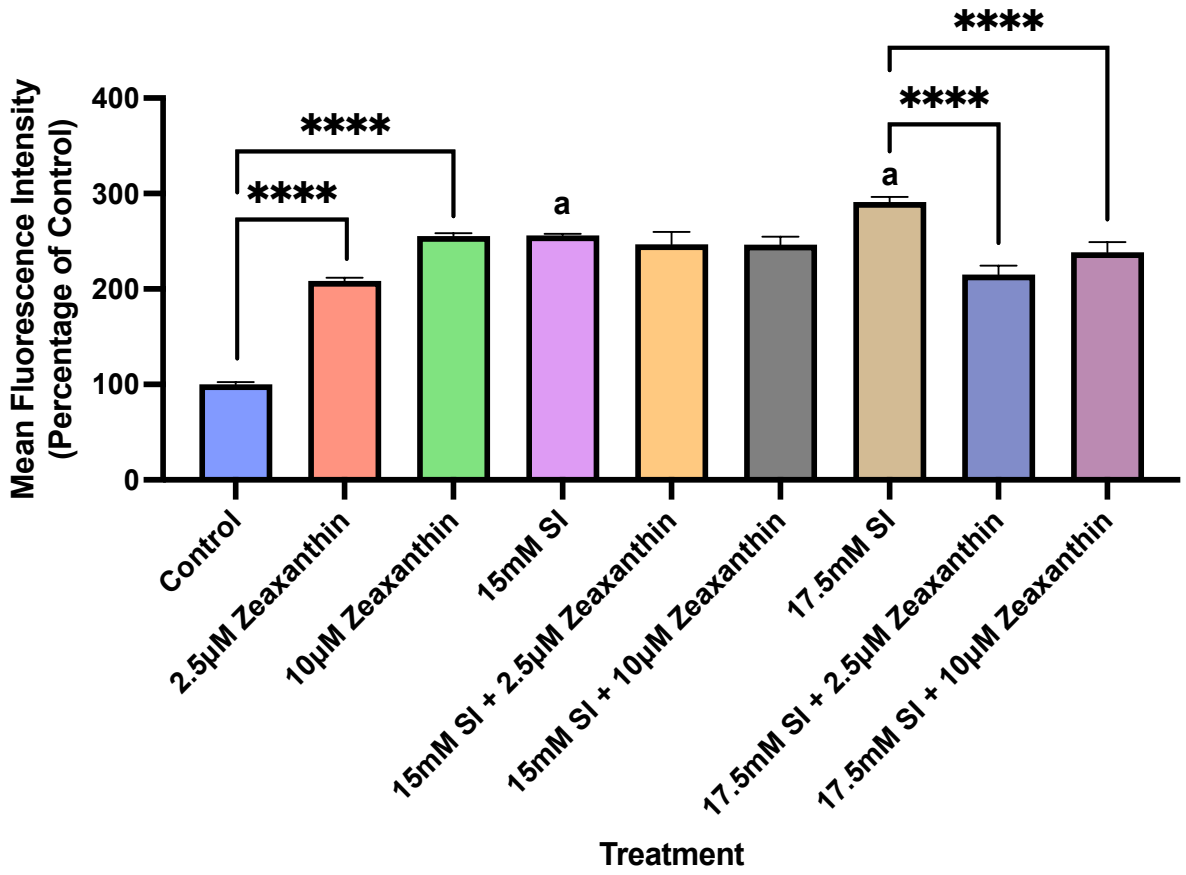

Figure 4.34 - Zeaxanthin and SI treatments on ARPE-19 SIRT-1 KO cells using CMH2-DCFDHA Assay.

ARPE-19 SIRT-1 KO cells were pre-treated with zeaxanthin in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing the CMH2-DCFDHA assay to quantify reactive oxygen species. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, **** indicates $p<0.0001$.)

4.6.3.3 ARPE-19 SIRT-1 KO CMH2-DCFDHA Assay – SI & Beta-carotene

Beta-carotene (5 and 20 μM) caused a significant ($p<0.0001$) increase in intracellular oxidative stress when compared to untreated control cells. Exposure of SI caused an increase of oxidative stress by 94% at 15 mM and 120% and 17.5 mM. Pre-treatment with beta-carotene illustrated a significant reduction ($p<0.05$) in intracellular ROS when compared to the non-treated SI controls at 17.5 mM of SI and 20 μM of beta-carotene. When pre-treated with beta-carotene at 5 μM and exposed to SI, intracellular ROS was reduced by 20% at 15 mM and 15% at 17.5 mM. When pre-treated with 20 μM of beta-carotene and exposed to SI, intracellular ROS was decreased by 21% at 15 mM and 41% at 17.5 mM (Figure 4.35).

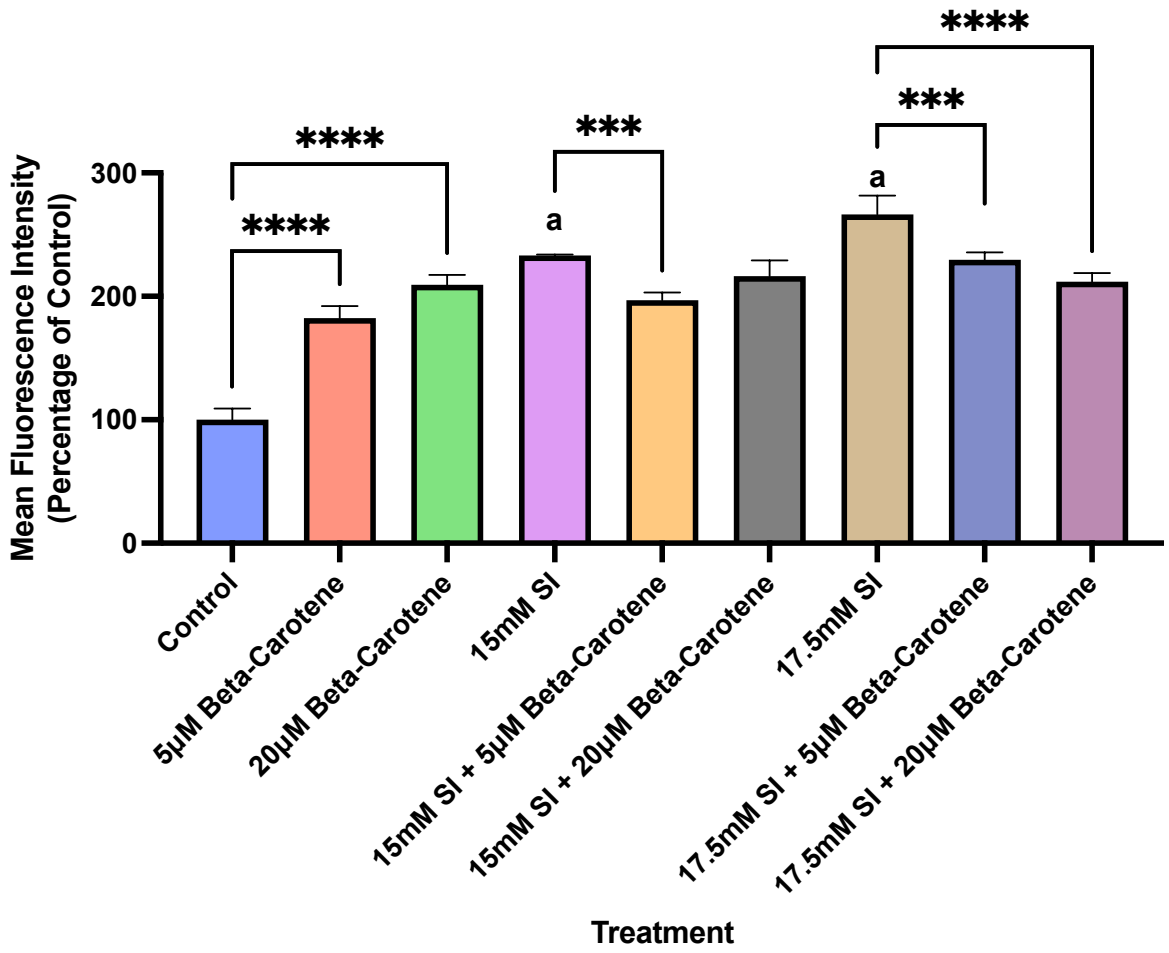

Figure 4.35 - Beta-Carotene and SI treatments on ARPE-19 SIRT-1 KO cells using CMH2-DCFDHA Assay.

ARPE-19 SIRT-1 KO cells were pre-treated with beta-carotene in supplemented media for 4 hours then exposed to SI for 18 hours prior to performing the CMH2-DCFDHA assay to quantify reactive oxygen species. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with post-hoc Tukey HSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$, **** indicates $p<0.0001$.)

4.6.4 CMH2-DCFDHA Summary

Pre-treatment with lutein, zeaxanthin, and beta-carotene resulted in significant reductions in intracellular ROS levels compared to untreated controls exposed to SI. Lutein and zeaxanthin showed significant decreases in ROS levels, with zeaxanthin exhibiting a stronger protective effect. Beta-carotene also led to a reduction in ROS levels, at the higher exposure to SI (17.5 mM) correlating with greater reductions of ROS. These results suggest the antioxidant potential of these compounds in alleviating oxidative stress in ARPE-19 WT cells.

4.7 Western Blot analysis

In order to investigate the protein expressions of MnSOD, catalase, and caspase-3, Western blot methodology was used. Western blot data is expressed as Western blot / loading control ratio, based on a loading control sample that remained consistent across all experiments. Western blot results are grouped first by cell line used (ARPE-19 WT, ARPE-19 BIRC5 KO, and ARPE-19 SIRT-1 KO), and by treatment condition and concentration (lutein, zeaxanthin, and beta-carotene).

4.7.1 ARPE-19 WT

4.7.1.1 ARPE-19 WT – Lutein

Protein expression of MnSOD, catalase, and caspase-3 were measured via Western blot following ARPE-19 WT pre-treatment with lutein (10 and 25 μM) for 4 hours followed by exposure to SI (15 and 17.5 mM) for 18 hours. MnSOD protein expression was significantly ($p<0.05$) reduced in hydrogen peroxide exposed cells. MnSOD protein expression remained unchanged in all the other conditions (Figure 4.36 A). H_2O_2 led to a significant ($p<0.0001$) decrease in catalase protein expression**.** SI exposure of 15 to 17.5 mM was shown to cause significant ($p<0.05$) reductions on catalase protein expression levels. Pre-treatment with 10 μM of lutein prior to 15 or 17.5 mM SI exposure had no significant change on catalase protein expression levels within ARPE-19 WT cells (Figure 4.36 B). SI exposure of 17.5 mM caused significant ($p<0.05$) reductions on caspase-3 protein expression levels when compared to control. Caspase 3 protein expression remained unchanged in all the other conditions (Figure 4.36 C).

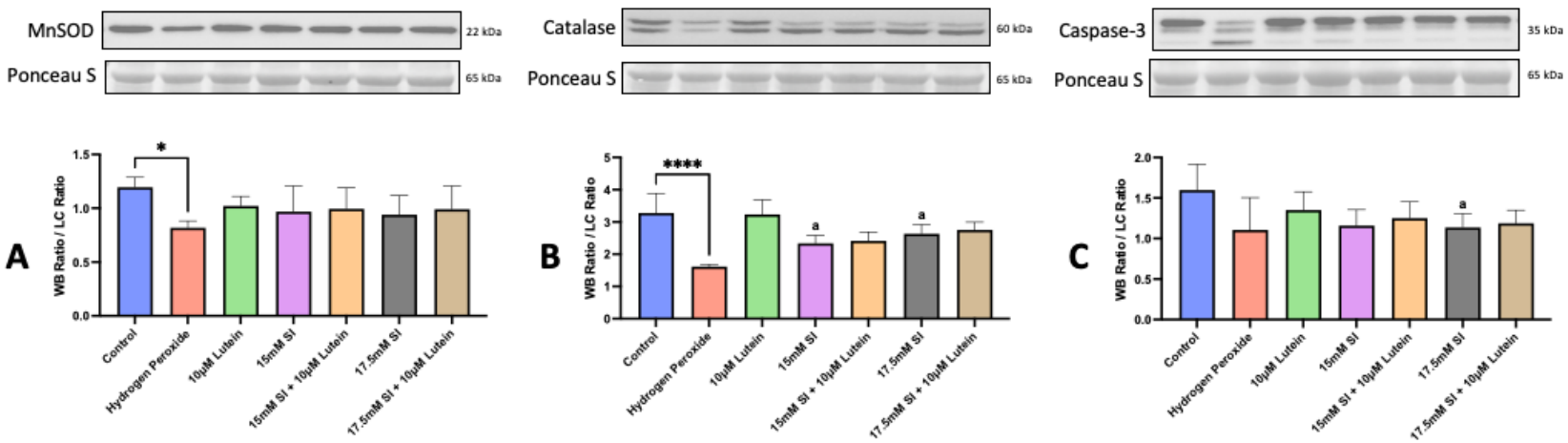

Figure 4.36 – Lutein 10 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 WT cells. Representative Western blots and quantified band densities of ARPE-19 WT cells pre-treated with lutein (10 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression. Data is expressed as Western blot / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, **** indicates $p<0.0001$.)

SI exposure of 15 to 17.5 mM caused significant ($p<0.05$) reduction in MnSOD protein expression levels on ARPE-19 WT cells. Pre-treatment with 25 μM of lutein prior to SI exposure had no significant change on MnSOD protein expression levels (Figure 4.37 A). SI exposure of 15 and 17.5 mM caused significant ($p<0.05$) increases of catalase protein expression levels. Pre-treatment with 25 μM of lutein prior to SI exposure had no significant change on catalase protein expression levels within ARPE-19 WT cells (Figure 4.37 B). A 25 μM treatment with lutein resulted in no significant change to caspase-3 protein expression levels. SI exposure of 15 or 17.5 mM caused no significant change to catalase protein expression levels when compared to control. Pre-treatment with 25 μM of lutein prior to SI exposure had no significant change on caspase-3 protein expression levels within ARPE-19 WT cells (Figure 4.37 C).

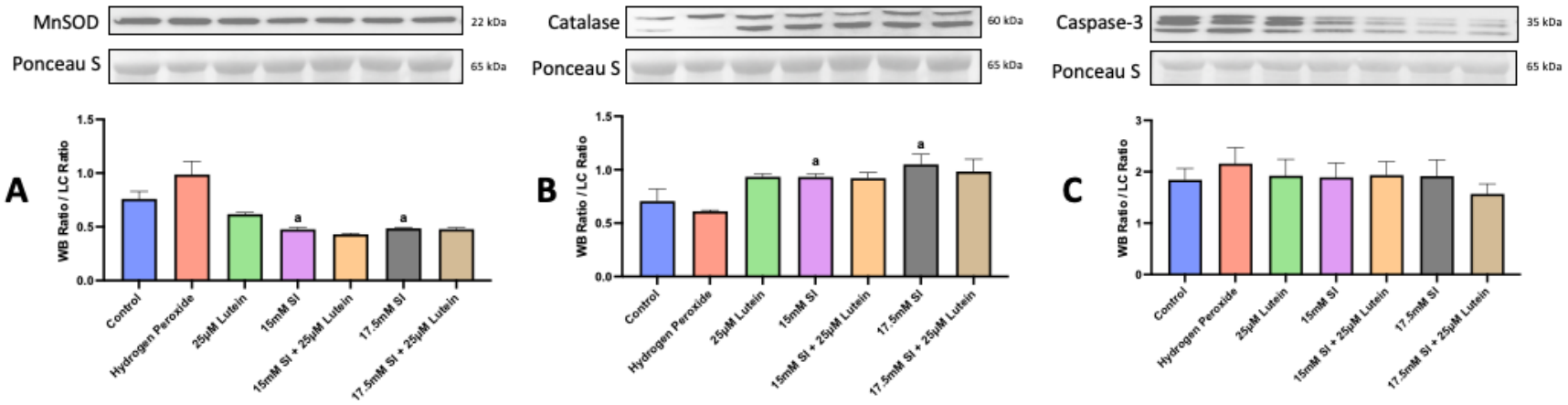

Figure 4.37 – Lutein 25 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 WT cells. Representative Western blots and quantified band densities of ARPE-19 WT cells pre-treated with lutein (25 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression. Data is expressed as Western blot / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control.)

4.7.1.2 ARPE-19 WT – Zeaxanthin

Results from the Western blots of MnSOD showed a significant decrease in protein expression levels when comparing exposure of 15 and 17.5 mM SI cells to untreated control cells. MnSOD protein expression was significantly ($p<0.0001$) reduced in hydrogen peroxide exposed cells. A significant increase ($p<0.01$) in MnSOD expression was found when pre-treated with 2.5 μM of zeaxanthin and exposed to 15 mM of SI and compared to non-pretreated controls. Conversely, a decrease in MnSOD level was detected when pre-treated with 2.5 μM of zeaxanthin then exposed to 17.5 mM of SI when compared to the non-pretreated controls (Figure 4.38 A). Catalase protein expression levels showed no significant change when comparing 15 and 17.5 mM SI exposure to control cells. Catalase levels were also unchanged when pretreated with 2.5 μM of zeaxanthin prior to exposing ARPE-19 WT cells to SI (Figure 4.38 B). Caspase-3 protein expression levels were significantly ($p<0.05$) reduced when exposed to both 15 and 17.5 mM of SI. Pre-treatment with 2.5 μM of zeaxanthin prior to exposure to SI indicated no significant change on caspase-3 protein expression level (Figure 4.38 C**).**

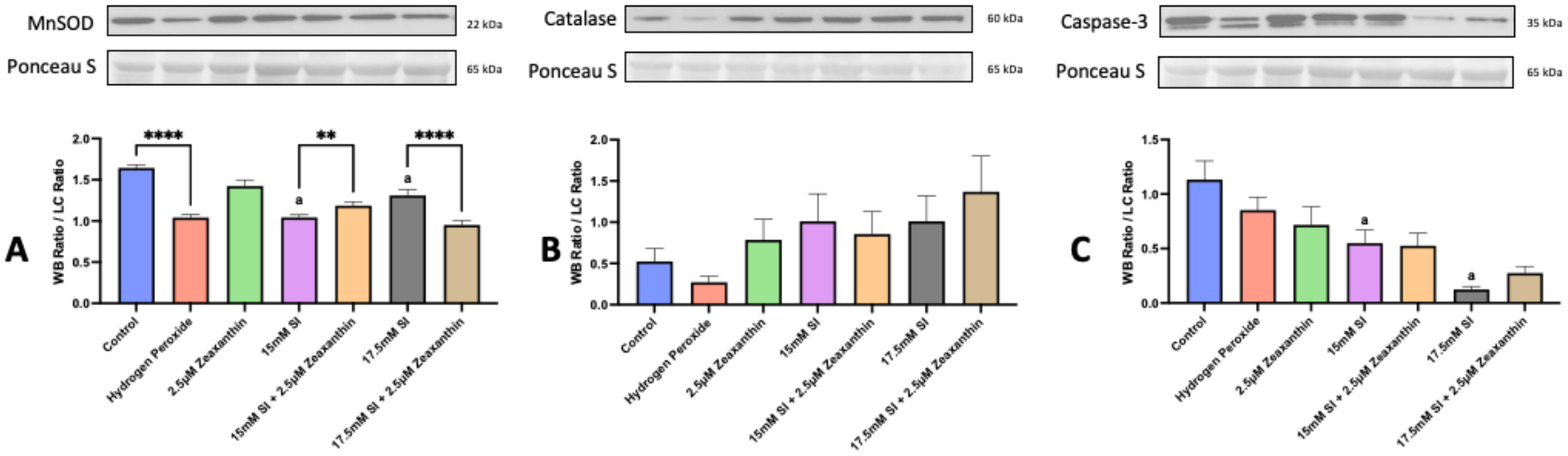

Figure 4.38 – Zeaxanthin 2.5 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 WT cells. Representative Western blots and quantified band densities of ARPE-19 WT cells pre-treated with zeaxanthin (2.5 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression. Data is expressed as Western blot / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, **** indicates $p<0.0001$.)

Western blot analysis of MnSOD protein expression levels with 15 and 17.5 mM of SI and hydrogen peroxide showed a significant ($p<0.05$) decrease in protein expression levels when compared to control. Pre-treatment with 10 μM of zeaxanthin prior to exposure to SI indicated no significant change on MnSOD protein expression level (Figure 4.39 A). Catalase protein expression levels showed no significant change when comparing 15 and 17.5 mM SI exposure to control ARPE-19 WT cells. Catalase levels were also unchanged when pretreated with 10 μM of zeaxanthin prior to exposure to 15 mM of SI. When pre-treated with 10 μM of zeaxanthin and exposed to 17.5 mM of SI a significant decrease ($p<0.001$) in catalase protein expression level was seen compared to the 17.5 mM SI exposure alone (Figure 4.39 B). Caspase-3 protein expression levels were significantly ($p<0.05$) reduced when exposed to 17.5 mM of SI. Pre-treatment with 10 μM of zeaxanthin prior to exposure to SI indicated no significant change on caspase-3 protein expression level (Figure 4.39 C).

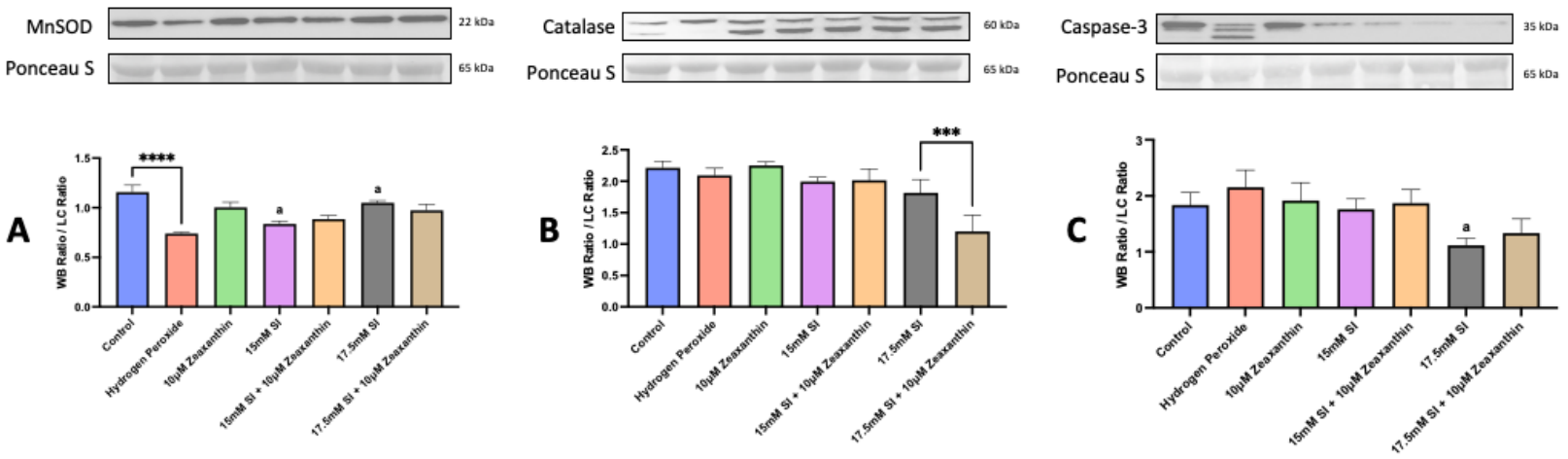

Figure 4.39 – Zeaxanthin 10 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 WT cells. Representative Western blots and quantified band densities of ARPE-19 WT cells pre-treated with zeaxanthin (10 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression. Data is expressed as Western blot / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, *** indicates $p<0.001$, **** indicates $p<0.0001$.)

4.7.1.3 ARPE-19 WT – Beta-carotene

Western blot analysis of MnSOD protein expression showed a significant ($p<0.05$) decrease in protein expression levels in the 15 mM SI treated cells when compared to control. MnSOD protein expression remained unchanged in all the other conditions (Figure 4.40 A). Catalase protein expression levels showed significant ($p<0.05$) reduction when comparing 15 and 17.5 mM SI exposure cells to control cells. Catalase levels were significantly ($p<0.05$) reduced when pretreated with 5 μM of beta-carotene prior to exposing ARPE-19 WT cells to 15 mM of SI (Figure 4.40 B). Caspase-3 protein expression levels were significantly ($p<0.05$) reduced when exposed to 15 and 17.5 mM of SI. Caspase 3 protein expression remained unchanged in all the other conditions (Figure 4.40 C).

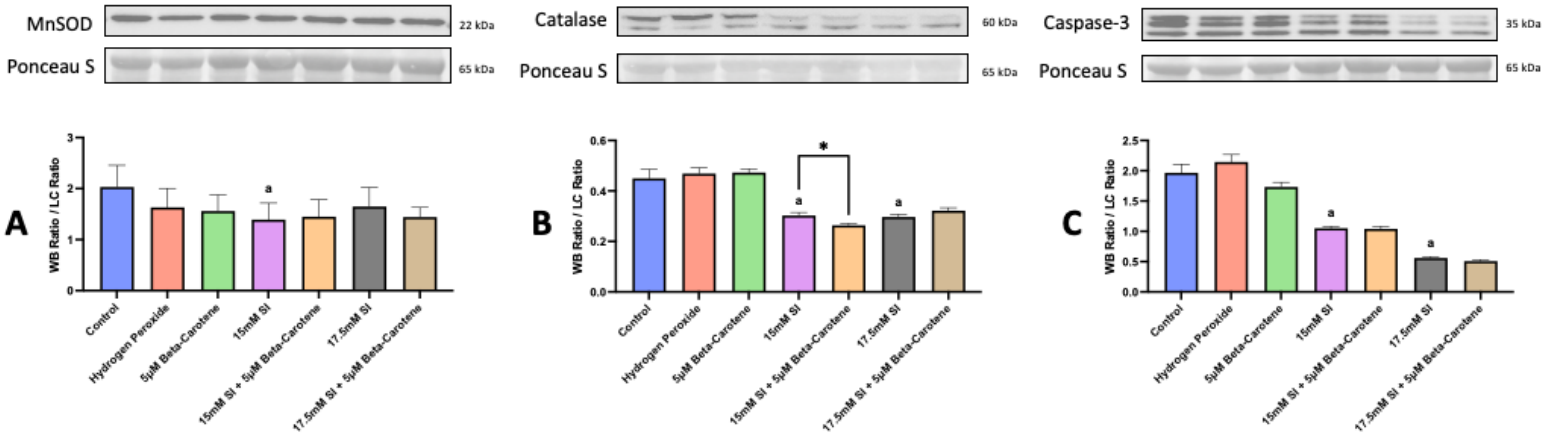

Figure 4.40 – Beta-carotene 5 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 WT cells. Representative Western blots and quantified band densities of ARPE-19 WT cells pre-treated with beta-carotene (5 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression. Data is expressed as Western blot / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$.)

Pre-treatment with 20 μM of beta-carotene prior to exposure to 15 mM SI illustrated a significant ($p<0.05$) decrease in MnSOD protein expression level when compared to the 15 mM SI exposure alone. No significant change was seen when pre-treated with 20 μM of beta-carotene prior to exposure to 17.5 mM of SI on ARPE-19 WT cells (Figure 4.41 A). Catalase protein expression levels showed significant ($p<0.05$) reduction when comparing both 15 and 17.5 mM SI exposure to control cells. Catalase levels were unchanged when pretreated with 20 μM of beta-carotene prior to exposure to 15 or 17.5 mM of SI (Figure 4.41 B). Caspase-3 protein expression levels were significantly ($p<0.05$) reduced when exposed to 15 and 17.5 mM of SI. Pre-treatment with 20 μM of beta-carotene prior to exposure to SI indicated no significant change on caspase-3 protein expression level at 15 mM, however, a significant ($p<0.001$) increase in caspase-3 levels were seen at 17.5 mM on ARPE-19 WT cells (Figure 4.41 C).

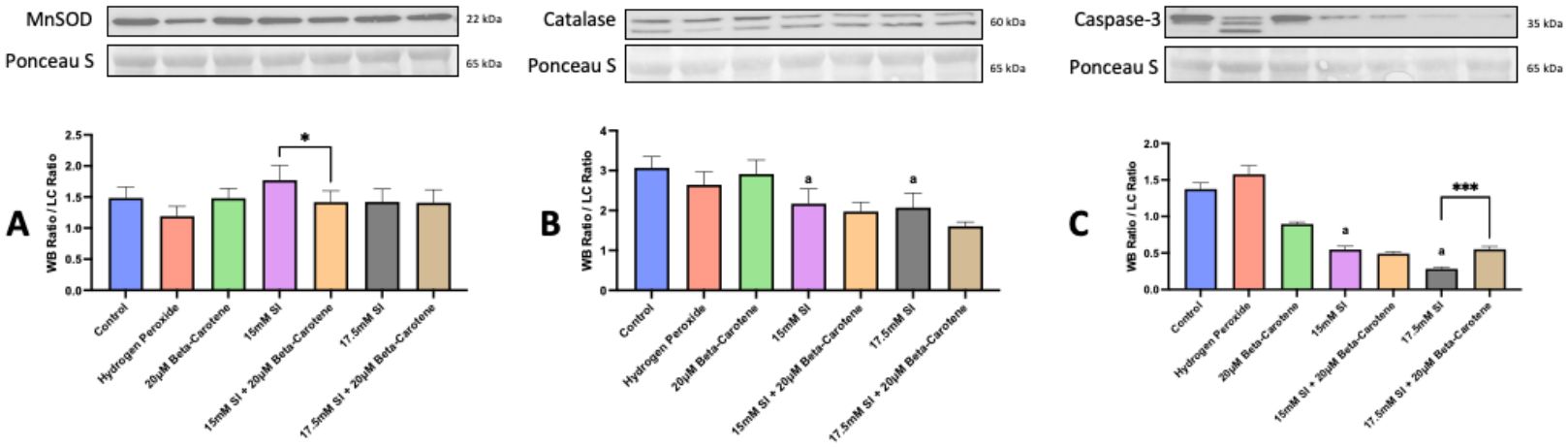

Figure 4.41 – Beta-carotene 20 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 WT cells. Representative Western blots and quantified band densities of ARPE-19 WT cells pre-treated with beta-carotene (20 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression. Data is expressed as Western blot / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$.)

4.7.2 ARPE-19 BIRC5 Knockout

4.7.2.1 ARPE-19 BIRC5 – Lutein

Lutein treatment of 10 μM both in the presence and absence of SI resulted in no significant change on MnSOD protein expression of ARPE-19 BIRC5 KO cells. SI exposure of 15 to 17.5 mM caused no significant change in MnSOD protein expression levels. MnSOD protein expression was significantly ($p<0.01$) reduced in hydrogen peroxide exposed cells (Figure 4.42 A). Hydrogen peroxide exposure and SI exposure of 15 to 17.5 mM caused significant ($p<0.05$) reductions on catalase protein expression levels. Pre-treatment with 10 μM of lutein prior to SI exposure had no significant change on catalase protein expression levels (Figure 4.42 B). Caspase-3 protein expression remained unchanged in all the treatment conditions of ARPE-19 BIRC5 KO cells (Figure 4.42 C).

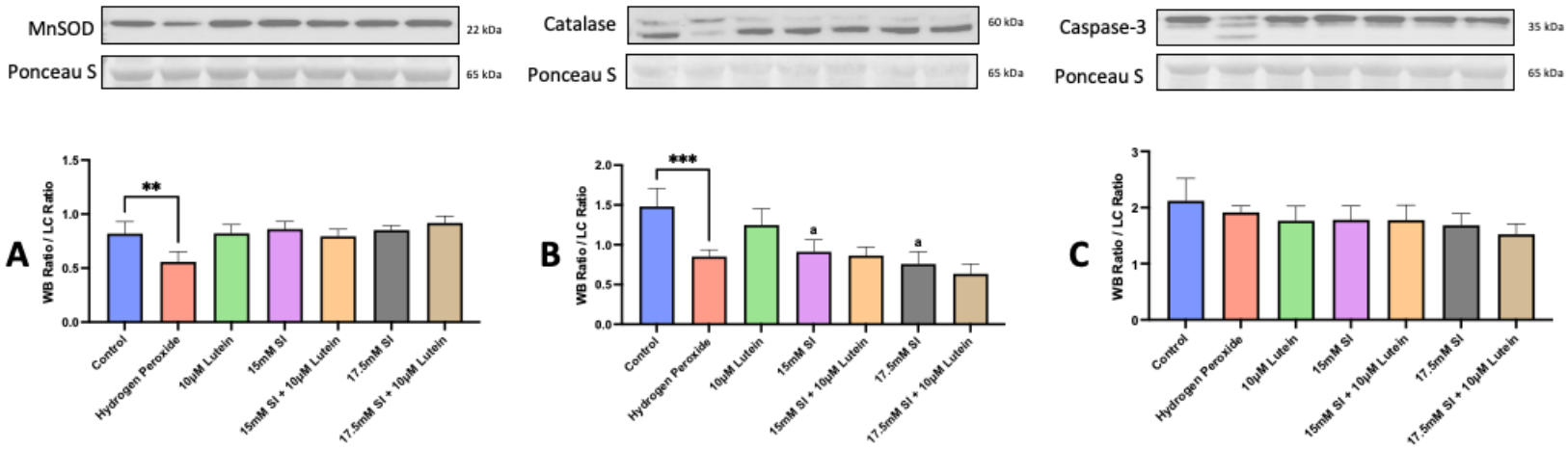

Figure 4.42 – Lutein 10 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 BIRC5 KO cells. Representative Western blots and quantified band densities of ARPE-19 BIRC5 KO cells pre-treated with lutein (10 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, ** indicates $p<0.01$, *** indicates $p<0.001$.)

Hydrogen peroxide exposure and SI exposure of 15 to 17.5 mM caused significant ($p<0.05$) reduction in MnSOD protein expression levels. Pre-treatment with 25 μM of lutein prior to SI exposure had no significant change on MnSOD protein expression (Figure 4.43 A). There was a significant decrease in catalase protein expression after hydrogen peroxide and SI exposure of 15 to 17.5 mM. Pre-treatment with 25 μM of lutein prior to SI exposure had no significant change on catalase protein expression levels (Figure 4.43 B). Caspase 3 protein expression remained unchanged in all the treatment conditions of ARPE-19 BIRC5 KO cells (Figure 4.43 C).

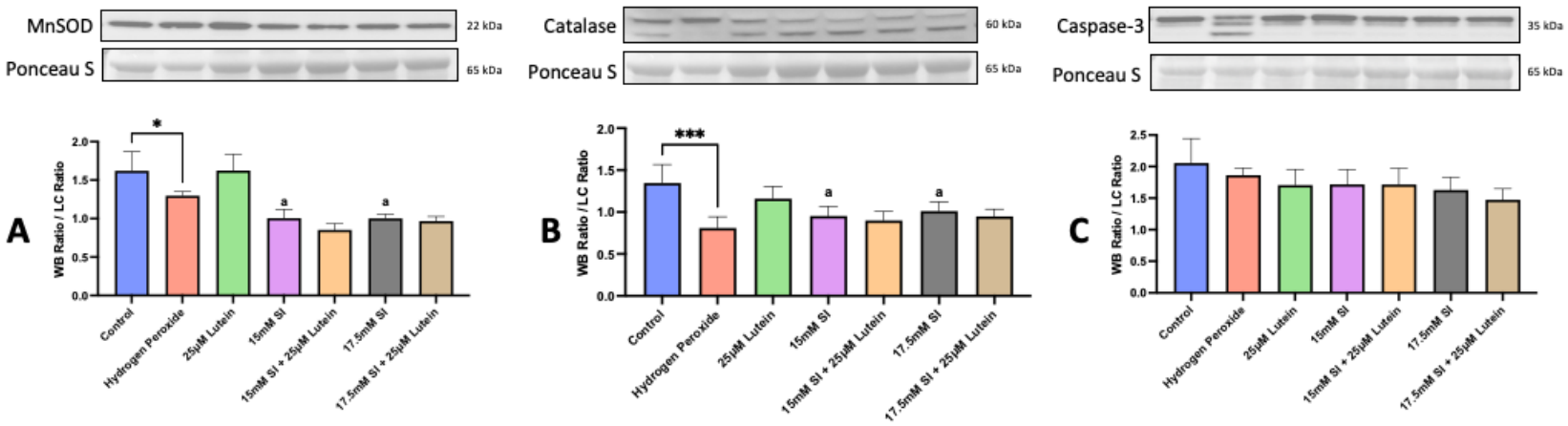

Figure 4.43 – Lutein 25 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 BIRC5 KO cells. Representative Western blots and quantified band densities of ARPE-19 BIRC5 KO cells pre-treated with lutein (25 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, *** indicates $p<0.001$.)

4.7.2.2 ARPE-19 BIRC5 – Zeaxanthin

Results from the Western blots of MnSOD on ARPE-19 BIRC5 KO cells showed a significant ($p<0.05$) decrease in protein expression levels when comparing 15 and 17.5 mM SI treated cells to untreated control cells. A significant ($p<0.0001$) decrease in MnSOD expression was also seen subsequent to hydrogen peroxide exposure. No significant change was found in MnSOD expression was found when treated with 2.5 μM of zeaxanthin and exposed to 15 or 17.5 mM of SI and compared to non-pretreated controls (Figure 4.44 A). Catalase protein expression levels showed a significant decrease when exposed to 15 mM of SI ($p<0.05$) and hydrogen peroxide ($p<0.0001$). Catalase levels were also significantly ($p<0.05$) increased when pretreated with 2.5 μM of zeaxanthin prior to exposure to 15 mM SI, however, no significant change was seen when exposed to 17.5 mM of SI (Figure 4.44 B). Caspase-3 protein expression levels were significantly ($p<0.05$) reduced when exposed to hydrogen peroxide and 15 and 17.5 mM of SI. Pre-treatment with 2.5 μM of zeaxanthin prior to exposure to 17.5 mM SI indicated a significant ($p<0.01$) decrease on caspase-3 protein expression level within ARPE-19 BIRC5 KO cells (Figure 4.44 C).

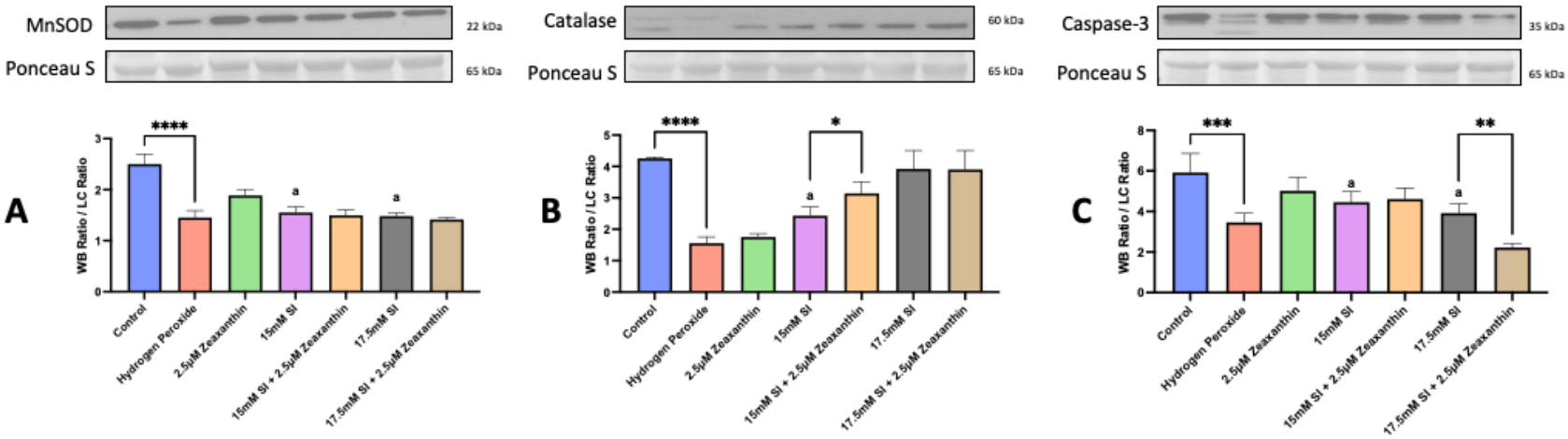

Figure 4.44 – Zeaxanthin 2.5 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 BIRC5 KO cells.

Representative Western blots and quantified band densities of ARPE-19 BIRC5 KO cells pre-treated with zeaxanthin (2.5 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$.)

Exposure to 15 and 17.5 mM of SI showed a significant ($p<0.05$) decrease in MnSOD protein expression levels when compared to untreated control ARPE-19 BIRC5 KO cells. Pre-treatment with 10 μM of zeaxanthin prior to exposure to 15 mM SI indicated a significant ($p<0.5$) increase in MnSOD protein expression level (Figure 4.45 A). Catalase protein expression levels showed a significant ($p<0.05$) decrease when comparing 15 and 17.5 mM SI exposure and hydrogen peroxide exposure to untreated control cells. Catalase levels were also unchanged when pretreated with 10 μM of zeaxanthin prior to exposure to 15 or 17.5 mM of SI (Figure 4.45 B). Caspase-3 protein expression levels were unchanged in all of the treatment conditions of ARPE-19 BIRC5 KO cells (Figure 4.45 C).

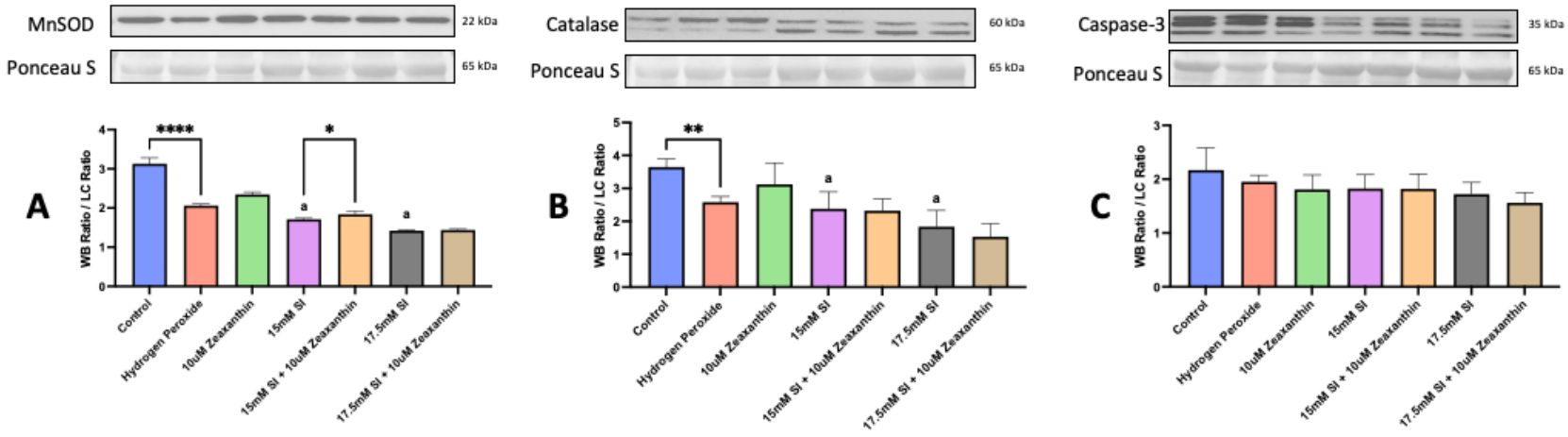

Figure 4.45 – Zeaxanthin 10 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 BIRC5 KO cells.

Representative Western blots and quantified band densities of ARPE-19 BIRC5 KO cells pre-treated with zeaxanthin (10 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$, **** indicates $p<0.0001$.)

4.7.2.3 ARPE-19 BIRC5 KO – Beta-carotene

MnSOD protein expression levels were significantly ($p<0.0001$) reduced with exposure to hydrogen peroxide. Exposure to 15 and 17.5 mM of SI showed no significant change in MnSOD protein expression levels when compared to untreated control cells. Pre-treatment with 5 μM of beta-carotene prior to exposure to 15 and 17.5 mM SI indicated a significant ($p<0.001$, $p<0.01$) reduction in MnSOD protein expression level (Figure 4.46 A). Catalase protein expression levels showed significant ($p<0.05$) reduction when comparing 15 and 17.5 mM SI exposure to control cells. Catalase levels were unchanged when pretreated with 5 μM of beta-carotene prior to exposure to 15 or 17.5 mM of SI (Figure 4.46 B). Caspase-3 protein expression levels were significantly ($p<0.05$) reduced when exposed to 17.5 mM of SI. Pre-treatment with 5 μM of beta-carotene prior to exposure to SI indicated no significant change on caspase-3 protein expression level of ARPE-19 BIRC5 KO cells (Figure 4.46 C).

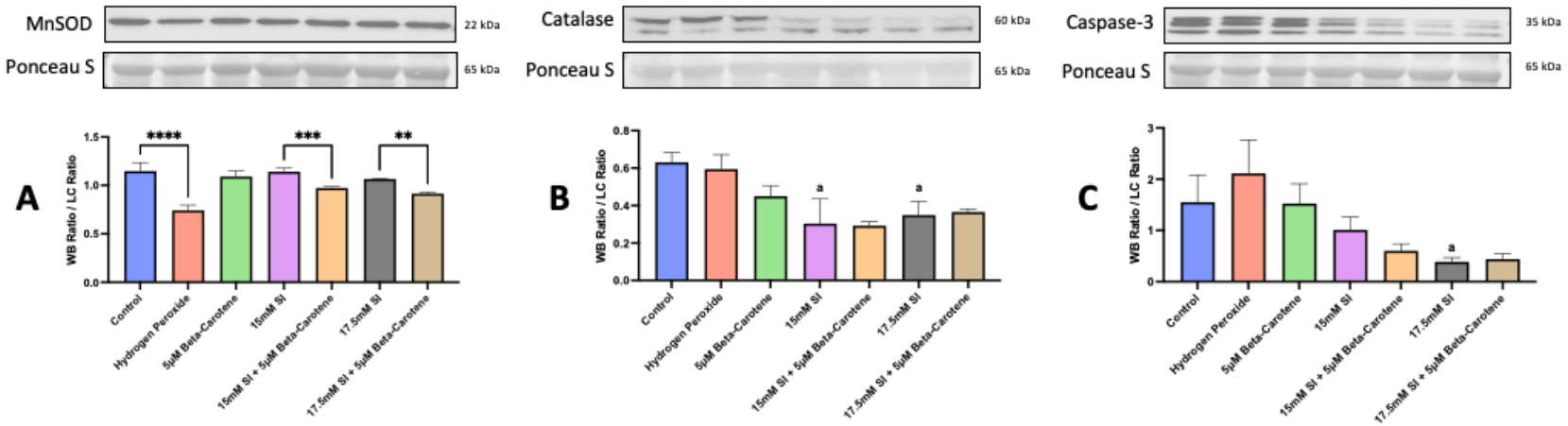

Figure 4.46 – Beta-carotene 5 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 BIRC5 KO cells.

Representative Western blots and quantified band densities of ARPE-19 BIRC5 KO cells pre-treated with beta-carotene (5 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$.)

Western blot analysis of MnSOD protein expression levels with exposure to 17.5 mM of SI showed a significant ($p<0.05$) decrease in MnSOD protein expression levels when compared to control. MnSOD protein expression levels were significantly ($p<0.0001$) reduced with exposure to hydrogen peroxide. Pre-treatment with 20 μM of beta-carotene prior to exposure to 15 mM SI indicated a significant ($p<0.05$) decrease in MnSOD protein expression level when compared to the 15 mM SI exposure alone. A significant ($p<0.001$) increase in MnSOD protein expression level seen when pre-treated with 20 μM of beta-carotene prior to exposure to 17.5 mM of SI (Figure 4.47 A). Catalase protein expression levels showed no significant change when comparing 15 and 17.5 mM SI exposure to control cells. Catalase levels were unchanged when pre-treated with 20 μM of beta-carotene prior to exposure to 15 mM of SI, however, when pre-treated with 20 μM of beta-carotene then exposed to 17.5 mM of SI a significant ($p<0.05$) increase in catalase protein expression levels was found with ARPE-19 BIRC5 KO cells (Figure 4.47 B). Caspase-3 protein expression levels were significantly ($p<0.05$) reduced when exposed to 15 and 17.5 mM of SI. Pre-treatment with 20 μM of beta-carotene prior to exposure to SI indicated no significant change on caspase-3 protein expression level at 15 mM, however, a significant ($p<0.001$) increase in caspase-3 levels were seen at 17.5 mM (Figure 4.47 C).

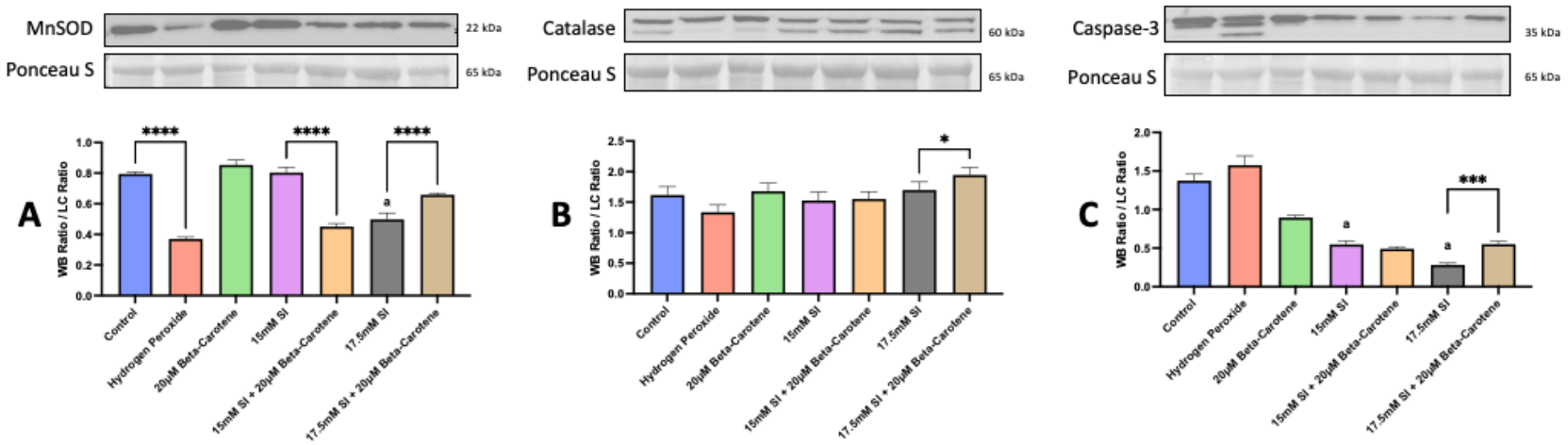

Figure 4.47 – Beta-carotene 20 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 BIRC5 KO cells.

Representative Western blots and quantified band densities of ARPE-19 BIRC5 KO cells pre-treated with beta-carotene (20 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, *** indicates $p<0.001$, **** indicates $p<0.0001$.)

4.7.3 ARPE-19 SIRT1 Knockout

4.7.3.1 ARPE-19 SIRT-1 – Lutein

Hydrogen peroxide and 15 mM SI exposure caused a significant ($p<0.05$) decrease in MnSOD levels. Pre-treatment with 10 μM of lutein prior to 17 mM SI exposure had a significant reduction ($p<0.01$) in MnSOD protein expression (Figure 4.48 A). 15 to 17.5 mM of SI exposure caused significant ($p<0.05$) reductions on catalase protein expression levels. Pre-treatment with 10 μM of lutein prior to SI exposure had a significant ($p<0.01$) reduction on catalase protein expression for both 15 and 17.5 mM SI exposures (Figure 4.48 B). Caspase 3 protein expression remained unchanged for all the treatment conditions for ARPE-19 SIRT-1 KO cells (Figure 4.48 C).

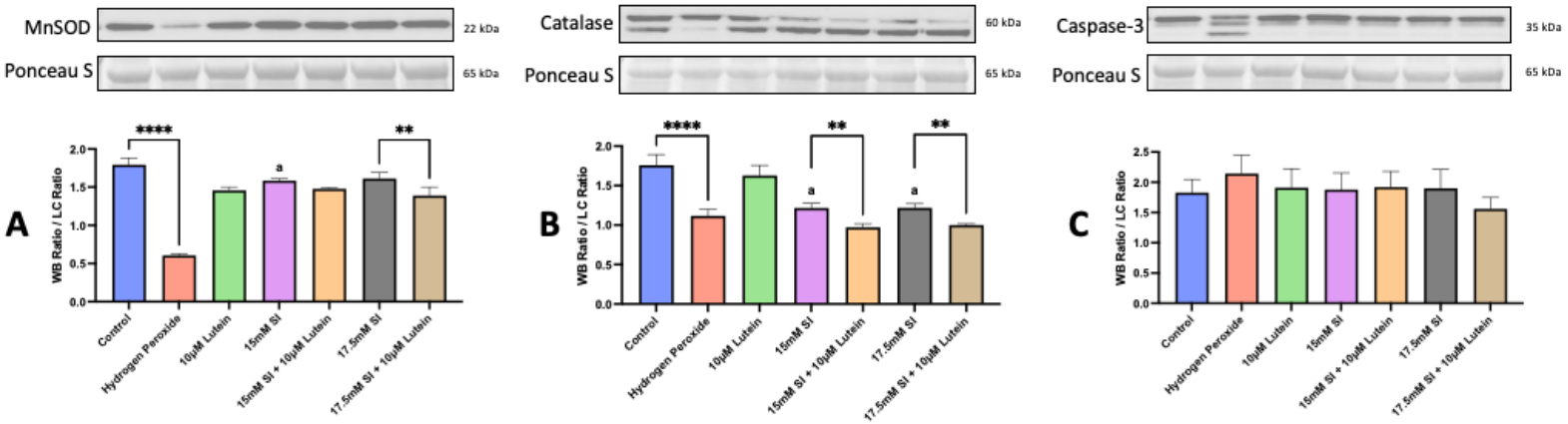

Figure 4.48 – Lutein 10 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 SIRT-1 KO cells. Representative Western blots and quantified band densities of ARPE-19 SIRT-1 KO cells pre-treated with lutein (10 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, ** indicates $p<0.01$, **** indicates $p<0.0001$.)

MnSOD protein expression remained unchanged both in the presence and absence of lutein in cells exposed to both concentrations of SI (Figure 4.49 A). Hydrogen peroxide exposure resulted in s significant ($p<0.0001$) reduction in catalase protein expression. SI exposure of 15 and 17.5 mM caused significant ($p<0.05$) increase of catalase protein expression levels within ARPE-19 SIRT-1 KO cells. Pre-treatment with 25 μM of lutein prior to SI exposure had a significant ($p<0.001$) increase on catalase protein expression levels at 15 mM of SI. However, a significant ($p<0.0001$) decrease of catalase protein expression was seen when pretreated with 25 μM of lutein prior to 17.5 mM SI exposure (Figure 4.49 B). Caspase 3 protein expression remained unchanged in all the treatment conditions for ARPE-19 SIRT-1 KO cells (Figure 4.49 C).

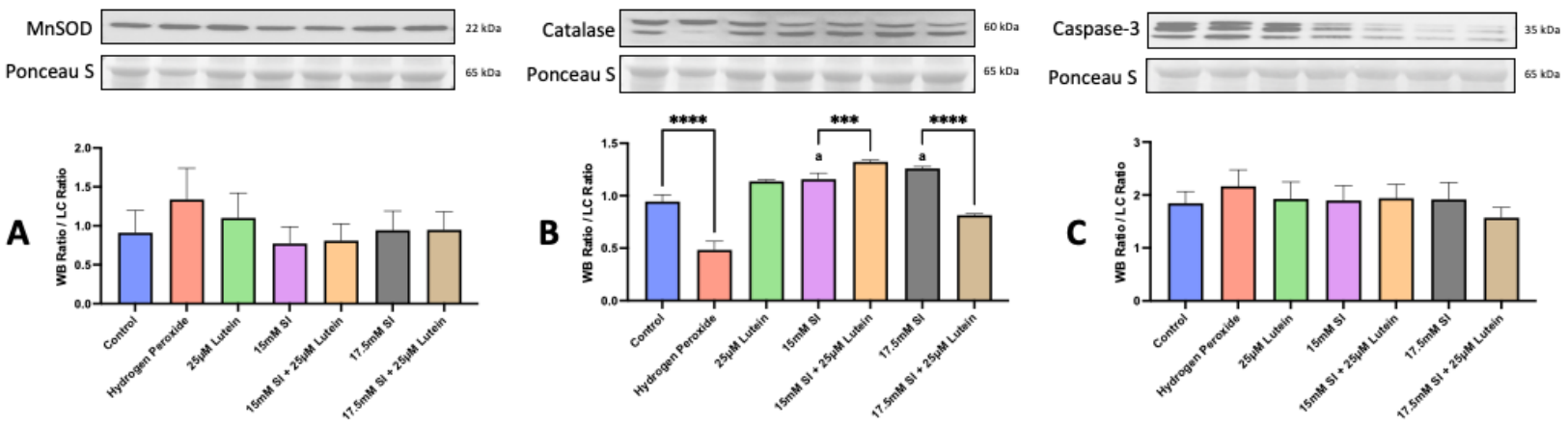

Figure 4.49 – Lutein 25 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 SIRT-1 KO cells. Representative Western blots and quantified band densities of ARPE-19 SIRT-1 KO cells pre-treated with lutein (25 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, *** indicates $p<0.001$, **** indicates $p<0.0001$.)

4.7.3.2 ARPE-19 SIRT-1 – Zeaxanthin

Results from the Western blots of MnSOD levels of ARPE-19 SIRT-1 KO cells showed a significant ($p<0.05$) decrease in protein expression levels when comparing hydrogen peroxide treated cells to control cells. Pretreatment with 2.5 μM of zeaxanthin and exposure to 15 mM of SI had a significant ($p<0.01$) reduction in MnSOD protein expression levels when compared to non-pretreated control cells. No significant change was found in MnSOD expression was found when treated with 2.5 μM of zeaxanthin and exposed to 17.5 mM of SI and compared to non-pretreated controls (Figure 4.50 A). Catalase protein expression levels showed a significant ($p<0.0001$) decrease in catalase protein expression when exposed to hydrogen peroxide and 15 and 17.5 mM of SI. Catalase levels were also significantly ($p<0.05$) decreased when pretreated with 2.5 μM of zeaxanthin prior to exposure to 15 mM SI, however, no significant change was seen when exposed to 17.5 mM of SI (Figure 4.50 B). Caspase-3 protein expression levels were significantly ($p<0.05$) reduced when exposed to hydrogen peroxide, 15 and 17.5 mM of SI. Pre-treatment with 2.5 μM of zeaxanthin prior to exposure to 15 or 17.5 mM SI indicated no significant change on caspase-3 protein expression level (Figure 4.50 C).

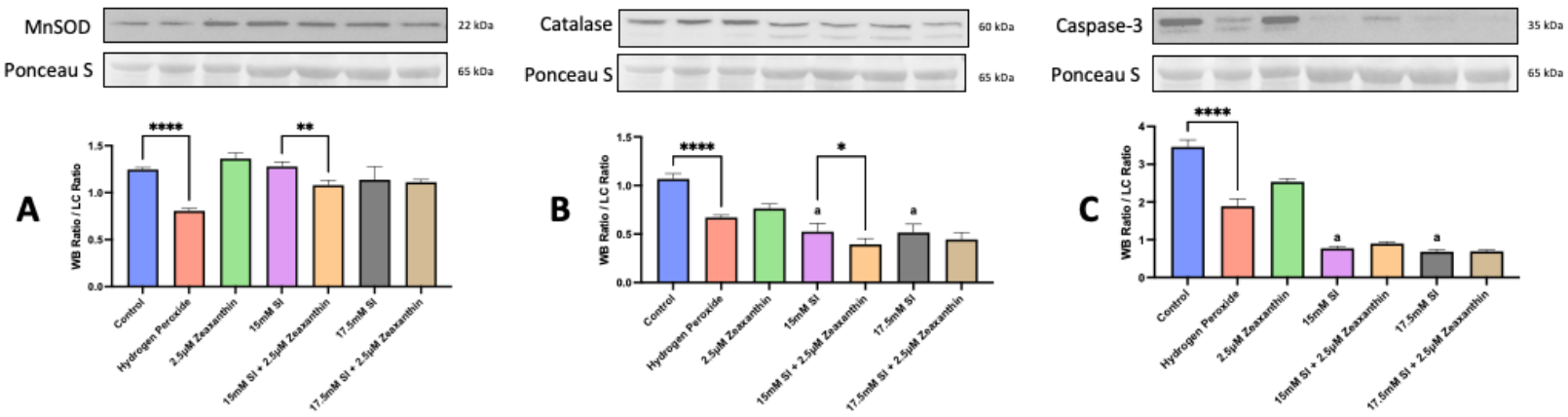

Figure 4.50 – Zeaxanthin 2.5 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 SIRT-1 KO cells.

Representative Western blots and quantified band densities of ARPE-19 SIRT-1 KO cells pre-treated with zeaxanthin (2.5 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, ** indicates $p<0.01$, **** indicates $p<0.0001$.)

Western blot analysis of MnSOD protein expression levels when exposed to hydrogen peroxide and 15 mM of SI showed a significant ($p<0.05$) reduction in MnSOD protein expression levels when compared to control. No significant change was seen when pre-treated with 10 μM of zeaxanthin prior to exposure to 15 or 17.5 mM SI (Figure 4.51 A). Catalase and caspase 3 protein expression levels showed no significant change in any of the treatment conditions of ARPE-19 SIRT-1 KO cells (Figure 4.51 B,C).

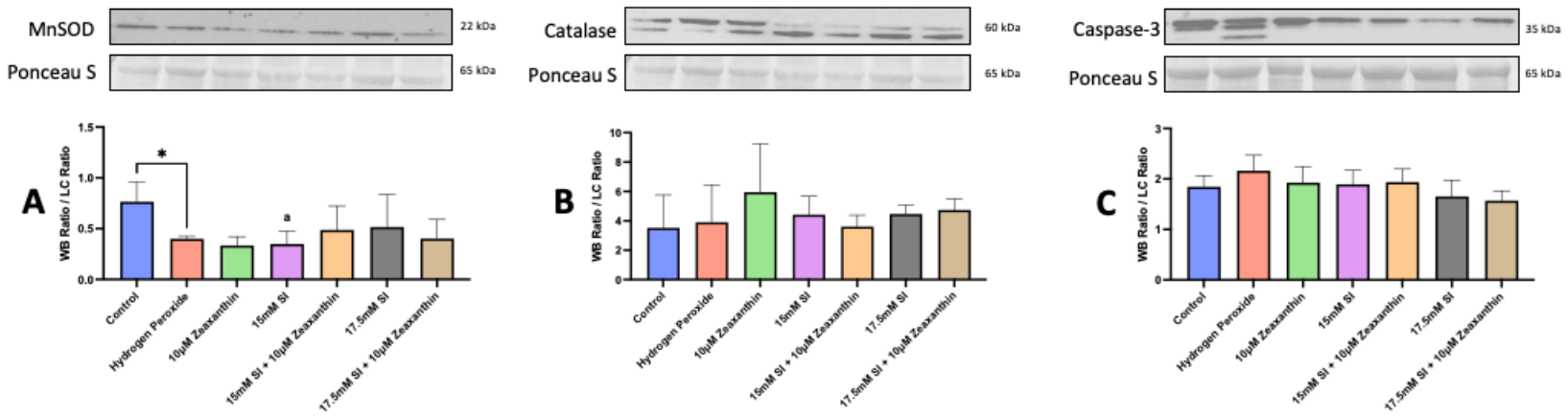

Figure 4.51 – Zeaxanthin 10 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 SIRT-1 KO cells.

Representative Western blots and quantified band densities of ARPE-19 SIRT-1 KO cells pre-treated with zeaxanthin (10 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$.)

4.7.3.3 ARPE-19 SIRT-1 KO – Beta-carotene

Western blot analysis of MnSOD protein expression levels with exposure to hydrogen peroxide and 17.5 mM of SI showed a significant ($p<0.05$) reduction in MnSOD protein expression levels when compared to control. Pre-treatment with 5 μM of beta-carotene prior to exposure to 15 and 17.5 mM SI indicated no significant change in MnSOD protein expression level (Figure 4.52 A). Catalase protein expression levels showed no significant change across all treatment conditions (Figure 4.52 B). Caspase-3 protein expression levels was significantly ($p<0.001$) increased in the presence of hydrogen peroxide. It was significantly ($p<0.05$) reduced when exposed to 15 or 17.5 mM of SI. Pre-treatment with 5 μM of beta-carotene prior to exposure to 15 mM SI indicated no significant change on caspase-3 protein expression level. Pre-treatment with 5 μM of beta-carotene prior to exposure to 17.5 mM SI indicated a significant ($p<0.05$) reduction on caspase-3 protein expression level of ARPE-19 SIRT-1 KO cells (Figure 4.52 C).

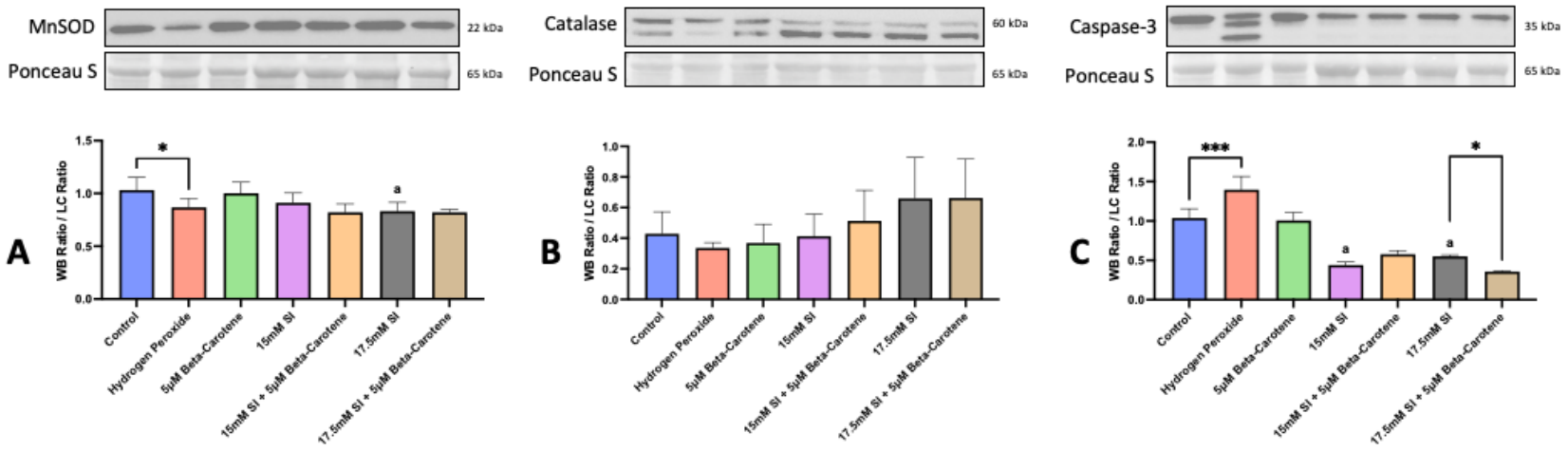

Figure 4.52 – Beta-carotene 5 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 SIRT-1 KO cells.

Representative Western blots and quantified band densities of ARPE-19 SIRT-1 KO cells pre-treated with beta-carotene (5 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, * indicates $p<0.05$, *** indicates $p<0.001$.)

Western blot analysis of MnSOD and catalase protein expression levels when exposed to hydrogen peroxide showed a significant ($p<0.01$) decrease as compared to control cells. MnSOD and catalase protein expression levels remained unchanged in all other treatment conditions (Figure 4.53 A,B). Caspase-3 protein expression levels were significantly ($p<0.05$) reduced when exposed to 15 and 17.5 mM of SI. Hydrogen peroxide led to a significant ($p<0.0001$) increase in caspase-3 protein expression. Pre-treatment with 20 μM of beta-carotene prior to exposure to SI indicated no significant change on caspase-3 protein expression level at 15 or 17.5 mM (Figure 4.53 C).

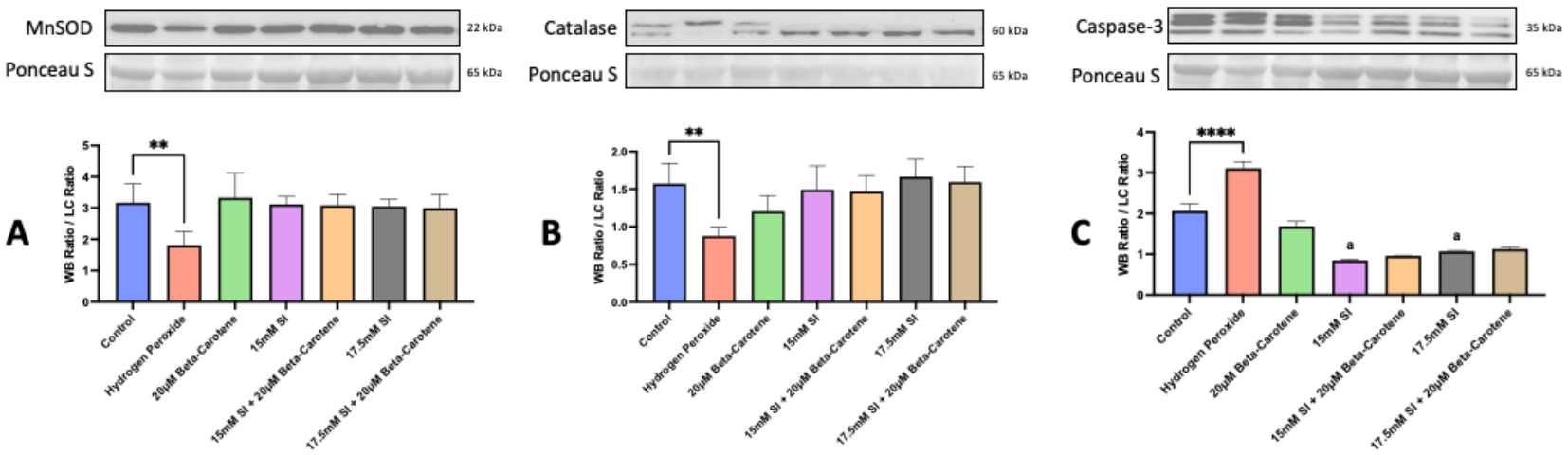

Figure 4.53 – Beta-carotene 20 μM – Western Blot Analysis of MnSOD, Catalase, and Caspase-3 on ARPE-19 SIRT-1 KO cells.

Representative Western blots and quantified band densities of ARPE-19 SIRT-1 KO cells pre-treated with beta-carotene (20 μM) in supplemented media for 4 hours then exposed to SI for 18 hours to measure protein expression and expressed as Western blot ratio / loading control ratio. MnSOD, Catalase, and Caspase-3 primary antibodies were incubated overnight at 1:1000 concentrations prior to incubating for 1 hour with anti-rabbit secondary antibodies at 1:1000 concentrations. Data presented as mean ± SEM of n=3 experiments. Data was compared using a one-way ANOVA with Fisher's LSD test. (a indicates $p<0.05$ significance from control, ** indicates $p<0.01$, **** indicates $p<0.0001$.)

Chapter 5: Discussion

5.1 Blue Light Study

The first set of experiments in this study were centered around developing and optimizing a model for damage to RPE cells similarly to AMD through blue light. While other studies have investigated the effects of blue light on retinal cells, specifics on the panel design are not fully disclosed. In development a model of blue light induced cell damage, some setbacks that were encountered was the overproduction of heat trapped between the culture vessel and the LED panel, resulting in a rise of temperature within the vessel. This rise in temperature likely resulted in more cell death caused by heat rather than the oxidative effects of blue light.

Once the panel was developed and optimized, the panel's suitability to induce oxidative damage, similar to AMD, could be assessed utilizing ARPE-19 cells. Investigating the impact of blue light exposure on the levels of specific oxidative stress related markers, including 4-HNE protein adducts, caspase-3, catalase, GPx-1, and MnSOD levels in ARPE-19 cells was measured in this study. Given the known protective effects of antioxidants in the eyes ("The Age-Related Eye Disease Study (AREDS)" 1999), the effects of pre-treatment with resveratrol and pterostilbene on these proteins was also assessed in response to blue light exposure on ARPE-19 cells. The findings from these studies are crucial for understanding the cellular response to oxidative stress induced by blue light exposure and the potential protective effects of resveratrol and pterostilbene for the eye.

Resveratrol has a variety of beneficial properties including antioxidative, anticarcinogenic, cardioprotective, anti-inflammatory, and anti-aging (Burns et al. 2002). Resveratrol has been studied for its antioxidant ability and has been noted to have a strong role in scavenging free radicals and mitigating oxidative stress (Frémont 2000; Bhat, KosmederII, and Pezzuto 2001). Pterostilbene has been studied and shows promise as a strong antioxidant (McCormack and McFadden 2013; Estrela et al. 2013), protective against cardiovascular disease (McCormack and McFadden 2013), and effective against cancer processes (Estrela et al. 2013). Both resveratrol and pterostilbene are possible supplements that would slow down the oxidative damage in the eyes caused by blue light.

It was hypothesised that blue light would induce oxidative stress increasing the levels of 4-HNE protein adducts, as well as increasing apoptosis signified by increases in caspase-3 levels. Antioxidant activity was expected to be increased due to the increased presence of ROS, this increase would be depicted as a decrease of MnSOD, GPx-1, and catalase due to the lower available concentration of unused intermediate. To investigate our hypothesis, there were two specific aims for the blue light study:

d. Measure cell viability of cells pre-treated with resveratrol and pterostilbene and exposed to blue light
e. Determine the protein expression of antioxidant enzymes, cell death markers, and lipid peroxidation with protein blotting techniques

An earlier study by Ogawa et al. (2014) explored the protective effects of bilberry and lingonberry extracts against blue light-induced retinal photoreceptor cell damage *in vitro*. This study provided insights into potential protective agents against blue light-induced damage within the eye (Ogawa et al. 2014). 4-HNE is a protein adduct which has been described as a stable marker of oxidative stress in cell culture models. This study found that after 6 hours of blue light exposure, ARPE-19 cells exhibited increased 4-HNE levels, indicating that the ARPE-19 cells experience an increase in oxidative stress when exposed to blue light. These findings are consistent with a study by Brandstetter et al. (2015) who also demonstrated an increase in 4-HNE levels in ARPE-19 cells when exposed to blue light for 6 hours (Brandstetter et al. 2015). This increased 4-HNE production could be mitigated when cells were pre-treated with 50 and 100 μM of resveratrol and then exposed to blue light conditions, suggesting that resveratrol may provide protection against lipid peroxidation induced by blue light. Conversely, when cells were pre-treated with pterostilbene and then exposed to blue light conditions, there was a significant increase in 4-HNE production when compared to control cells, indicating that pterostilbene may exacerbate oxidative stress levels in ARPE-19 cells. The vast difference in the ARPE-19 cells' response to resveratrol compared to pterostilbene when exposed to blue light could be due to the structural differences between pterostilbene and resveratrol. Exposure to blue light could be creating

oxidative intermediates from the methyl groups of pterostilbene which may be toxic to cells and causing the increase in cell death.

Caspase-3 is an apoptotic protein released by cells undergoing cell death. In this study, Caspase-3 concentrations were seen to slightly increase in ARPE-19 cells exposed to blue light for 12 hours. In an earlier study by Sparrow et al. (2001), blue light caused a significant increase in caspase-3 activity in ARPE-19 cells (Sparrow and Cai 2001). While the increase in caspase-3 observed in this study was not significant, the trend of increased apoptotic proteins when exposed to blue light was observed. When pre-treated with 50 μM of resveratrol, the effect of this increase in caspase-3 was slightly reduced when comparing the blue light treated cells to the control cells. This indicated that resveratrol may be offering some protective effects to the ARPE-19 cells when they are exposed to blue light.

Catalase plays an important role in the reduction of H_2O_2 to water and oxygen (Aebi 1974). This study investigated protein expression of catalase and observed a non-significant increase in Catalase levels in ARPE-19 cells exposed to blue light for 12 hours. In a recent study, a significant increase in catalase concentration in ARPE-19 cells exposed to H_2O_2 has been reported (Arumugam et al. 2019). The discrepancy in findings may be attributed to the different sources of oxidative stress (blue light vs. H_2O_2) and the distinct cellular responses to these stressors.

This study also examined GPx-1 levels in ARPE-19 cells exposed to blue light. GPx-1 is expressed in tissues and has been found to be cytoprotective against oxidative stressors by catalyzing the reduction of H_2O_2 to water and oxygen (Lubos, Loscalzo, and Handy 2011). It was found that GPx-1 levels overall were similar between blue light and control conditions. In a study by Pilat et al. (2013), GPx-1 levels in RPE cells were elevated following increased exposure to blue light (Pilat et al. 2013). However, the study by Pilat et al. (2013) did not report on enzyme levels post confluency, which suggests interpreting their results should be done so cautiously. In this study, there was a significant reduction in GPx-1 enzyme levels when cells were pre-treated with 25 μM of pterostilbene. The findings from this study suggest that the GPx-1 protein may not be implicated in the oxidative stress pathway in ARPE-19 cells when they are exposed to blue light, however, 25 μM pre-treatments of pterostilbene have been shown to reduce GPx-1 protein levels.

Finally, this study also investigated MnSOD levels in ARPE-19 cells exposed to blue light. MnSOD is a major ROS scavenging molecule and catalyzes the detoxification of superoxide radical (Miriyala et al. 2012). Similarly to the GPx-1 results, no significant change in MnSOD levels in ARPE-19 cells exposed to blue light for 12 hours. Arumugam et al. (2019) reported a significant increase in MnSOD concentrations in ARPE-19 cells exposed to H_2O_2 (Arumugam et al. 2019). The differences in MnSOD

responses to different oxidative stressors highlights the complexity of cellular responses to oxidative stress and the need for further research to elucidate these mechanisms.

In conclusion, the findings from the blue light study provide valuable insights into the cellular response to blue light-induced oxidative stress and the potential protective effects of resveratrol and pterostilbene in ARPE-19 cells. With oxidative stress marker 4-HNE significantly increasing and caspase-3 non-significantly increasing in response to blue light, but then partially rescued by a pre-treatment of resveratrol, this study suggests that this antioxidant may be beneficial to the eye under certain conditions. This study contributes to the existing body of knowledge on oxidative stress and antioxidant responses in RPE cells, shedding light on potential therapeutic strategies for mitigating oxidative damage in the retina.

These findings report on major differences in response to blue light exposure seen between the analogs of resveratrol and pterostilbene. The differences in protective effect of the two compounds could be due to structural differences, bioavailability, and half-life. Pterostilbene demonstrates superior bioavailability, a longer half-life, and increased cellular uptake compared to resveratrol due to its enhanced lipophilicity from the presence of methoxy groups, making it more easily absorbed orally and metabolically stable (V. C. Lin et al. 2012; H. Lin, Yue, and Ho 2009; Dellinger, Garcia, and Meyskens 2014).

5.2 Sodium Iodate Model

In order to effectively investigate the protective role of antioxidant compounds on ARPE-19 cells in cell culture models, an established model of eye damage induction has to be used. Originally, cells were exposed to a blue light model of AMD, however after significant difficulties with reproducible results and fatigue of the LEDs in the panel, experiments were adapted towards a more commonly used chemical model of stress induction. Many studies have used A2E, a oxidative by-product of the visual pathway, however, A2E is not easily accessible and tedious biochemical experiments and equipment were needed for its synthesis (Wielgus et al. 2010; Sparrow and Cai 2001). SI was the second most common model of stress induction in ARPE-19 cells. The use of SI as a method of retinal degeneration dates back to 1941, where Sorsby described the effect of SI injected into a rabbit retina (Sorsby 1941). Since the early days of testing with SI, many *in vivo* models have been used including mice, sheep, rabbits, and rats, with mice being the most common reviewed by Kannan et al., and Hanus et al (Kannan and Hinton 2014; Hanus et al. 2016). Many cell culture models have also utilized SI, however, the most common *in vitro* model is in ARPE-19 cells (Kannan and Hinton 2014; Hanus et al. 2016). In both animal models and cell culture models it is challenging to accurately simulate what occurs in the eye as AMD progresses due to the disease's multifactorial progression, however, SI is a dependable method for mimicking

conditions within the eye undergoing AMD and there are several published articles using this *in vitro* model.

It was hypothesized that SI would induce a linear dose response curve with cell viability decreasing as the SI concentration increases. In order to test this hypothesis, a reproducible model of oxidative stress with SI that would be similar to the conditions seen within the eye of patients with AMD had to be established. After optimization, experiments would be able to move forward with the model for the remainder of the study. In order to investigate the hypothesis, there were four specific aims:

a. Create a method of exposing cells to SI and inducing oxidative damage
b. Measure cell viability at various doses of SI on ARPE-19 cells
c. Determine levels of oxidative stress produced by SI exposure
d. Determine levels of cell death produced by SI exposure

Initially, when the literature was reviewed, it was discovered that there was no consensus amongst the scientific community for which concentration of SI would induce AMD-related effects in ARPE-19 cells. Hanus et al. (2016) demonstrated that the EC50 of SI in ARPE-19 cells was calculated to be 10.5 mM, with 10 mM SI selected for subsequent *in vitro* experiments (Hanus et al. 2016). Additionally, Zhang et al. (2016) found that treatment of ARPE-19 cells with 15.12 mM SI for 24 hours induced massive cell death, which was not observed in lower doses of SI (1-5 mM) (X.-Y. Zhang et al.

2016). Another study reported that treatment of ARPE-19 cells with 7.5 mM SI for 24 hours resulted in 44% of cells being nonviable (Sundararajan et al. 2022). These studies collectively indicate that SI concentrations ranging from 7.5 mM to 15.12 mM have been used to induce cell death in ARPE-19 cells, with no concentration being consistently utilized for *in vitro* studies.

From the initial testing of SI on ARPE-19 WT cells with MTS assay, a significant reduction in cell viability was observed at 15 mM SI and for any higher concentrations when cells were exposed for 18 hours. At 15 mM SI exposure, ARPE-19 WT cells saw a significant reduction in cell viability when compared to the control. At 17.5 mM SI exposure, ARPE-19 WT cells saw even greater reduction in cell viability when compared to the control. At 20 and 30 mM SI exposure, ARPE-19 WT cells were almost completely unviable with significant decreases in cell viability. From the results of the dose response testing, for further experiments 12.5 mM, 15 mM and 17.5 mM would be the chosen treatments of SI for ARPE-19 cells. The higher concentrations of SI (20 mM and 30 mM) were found to have caused significant changes to cell morphology and appearance and added an unnecessary variability to the experiment. These observable changes were also noted by Zhang et al. (2016) when they described that that the continuous exposure to non-toxic doses of SI (15.12mM) induces cellular dysfunction in ARPE-19 cells (X.Y. Zhang et al. 2016). These results further emphasize the importance of understanding the cellular responses to sublethal SI exposure.

Results from these experiments suggest that the range of SI concentrations optimized in our ARPE-19 WT cells are in alignment with the upper range of the literature reported values for similar experiments. The deviation from the lower range of the published values could be due to differences in cell viability reagent used, and various medias and culture conditions used. Overall, SI exhibits promise as a linear and reproducible model of AMD in ARPE-19 cells.

To investigate the ability of SI to induce apoptosis, caspase-3 levels were measured. Caspase pathways are one of many pathways by which SI induces damage to RPE cells. Balmer et al (2015), investigated the involvement of multiple caspase-dependent and caspase-independent cell-death pathways in retinal cell death caused by SI, further emphasizing the relevance of SI in inducing cell death in retinal cells (Balmer et al. 2015). From their study, caspase levels and overall apoptosis were increased with treatments of SI on photoreceptor cells. Additionally, they found that RPE cells had increased necrosis with SI treatments. Hanus et al. (2016) investigated the role of caspase-1 in SI-induced necroptosis in ARPE-19 cells, and illustrated the potential for caspase inhibitors to mitigating SI-induced cell death (Hanus et al. 2016). Although the mechanism of SI induced cellular damage is not fully understood, the literature suggests the strong involvement of caspase pathways through apoptosis and necroptosis. Further investigations need to mechanistically understand the interconnected pathways of SI induced cell death.

Caspase 3/7 Fluorescence Dye was used to investigate caspase levels within ARPE-19 WT cells following 18 hours of exposure to SI through a cell imaging methodology. Results illustrate larger amounts of green fluorescently tagged cells as the concentrations of SI increased from 0 mM to 20 mM. At higher concentrations of SI, large black patches can be observed on the fluorescent images. The black patches are areas where cells have completely detached and were removed from media prior to adding the fluorescent dye. The presence of these black patches indicates nonadherent cells and further showcases the damaging effects of SI on the ARPE-19 cells at high concentrations. Results from these images illustrate that caspase 3/7 activity is significantly increased compared to control cells when exposed to SI.

To investigate the ability of SI treatment on ARPE-19 WT cells to induce oxidative stress, CMH2-DCFDHA assay was used to measure intracellular reactive oxygen species (ROS). When compared to control cells with no treatment, 12.5, 15, and 17.5 mM treatments of SI exhibited significant increases in intracellular ROS levels. A 12.5 mM SI exposure resulted in a 64% increase in ROS levels whereas 15 mM and 17.5 mM of SI exposure resulted in 115% and 285% increases in ROS levels respectively. The results from this study agree with the literature in that increasing levels of SI exposure result in increased levels of intracellular ROS within ARPE-19 cells (Kannan and Hinton 2014; X.-Y. Zhang et al. 2016). In a study by He et al (2019), 1200 μg/mL treatments of SI

for 24 hours induced a 2-fold increase in ROS levels measured by CMH2-DCFDHA assay in ARPE-19 WT cells (He et al. 2019).

In summary, a model of SI induced retinal pigment damage in ARPE-19 WT cells was optimized. The results from the optimization indicated that concentrations above 15 mM for 18 hours induced significant decreases in cell viability via MTS assay. The analysis of Caspase 3/7 activation results indicate that Caspase 3/7 levels are increased with increasing concentrations of SI as illustrated through fluorescent microscopy. Intracellular ROS levels were also investigated using a CMH2-DCFDHA assay probe, and results illustrate significant increases in ROS levels across 12.5, 15, and 17.5 mM treatments of SI. These results confirm that SI is a robust and linear model of inducing cellular death and oxidative stress within an *in vitro* model of AMD in ARPE-19 cells. Furthermore, this optimized model will be utilized all experiments moving forward in this dissertation.

5.3 Antioxidant compounds

In order to investigate the protective effects of antioxidant compounds on SI induced RPE cell damage, optimization of concentrations for each the desired compounds needed to be established. Antioxidants are substances which can slow down the oxidative damages caused by free radicals (Halliwell 1996). Naturally occurring antioxidants are commonly found in plants and the fruit of plants. These plant compounds can be broken down into groups of flavonoids, tannins, phenols, and

lignans. Extensive research has explored the potential role of antioxidants in treating a variety of diseases. The medical field has shown an increased interest in investigating the use of antioxidant supplements for preventing mortality and managing a range of health conditions. In the context of eye diseases, combinations of antioxidants with additional vitamins and minerals have undergone testing ("The Age-Related Eye Disease Study (AREDS)" 1999). These studies demonstrate a lowered risk of developing an advanced form of AMD (Chew et al. 2014).

For this study the compounds of interest are carotenoids (lutein, zeaxanthin, and beta-carotene), iridoid glycoside (loganin), tannins (punicalagin and chebulagic acid) and stilbenes (resveratrol and pterostilbene). In order to accurately test antioxidant's effectiveness against a stressor, baseline measurements for each have to be performed to determine the cytotoxicity on ARPE-19 WT cells. To do this, an MTS assay was utilized as a means of quantifying cell viability of ARPE-19 WT cells with 22-hour treatments of each antioxidant. It was hypothesised that the antioxidant compounds would have a non-toxic effect on the ARPE-19 WT cells. To test this hypothesis, there were four specific aims:

a. Determine the dose response and optimal treatment concentrations of carotenoids (lutein, zeaxanthin, and beta-carotene), iridoid glycoside (loganin), tannins (punicalagin and chebulagic acid) and stilbenes (resveratrol and pterostilbene) on ARPE-19 WT cells.

b. Investigate cell viability of selected compounds alone and in the presence of SI on ARPE-19 WT cells
c. Investigate ROS levels of selected compounds alone and in the presence of SI on ARPE-19 WT cells
d. Determine the protein expression of antioxidant enzymes and cell death markers with protein blotting techniques on ARPE-19 WT cells

5.3.1 Resveratrol – ARPE-19 WT – MTS Assay

Resveratrol is a polyphenol produced by a variety of plants in response to injury, stress, UV irradiation, or fungal attack (Frémont 2000; Burns et al. 2002; Shishodia and Aggarwal 2005). Resveratrol is found in the skins of some fruits such as grapes, blueberries, mulberries, cranberries, as well as certain teas (Frémont 2000; Burns et al. 2002). Resveratrol has a variety of therapeutic properties including antioxidative, anticarcinogenic, cardioprotective, anti-inflammatory, and anti-aging (Burns et al. 2002). Resveratrol has previously been investigated within *in vitro* and *in vivo* models of eye diseases and illustrates promise in mitigating the damaging effects of oxidative stress.

Resveratrol has been heavily studied across many different cell lines, including ARPE-19 WT cells. Josifovska et al. (2020) investigated ARPE-19 cells exposed to 10 and 50 μM of resveratrol and found that it is an inducer of autophagy, pro-survival, and anti-inflammatory stimuli, contributing to overall cell survival (Josifovska et al. 2020). In

another study by Nashine et al. (2020), ARPE-19 cells were treated with a wide range of delivery methods and extract components of a single 100 μM dose of resveratrol, noting that resveratrol causes an increase in cell viability when compared to control cells (Nashine et al. 2018). Nashine et al. (2020) also investigated resveratrol's effect on ROS and found that treatments of resveratrol resulted in significant decreases in cellular levels of ROS (Nashine et al. 2018), indicating a strong antioxidant ability in conditions where oxidative stress is a driving force.

Initially a wide range of concentrations was selected to begin testing with and created a dose response curve for resveratrol (25, 50, 75, 100, 150, 200, and 250 μM) on ARPE-19 WT cells for 22 hours using the MTS assay. From the results of the MTS assay, resveratrol was found to not reduce cell viability, indicating that the compound itself was not toxic to the cells and could be tested for its effect on oxidative stress and cell death parameters. Based off the results from the MTS assay 50 μM and 100 μM treatments of resveratrol were selected for continued testing with SI.

5.3.2 Pterostilbene – ARPE-19 WT – MTS Assay

Pterostilbene is a naturally occurring di-methylated derivative of resveratrol primarily found in blueberries (Estrela et al. 2013; McCormack and McFadden 2013). Although the mechanism of action of pterostilbene is not fully understood, results from both *in vitro* and *in vivo* studies suggest that its strong antioxidant ability presents great

promise in diseases where oxidative stress is a driving factor (McCormack and McFadden 2013). In order to test the effects of pterostilbene on ARPE-19 WT cell viability, an MTS assay was used to create a dose response curve for pterostilbene (5, 10, 20, 30, 40 μM) for 22 hours. From the results of the MTS assay, pterostilbene exhibited a significant cytotoxic effect on ARPE-19 WT cells. Across all concentrations, cell viability was reduced by over 50%. These results were not what was initially expected, however, in earlier studies from our group, exposing pterostilbene to blue light resulted in cytotoxicity on ARPE-19 WT cells resulting in significant decreases in cell viability (Bel 2020). From the preliminary results, it was decided to not proceed with SI exposure on ARPE-19 WT cells pretreated with pterostilbene due to these findings. There is a lack of evidence of studies using pterostilbene as a treatment on ARPE-19 cells as a protective agent against oxidative stress and this leaves an opportunity for further investigation into the toxic effect pterostilbene can exhibit within these cells.

5.3.3 Lutein – ARPE-19 WT – MTS Assay

Lutein is a naturally occurring carotenoid which belongs to the xanthophylls group and is found primarily in various fruits, vegetables, and egg yolks (Marse-Perlman et al. 2001; Gong et al. 2017). Lutein, is selectively taken up into eye tissues, particularly the macula and lens, where it accumulates at much higher concentrations than in other tissues (Johnson 2014; Norkus et al. 2010). Lutein is known to have strong

antioxidant properties, which has led to increased attention to its potential benefits in promoting eye health and prevention of progression of AMD. The AREDS study ("The Age-Related Eye Disease Study (AREDS)" 1999) was one of the first to highlight lutein's benefits in eye health. The results of the MTS assay found that lutein (10, 25 μM) treated ARPE-19 WT cells illustrated a significant reduction in cellular viability.

Du et al. (2018) reported that lutein (1-50 μM) treated ARPE-19 cells are less susceptible to reductions in cell viability when exposed to H_2O_2 (Du et al. 2018). Although no other literature has found the same degree of reduction in cellular viability that was observed in this study when coupled with a stressor like H_2O_2 or SI, lutein still shows superior antioxidant ability to mediate the damages caused by oxidative stress.

5.3.4 Punicalagin – ARPE-19 WT – MTS Assay

Punicalagin is a tannin commonly found in the seeds of pomegranates which has shown significant promise due to its antioxidant potential, and role in mitigating various diseases (Zhong, Reece, and Yang 2015). Punicalagin has been found to promote cell autophagy and protect cells from stress-induced cellular apoptosis, indicating its strong potential for cellular protection (Ying Wang et al. 2016). There are only a few studies on the effects of punicalagin in ARPE-19 cells. Clementi et al. (2021) reported a decreases in ROS levels and increases in cell viability when ARPE-19 cells were pre-treated with punicalagin and exposed to H_2O_2 (Clementi et al. 2021). Results from this study provide promise into the possible protective mechanism of punicalagin for AMD

where oxidative stress is a factor in progression. Using an animal model, Lin et al. (2001) has investigated the protective effects of punicalagin on rat livers and reported that at low doses, punicalagin offers protective effects but at higher doses it proved to be cytotoxic and enhanced liver damage (C. Lin et al. 2001).

The results using the MTS assay showed no significant differences in cell viability for all concentrations (5, 10, 20 μM) of punicalagin. This indicates that the compound itself illustrated no toxic effects to the ARPE-19 WT cells.

5.3.5 Loganain – ARPE-19 WT – MTS Assay

Loganin is an iridoid glycoside, originally found in *Cornus officinalis* (a Japanese cornelian cherry tree) which has been the subject of extensive research due to its potential antioxidant properties and role in mitigating oxidative stress in various diseases (Park et al. 2021). Loganin has shown promise in offering biological activities including immunomodulation, antioxidant, and anti-inflammation (X. Chen et al. 2023). To our knowledge, there is no published data investigating the biological activity of loganin in ARPE-19 cells.

The results of the MTS assay with loganin (20, 40 μM) illustrated that there were no significant differences in cell viability between the treatments and the control cells. These results indicate that the compound itself illustrates no toxic effects to the ARPE-19 WT cells. However, in other *in vitro* studies, loganin has been linked to proliferation and

cytoprotective effects (C. Gao et al. 2017; Park et al. 2022; Cheng et al. 2020). Park et al. (2022) investigated the protective effects of loganin on HaCaT keratinocytes and found that 7.5 and 12.5 μM treatments of loganin resulted in no significant reduction in cell viability, however, at 15 μM, a reduction in cell viability was observed (Park et al. 2022). Differences in the concentrations and toxicity of loganin are likely due to the difference in cell lines used. Additionally, HaCaT keratinocytes may have more sensitivity to compounds when compared to ARPE-19 WT cells, however, this has not been tested.

5.3.6 Chebulagic Acid – ARPE-19 WT – MTS Assay

Chebulagic acid is a tannin found in the fruit of *Terminalia chebula*, a native tree to parts of South Asia, which is commonly referred to as chebulic myroblan (H. Gao et al. 2008). Chebulagic acid has been identified as an active inhibitor of xanthine oxidase with antibacterial and antifungal effects (Kongstad et al. 2014). Chebulagic acid has been investigated for its anti-fungal and neuroprotective effects at a wide range of concentrations (Kongstad et al. 2014; Kim et al. 2014). Although no study has investigated the effects of chebulagic acid on ARPE-19 cells, investigations in other cell lines have taken place. Cell viability assays of chebulagic acid at low concentrations (0.5 to 5 μg/μL) on vero cells (African green monkey kidney cells) were investigated and exhibited a significant reduction in cell viability (Kesharwani et al. 2017). A dose response curve was created for chebulagic acid (5, 20, 40 μM) for 22 hours to study cell

viability. All concentrations of chebulagic acid illustrated a significant reduction in the cellular viability of ARPE-19 WT cells. The slight reduction in cell viability could be due to the strong antioxidant capabilities of the compound and requires further experimentation on ARPE-19 WT cells in order to fully understand the mechanisms occurring within the cells. The results from the study by Kesharwani et al. (2017) and this current study suggest that the reduction in cell viability is a common response with this antioxidant.

5.3.7 Zeaxanthin – ARPE-19 WT – MTS Assay

Zeaxanthin is a naturally occurring carotenoid which belongs to the xanthophylls group and is found primarily in various fruits, vegetables, and egg yolks (Marse-Perlman et al. 2001; Gong et al. 2017). As an analog of lutein, zeaxanthin offers strong potential as an antioxidant in diseases like AMD where oxidative stress plays a critical role. In the literature, zeaxanthin has shown great promise as an antioxidant in cell culture conditions. Rozanowska et al. (2022) examined the efficacy of zeaxanthin, in protecting RPE cells against light-induced toxicity of vitamin A aldehyde (Różanowska, Czuba-Pełech, and Różanowski 2022). The supplementation of cells with zeaxanthin increased cell survival, indicating a potential protective effect against light-induced toxicity (Różanowska, Czuba-Pełech, and Różanowski 2022). A dose response curve of zeaxanthin (1, 2.5, 5, 10, 20, 30 μM) was created and the results illustrate that zeaxanthin

significantly increases the ARPE-19 WT cell viability at concentrations 10, 20, and 30 μM. Increases in cell viability were seen across all concentration, however, these higher concentrations illustrated the most significant results compared to that of the control cells. The results from this study are in alignment with the results from the study on RPE cells by Różanowska, Czuba-Pełech, and Różanowski (2022), further supporting the idea that zeaxanthin has strong antioxidant capacity and that it could offer promise as a therapeutic in conditions where oxidative stress is a key player.

5.3.8 Beta-Carotene – ARPE-19 WT – MTS Assay

Beta-carotene, a precursor to vitamin A, has been extensively studied for it's role as a potential antioxidant in eye diseases ("The Age-Related Eye Disease Study (AREDS)" 1999) as well as other pathophysiological conditions. The initial AREDS study had beta-carotene as a main component ("The Age-Related Eye Disease Study (AREDS)" 1999). Following conclusion of the AREDS study, beta-carotene was associated with an increased risk of lung cancer and other harmful outcomes in persons at high risk of lung cancer and later replaced in AREDS2 formulation with lutein and zeaxanthin (O'Connor et al. 2022; Chew et al. 2014). In a recent study, the oxidative product of beta-carotene, beta-apo-8'-carotenal, was shown to induce DNA damage on A549 cells (a lung epithelial cell line) that were pre-treated with beta-carotene then exposed to benzo[a]pyrene resulting in increased cellular apoptosis (Yeh and Wu 2006).

Furthermore, in cell culture models Meganathan et al. (2022) investigated the effect of carotenes, including beta-carotene, against oxidative stress-induced AMD in human RPE cells (Meganathan et al. 2022). Their study found that carotenoids at concentrations between 1.56 and 25 μg/mL were successful in protecting ARPE-19 WT cells against H_2O_2 induced RPE cell damage (Meganathan et al. 2022). Beyond its antioxidant potential, Choo et al (2022) highlighted beta-carotene's potential in inhibiting DNA damage which is typically associated with diseases of the eye (Choo et al. 2022).

From the results of the MTS assay, beta-carotene (2.5, 5, 10, 20, 40 μM) had no significant effects on cell viability in ARPE-19 WT cells. This indicates that beta-carotene itself is not cytotoxic to the cells and can be confidently used as a possible source of antioxidant in further experiments.

5.3.9 Optimization Summary and Conclusion

In an effort to determine possible antioxidant compounds that would be of therapeutic benefit to conditions of AMD, this study started with eight compounds of various concentrations (selected based of existing literature mentioned previously). The compounds of interest were resveratrol, pterostilbene, punicalagin, loganin, chebulagic acid, lutein, zeaxanthin, and beta-carotene. From these eight compounds, pterostilbene illustrated strong cytotoxic effects which resulted in significant reductions ($p<0.05$) in cell viability across all concentrations tested. Chebulagic acid illustrated slight reductions in cell viability across all concentrations tested, which aligned with what was

previously reported in the literature for this compound. Lutein at 10 and 25 μM concentrations illustrated slight reductions in cell viability, also in alignment with what has been previously published in the literature. Beta-carotene illustrated cytotoxic effects and a reduction in cell viability at the two higher concentrations tested (20 and 40 μM). At lower concentrations (2.5, 5, and 10 μM) beta-carotene illustrated cellular proliferation effects and resulted in an increase in cell viability. Zeaxanthin illustrated proliferative effects and an increase in cell viability when ARPE-19 WT cells were treated with 10, 20, and 30 μM concentrations of zeaxanthin. Resveratrol, punicalagin, and loganin illustrated no significant differences between the treatment and the control cells, indicating that no proliferative or cytotoxic effects were observed in the ARPE-19 WT cells. From the results of the compound optimization, all compounds, with the exception of pterostilbene, were tested as a pre-treatment on ARPE-19 cells for 4 hours prior to exposing the cells to SI for 18 hours in order to test the protective effects of these compounds in an *in vitro* model for AMD.

5.4 Selected Compounds and Sodium Iodate

As mentioned prior, test compounds were optimized on ARPE-19 WT cells alone to determine toxicity levels prior to exposing pre-treated cells to SI. From the optimization it was determined that resveratrol, punicalagin, loganin, chebulagic acid, lutein, zeaxanthin, and beta-carotene would be subject to testing with SI. When testing

with SI it was hypothesized that the strongest antioxidants will significantly increase cell viability over the corresponding control of SI.

5.4.1 Resveratrol and Sodium Iodate – MTS Assay

Resveratrol illustrated no cytotoxic effects on ARPE-19 WT cells when treated alone. When ARPE-19 WT cells were pre-treated for 4 hours with resveratrol and then exposed to 18 hours of SI, surprisingly, resveratrol had no significant effect on cell viability. Qin et al. (2014) demonstrated that resveratrol (5, 10, and 25 μM) protects RPE cells from SI (5 mM) (Qin, Lu, and Rodrigues 2014). The results found by Qin et al. (2014) were measurements from an WTS1 cell viability assay, possibly explaining the discrepancy between this study's results and theirs. In their study, SI was used in low concentrations (2.5 mM and 5 mM), and they found significant reduction in cell viability when cells were treated with 5 mM of SI (Qin, Lu, and Rodrigues 2014). In this study, the results indicate that a 15 mM SI exposure results in significant decreases in cell viability via MTS assay. Some of the differences observed between the study by Qin et al. (2014) and this study demonstrate the importance of accurately reporting cell culture conditions when investigating antioxidant enzymes and stress susceptibility, particularly with ARPE-19 cells.

5.4.2 Lutein and Sodium Iodate – MTS Assay

Lutein's antioxidant potential has been well documented in both *in vitro* and *in vivo* studies. ARPE-19 WT cells have been shown to have a 2–14-fold concentration of lutein within the cells compared to concentrations detected in the medium, indicating active uptake by the cells (Bian et al. 2012a). When ARPE-19 WT cells were pre-treated for 4 hours with lutein (10 and 25 μM), then exposed to SI for 18 hours, cell viability was maintained when compared to the SI treated control ARPE-19 WT cells. SI exposure resulted in significant decreases in cell viability. From these findings, lutein showed promising protective effects in an ARPE-19 model of SI induced retinal cell damage.

In the study by Bian et al. (2012) in a model of blue light exposure, 10 μM pre-treatment of lutein resulted in attenuation of significant cell death in control cells (Bian et al. 2012a). In a model of H_2O_2 induced ARPE-19 cellular damage, 10 μM treatments of lutein significantly increased cellular viability when compared to the H_2O_2 treated control cells (V. B. Toragall and V 2020).

5.4.3 Punicalagin and Sodium Iodate – MTS Assay

When ARPE-19 WT cells were pre-treated for 4 hours with punicalagin then exposed to SI for 18 hours, across both concentrations (10 and 25 μM) of punicalagin,

cell viability was significantly decreased when compared to SI treated control cells. Across all concentrations of punicalagin and SI, cell viability was reduced by roughly 80%. The reduction in cell viability could be due to a significantly toxic by-product emitted by the reaction of punicalagin and SI. The large structure of punicalagin offers the possibility for side reactions and decomposition reactions, which could lead to this toxic effect. Although not confirmed in literature, the possibility of interaction between SI and punicalagin could result in formations of oxidation by-products more toxic than punicalagin on its own.

In the literature, punicalagin has been investigated in ARPE-19 WT cells,; however, SI has not been used as a stressor in these experiments. One study investigated the effects of a 24 hour pre-treatment with 1 to 40 μM of punicalagin then exposure to H_2O_2 for 24 hours which resulted in decreases in ROS levels and increases in cell viability indicating its protective effect (Clementi et al. 2021). However, in this study, the results do not align. The differences between stressors used and timepoints of pre-treatment could hold clues into success of punicalagin as an antioxidant on ARPE-19 cells. However, further experiments and testing will need to be conducted to determine if this antioxidant truly does have protective effects in models of AMD.

5.4.4 Loganin and Sodium Iodate – MTS Assay

Loganin has shown strong antioxidant properties in mitigating oxidative stress in numerous cell models. In this study, when ARPE-19 WT cells were pre-treated for 4 hours with loganin (20 and 40 μM) then exposed to SI for 18 hours, the combination of 40 μM of loganin and 17.5 mM of SI was the only condition which illustrated an maintenance of cell viability. Loganin has a relatively low water solubility which would directly translate to relatively low bioavailability, rendering higher concentrations minimally beneficial for therapeutic effects (R. Xu et al. 2018).

Park et al. (2022) investigated the protective effects of loganin on HaCaT keratinocytes from H_2O_2 induced cellular injury. In their study, 7.5 and 12.5 μM treatments of loganin were able to attenuate damages caused by exposure to 250 μM of H_2O_2 for 24 hours (Park et al. 2022). Gao et al. (2017) demonstrated that treatments of 10 to 60 μg/mL of loganin can promote Schwann cell proliferation and attenuate TNF-α-mediated reductions in cell viability measured via MTS assay (C. Gao et al. 2017). Results from these studies offered promise to loganin's ability to attenuate cellular damages caused by diseases where oxidative stress plays a role.

5.4.5 Chebulagic Acid and Sodium Iodate – MTS Assay

In the literature, a large gap in the antioxidant ability of chebulagic acid pre-treatments prior to oxidative injury exists. No studies could be found investigating the effects of chebulagic acid against H_2O_2 or SI induced injury in ARPE-19 WT cells or any

other cell type. When ARPE-19 WT cells were pre-treated for 4 hours with chebulagic acid then exposed to SI for 18 hours, across all concentrations (5, 20, 40 μM) cell viability was significantly reduced when compared to control cells treated with SI. SI exposure caused a linear decrease in cell viability as observed across all experiments. Control treatments of chebulagic acid illustrated no significant deviation from the preliminary optimization testing. However, when pre-treated with chebulagic acid and then exposed to SI, cell viability was decreased significantly across all treatments and conditions. Again, as with punicalagin, there may be possible side reactions or decompositions of the compound due to its large structure and interaction with SI. Although not confirmed in literature, the possibility of interaction between SI and chebulagic acid could result in formations of oxidation by-products more toxic than chebulagic acid on its own. Further research should be conducted to better understand if chebulagic acid is a possible antioxidant compound for diseases where oxidative stress plays a strong role.

5.4.6 Zeaxanthin and Sodium Iodate – MTS Assay

In the literature, zeaxanthin has shown strong antioxidant abilities on ARPE-19 WT cells by increasing cell viability when oxidative stressors have been applied. Zeaxanthin has not been investigated in a study using SI as a stressor, however, Zou et al. (2014) treated ARPE-19 cells to 10 μM of zeaxanthin prior to inducing oxidative

damage through a tert-butyl hydroperoxide model of cellular injury (Zou et al. 2014). From their results, 10 μM treatments of zeaxanthin resulted in a cytoprotective result on ARPE-19 cells. Their findings concluded that zeaxanthin was able to activate Nrf2-mediated phase 2 enzymes, enhancing the anti-oxidative capacity and preventing cell death (Zou et al. 2014). Kalariya et al. (2008) investigated the possibility of carotenoids undergoing oxidation in ocular tissue forming carotenoid derived aldehydes (CDA) (Kalariya et al. 2008). From this study, CDA derived from zeaxanthin can result in a build-up of reactive oxygen species and result in an increase in cellular apoptosis on ARPE-19 WT cells (Kalariya et al. 2008) possibly explaining the decrease in cell viability on cells pre-treated with the higher concentrations of zeaxanthin then exposed to SI. From these results, zeaxanthin holds tremendous promise as a supplement for diseases where oxidative stress plays a crucial factor.

When ARPE-19 WT cells were pre-treated for 4 hours with zeaxanthin then exposed to SI for 18 hours, cell viability was significantly increased in concentrations lower than 10 μM (1, 2.5, 5 and 10 μM) when compared to control cells treated with SI alone. SI exposure caused a linear decrease in cell viability in these concentrations and zeaxanthin was able to increase the cellular viability beyond the controls treated with SI. At higher pre-treatment concentrations of zeaxanthin (20 and 30 μM) followed by exposure to SI, the protective effects of zeaxanthin were lost. Specifically, 20 and 30 μM pre-treatments of zeaxanthin saw significant reductions in cellular viability when

exposed to 12.5, 15, and 17.5 mM SI. Results from the MTS assay of zeaxanthin suggest a fine balance between the beneficial concentration to toxic concentration of zeaxanthin. Based on the results found in the cell viability study, concentrations of 2.5 and 10 μM zeaxanthin were selected to move forward with future experiments as these concentrations illustrated a range of a low dose and a high dose of zeaxanthin without causing toxic cellular effects offering a maintained cellular viability.

5.4.7 Beta-carotene and Sodium Iodate – MTS Assay

Beta-carotene exhibited an increase in cellular viability at 2.5 and 5 μM on ARPE-19 WT cells alone, but a decrease in cell viability at the higher concentrations (20 and 40 μM). When ARPE-19 WT cells were pre-treated for 4 hours with beta-carotene then exposed to SI for 18 hours, 2.5 and 5 μM concentrations of beta-carotene resulted in a significant cytoprotective maintenance of cell viability when compared to control cells treated with SI alone. At higher concentrations of beta-carotene, cell viability was reduced as observed in the earlier MTS experiments and supported by the study described by Kalaryia et al. (2008). 5 μM pre-treatments of beta-carotene resulted in a 43% increase in maintenance of cell viability when compared to an exposure of 15 mM of SI alone.

Meganathan et al. (2022) investigated the effect of beta-carotene, against oxidative stress-induced AMD in human RPE cells (Meganathan et al. 2022). Their

study found that beta-carotene at concentrations between 1.56 and 25 μg/mL were successful in protecting ARPE-19 WT cells against a model of H_2O_2 induced RPE cell damage (Meganathan et al. 2022). In a study by Yeh et al (2006) an oxidative product of beta-carotene, beta-apo-8′-carotenal, was shown to induce DNA damage on A549 cells resulting in more cellular apoptosis (Yeh and Wu 2006). Furthermore, Kalariya et al. (2008) investigated the possibility of carotenoids undergoing oxidation in ocular tissue forming carotenoid derived aldehydes (CDA) (Kalariya et al. 2008). From their study, CDA derived from beta-carotene was found to result in a build-up of ROS and ultimately lead to an increase in cellular apoptosis on ARPE-19 WT cells (Kalariya et al. 2008).

5.4.8 Compound and Sodium Iodate Summary and Conclusion

In conclusion, the effects on cellular viability of seven compounds were investigated across multiple concentrations. Resveratrol, punicalagin, loganin, chebulagic acid, lutein, zeaxanthin, and beta-carotene were tested on ARPE-19 WT cells with pre-treatments for 4 hours and exposure to SI concentrations (12.5, 15, 17.5 mM) for 18 hours. From the results, the compounds of lutein, zeaxanthin, and beta-carotene were selected as the most promising supplements to further investigate. Concentrations of SI were focused to 15 mM and 17.5 mM due to the significant decrease in cell viability compared to their controls. Lutein concentrations were narrowed down to 10 and 25

μM, zeaxanthin was selected as 2.5 and 10 μM, and beta-carotene was used at 5 and 20 μM. These concentrations would be the subject of all future experiments and encompass both a high and a low concentration for each compound of interest.

5.5 Measurement of Intracellular ROS – ARPE-19 WT – CMH2-DCFDHA Assay

A CMH2-DCFDHA assay was used to measure intracellular ROS levels in ARPE-19 cells pre-treated with either lutein, zeaxanthin, or beta-carotene in the presence and absence of SI.

5.5.1 Lutein and Sodium Iodate – CMH2-DCFDHA Assay

Results from the pre-treatment with lutein resulted in a significant reduction in intracellular ROS when compared to the non-treated SI controls. These results indicate that lutein's antioxidant ability is working to detoxify Reactive Oxygen Intermediates (ROI) within the ARPR-19 WT cells exposed to SI. The results are supported by the literature whereby lutein offers protective effects in SI induced models of RPE cell damage (V. B. Toragall and V 2020). They also reported a significant decrease in ROS levels within the lutein treated cells. Chae et al. (2018) also confirmed similar findings to this studies CMH2-DCFDHA assay where lutein (5, 10, and 20 μM) was able to mitigate the oxidative damages caused by oxidative stress induced by H_2O_2 (Chae, Park, and Park 2018). All of these results offer strong proof that the antioxidant potential of lutein especially in cells treated with an oxidative agent.

5.5.2 Zeaxanthin and Sodium Iodate – CMH2-DCFDHA Assay

To investigate intracellular ROS within ARPE-19 WT cells were pre-treated with zeaxanthin for 4 hours then exposed to SI for 18 hours, and a CMH2-DCFDHA assay was performed. Results from the pre-treatment with 2.5 and 10 μM of zeaxanthin and 15 mM of SI resulted in a significant increase in cellular viability over the control. At 17.5 mM exposure to SI, 10 μM of zeaxanthin resulted in a significant increase in cellular viability when compared to the control. At 17.5 mM SI exposure, 2.5 μM of zeaxanthin pre-treatment was able to decrease intracellular ROS levels by 89% and 10 μM pre-treatment of zeaxanthin was able to reduce ROS levels by 90% when compared to the 17.5 mM SI control.

Results from the intracellular ROS levels agree with the findings of Zou et al. (2014) that concluded that zeaxanthin was able to enhance the anti-oxidative capacity and prevent cell death (Zou et al. 2014). Results are also in agreement with Różanowska et al. (2022) who found that supplementation of cells with zeaxanthin increased cell survival, indicating a potential protective effect against light-induced toxicity (Różanowska, Czuba-Pełech, and Różanowski 2022). Although both models use different stressors to the SI model, mitigation of oxidative stress is a characteristic of zeaxanthin and offers great promise as a treatment for oxidative stress induced diseases in the future.

5.5.3 Beta-carotene and Sodium Iodate – CMH2-DCFDHA Assay

Results from the pre-treatment with beta-carotene illustrated a significant reduction in intracellular ROS when compared to the non-treated SI controls. In control cells treated with just beta-carotene (5 or 20 μM) a significant increase in ROS levels was observed. In the literature, Kalariya et al. (2008) investigated the possibility of carotenoids undergoing oxidation in ocular tissue forming carotenoid derived aldehydes (CDA) through the process of oxidation-reduction and isomerization reactions (Kalariya et al. 2008; Mein et al. 2011). From this study, CDA derived from beta-carotene can result in a build-up of reactive oxygen species and result in an increase in cellular apoptosis on ARPE-19 WT cells (Kalariya et al. 2008). The mechanism by which CDA's cause cell death is not fully known, however it could be through a combination of apoptosis and necrosis resulting in genotoxic effects on cell membrane structure (Kalariya et al. 2009).

In the experiments with beta-carotene, the decrease in cell viability at higher concentrations could be due to formation of CDA's resulting in higher toxicity to cells represented by MTS assay. When ARPE-19 WT cells were treated with beta-carotene alone, CMH2-DCFDHA assay indicated increases in intracellular ROS. Increases in intracellular ROS can suggest the formation of CDA's in control settings, however excess stress present from external factors, like SI, may cause increased detoxification of ROS from the external factors rather than production of CDA's. To investigate this

possibility, time course studies investigating the deterioration and breakdown of beta-carotene need to be performed.

5.5.4 Measurement of Intracellular ROS – ARPE-19 WT – Summary and Conclusion

By investigating intracellular ROS via CMH2-DCFDHA assay, pre-treatments of lutein, zeaxanthin, and beta-carotene were able to reduce levels of ROS when exposed to SI. Development of higher intracellular ROS levels in control treatments of the compounds could be due to the formation of CDA's and by-products formed in the process of oxidation-reduction and isomerization reactions of the carotenoid compounds (Kalariya et al. 2009; Mein et al. 2011). Lutein concentrations of 10 μM and 25 μM illustrated successful reductions in ROS levels when exposed to 17.5 mM of SI. However, when exposed to 15 mM of SI, the 25 μM lutein pre-treatment illustrated a significant reduction in ROS. Across all concentrations (2.5 μM and 10 μM) zeaxanthin significantly reduced ROS levels when compared to corresponding controls. Beta-carotene offered maintenance of cell viability and reduction in ROS levels when pre-treated with 20 μM and exposed to 17.5 mM of SI. These findings indicate the strong antioxidant ability of lutein and zeaxanthin; however, they also open the door for questions pertaining to the possibility of the generation of more reactive intermediates and the generation of more cellular stress. Further experiments into these mechanisms

will need to be conducted in order to truly understand the mechanisms of these antioxidants.

5.6 Protein Analysis of ARPE-19 WT Via Western Blot

In order to investigate markers of antioxidants (MnSOD and catalase) and cellular apoptosis (caspase-3), Western blots were performed on cellular lysates prepared from ARPE-19 WT cells. Cells were treated for 4 hours with specific concentrations of each antioxidant compound, then exposed to 18 hours of SI at either 15 or 17.5 mM.

5.6.1 Lutein – ARPE-19 WT – Western Blot

Pre-treatments of lutein (10 and 25 μM) were investigated on ARPE-19 WT cells with respect to protein concentrations of MnSOD, Catalase, and Caspase-3. From the results, protein concentrations of MnSOD, Catalase, and Caspase-3 had no significant findings when pre-treated with lutein (10 and 25 μM) prior to exposure to SI (15 and 17.5 mM).

MnSOD is a major ROS scavenging molecule and catalyzes the detoxification of superoxide radical (Miriyala et al. 2012). MnSOD levels were found to be unchanged in the experiments with 10 μM of lutein whereas in the 25 μM treatments of lutein, protein levels were found to decrease from the control when treated with SI. This result has not been observed in the literature. Conversely, Chang et al. (2021) noted an increase in MnSOD levels when ARPE-19 cells were exposed to 6 mM of SI. Differences in the

experiments between this studies testing methodologies and those utilized by Chang et al. (2021) include both the timing of exposure and the concentration of SI. These differences could indicate the need for further investigation into the optimal timing to investigate SI induced stress response.

Catalase is a major enzyme in the detoxification of H_2O_2 from cells. Catalase levels were found to decrease with exposure to SI and H_2O_2. These results indicate that SI can cause an accumulation of ROS, and more specifically H_2O_2, within the cells. This reduction in catalase levels was also documented by another group who investigated the protective effects or quercetin (a natural antioxidant) on SI induced RPE cell damage (Y.-Y. Chang et al. 2021). It is also possible that optimal concentrations of stressors (SI or H_2O_2) or the timing of treatment depleted the available catalase intracellularly within the ARPE-19 WT cells. Furthermore, protein expression levels of MnSOD, Catalase, and Caspase-3 following treatments of lutein are not well documented in the literature, indicating the need for further understanding of the linkages between this compound and its antioxidant response.

Caspase-3 protein expression levels had no significant change in any of the treatment conditions. A recent study reported a significant increase in Caspase-3 protein expression levels in ARPE-19 cells subsequent to SI exposure (Y.-Y. Chang et al. 2021).

5.6.2 Zeaxanthin – ARPE-19 WT – Western Blot

The effects of pre-treatments with zeaxanthin (2.5 and 10 μM) were investigated on ARPE-19 WT cells with respect to protein expression of MnSOD, Catalase, and Caspase-3. Protein expression of MnSOD was increased when cells were treated with SI, and the effects of SI were reduced with pre-treatments of 2.5 μM of zeaxanthin. No changes were found in the treatments with 10 μM of zeaxanthin, indicating that it was contributing to the ROS detoxification. MnSOD upregulation may be an adaptive response to SI induced oxidative stress.

Catalase and Caspase-3 activity has no significant changes when comparing pre-treatments of zeaxanthin followed by exposure to SI with SI exposure alone. A recent study reported that H_2O_2 induced a decrease in catalase when exposed to ARPE-19 cells (V. Toragall, Muzaffar, and Baskaran 2023). Additionally, a study conducted by Chiang et al. (2020) found that Caspase-3 levels were increased with exposure to a stressor of hyperglycemia in ARPE-19 cells (Chiang et al. 2020). The contradicting results may indicate that the cells could be in a dormant stage following the 22 hours of treatment and exposure, resulting in the inability to differentiate changes in protein expression.

5.6.3 Beta-Carotene – ARPE-19 WT – Western Blot

Pre-treatments of beta-carotene (5 and 20 μM) were investigated on ARPE-19 WT cells with respect to protein concentrations of MnSOD, Catalase, and Caspase-3 and results are displayed in. No significant changes in protein expression levels were

observed for MnSOD, Catalase, or Caspase when comparing pre-treated to non-pre-treated ARPE-19 WT cells. Caspase-3 levels were found to decrease with exposure to SI when compared to the control. This decrease is not documented in literature, however, this study only looked at protein expression data, future investigation should look at enzyme activity to provide a holistic understanding of the changes in antioxidants. An increases in caspase-3 levels in ARPE-19 cells exposed to H_2O_2 have been reported (Du et al. 2018). An increase in caspase-3 levels on ARPE-19 cells exposed to SI has also been reported (Y.-Y. Chang et al. 2021). The literature investigating the impact of beta-carotene on MnSOD, Catalase, and Caspse-3 levels is very sparsely documented. Further studies should divulge into the mechanistic interties of beta-carotene and antioxidative and cellular apoptosis pathways.

5.7 ARPE-19 WT Summary

As summarized by results discussed above, SI exposure at both concentrations was established as an inducer of cell death and oxidative stress on ARPE-19 WT cells. Eight compounds were selected and prepared in cell culture media prior to treating cells. Resveratrol, pterostilbene, punicalagin, loganin, chebulagic acid, lutein, zeaxanthin, and beta-carotene were tested for 22 hours on ARPE-19 cells and their toxicities were determined via MTS assays. From the initial results, resveratrol, punicalagin, loganin, chebulagic acid, lutein, zeaxanthin, and beta-carotene displayed minimal toxic effects on ARPE-19 cells and moved forward to testing with SI co-

treatments. ARPE-19 WT cells were pre-treated for 4 hours with two concentrations of each compound. Following the pre-treatment, cells were exposed to either 15 mM or 17.5 mM of SI for 18 hours. Following the 18 hours, MTS assay was performed to quantify cell viability. From the co-treatments of antioxidant and SI, lutein, zeaxanthin, and beta-carotene offered the strongest protective effect on ARPE-19 WT cells and would be the main compounds moving forward in this study.

Intracellular ROS was measured via CMH2-DCFDHA assay and results suggested in co-treatment conditions, all three compounds (lutein, zeaxanthin, and beta-carotene) offered significant reduction in ROS levels when compared to the corresponding controls. Increased levels of ROS can be attributed to the possible formation of CDA's being toxic to the ARPE-19 cells without increased stressors like H_2O_2 or SI present.

Protein levels of antioxidant and apoptosis related proteins were also investigated. It was determined that lutein had no significant changes on MnSOD, catalase, or caspase-3 levels when compared to control. Zeaxanthin pre-treatment resulted in reductions of MnSOD levels, but showed no effect on the catalase or caspase-3 levels. Beta-carotene had no notable effect on levels of MnSOD, catalase, or caspase-3 levels when compared to the controls. From the studies with ARPE-19 WT cells, pre-treatments of lutein and zeaxanthin show the most promising cytoprotective

properties against SI induced RPE cell damage across MTS assay, CMH2-DCFDHA assay, and Western blots investigating antioxidant and cell death markers.

5.8 BIRC5 Knockout – ARPE-19 cell line

Following optimization of and selection of successful antioxidant compounds, as well as optimization of the SI induced *in vitro* model of RPE cell damage, investigation into possible mechanisms of action for each compound were conducted. The BIRC5 pathway has been identified as a major target of many cancer signaling pathways (Cao et al. 2019). It is primarily involved in cell proliferation and immune response related signaling pathways (Jiang et al. 2021; J. Wang et al. 2021). BIRC5 has been identified as a regulator of autophagy, mitosis, apoptosis, migration and invasion in many cancers as reviewed by Adinew et al. 2022 and T. Lin et al. 2019. BIRC5 KO models are commonly used in studying cancer due to its regulation of cell division and inhibition of apoptosis (T. Lin et al. 2019). Although the BIRC5 pathway is not fully understood, its involvement in cell proliferation and immune response pathways makes it a possible mechanism of action for ocular diseases like AMD. Due to the lack of research on ARPE-19 CRISPR Cas-9 KO cells, BIRC5 was selected as a target to study its role in SI induced ARPE-19 cell damage.

An ARPE-19 CRIPSR Cas 9 KO cell line of the BIRC5 (survivin) gene was created by Synthego. Further testing using PCR amplification and restriction enzyme digest was

completed to verify success of the knockout**.** Results indicate that in the cell pool, over 50% have successful KOs of BIRC5.

It is hypothesised that if the antioxidant compound is utilizing the BIRC5 pathway for its antioxidant response, in a KO model, the viability results will exhibit significant reductions compared to the WT controls tested earlier. The specific aim of this study is to investigate how a KO of the BIRC5 gene plays a role in cytoprotective effects of SI on ARPE-19 cells with relation to cell viability, ROS accumulation, and antioxidant ability.

a. Investigate cell viability of selected compounds alone and in the presence of SI on ARPE-19 BIRC5 KO cells
b. Investigate ROS levels of selected compounds alone and in the presence of SI on ARPE-19 BIRC5 KO cells
c. Determine the protein expression of antioxidant enzymes (MnSOD and catalase) and cell death markers (caspase-3) with protein blotting techniques on ARPE-19 BIRC5 KO cells.

5.9 Cell Viability Assay – BIRC5 KO ARPE-19 Cells – MTS Assay

5.9.1 ARPE-19 BIRC5 KO – Lutein – MTS Assay

SI treatments of 15 and 17.5 mM caused a significant reduction of cell viability. The 4-hour pre-treatments with both 10 and 25 μM concentrations of lutein before

exposure to both concentrations of SI caused a significant maintenance of cellular viability.

Based on the results of the MTS assay, it can be inferred that SI has less of a toxic effect on ARPE-19 BIRC5 KO cells compared to ARPE-19 WT cells. This lack of susceptibility may be due to the known involvement of BIRC5 as a inhibitor of autophagy and apoptosis (Adinew et al. 2022; T. Lin et al. 2019). With successful KO of the gene, apoptosis signals cannot be activated by the BIRC5 pathway, resulting in less overall cell death when exposed to stressors such as SI. Protective effects from lutein on ARPE-19 BIRC5 KO cells were found to be in similar proportions to the WT cells, indicating lutein is still offering protection even without the BIRC5 pathway functioning. These results suggest that lutein does not rely solely on the BIRC5 pathway but may work through alternative pathways to confer antioxidant protection and increase cell viability.

5.9.2 ARPE-19 BIRC5 KO – Zeaxanthin – MTS Assay

Both SI treatments of 15 and 17.5 mM caused a significant reduction of cell viability in ARPE-19 BIRC5 KO cells. Cell viability of ARPE-19 BIRC5 KO cells was significantly reduced by pre-treatments of 10 μM zeaxanthin prior to exposure of SI. Although not published in literature, these results suggest that zeaxanthin could be utilizing the BIRC5 pathway as a mechanism of antioxidant protection through

regulation of apoptosis and autophagy. Due to the significant reduction in viability when compared to WT cells treated in the same manor, these results suggest that the BIRC5 pathway is important for zeaxanthin to render its antioxidant capabilities. More investigations into the role of the BIRC5 pathway and zeaxanthin protection needs to be conducted in order to fully determine if zeaxanthin activates the BIRC5 pathway, however our results provide a baseline for future studies.

5.9.3 ARPE-19 BIRC5 KO – Beta-Carotene – MTS Assay

To investigate the effects of beta-carotene on the viability of ARPE-19 BIRC5 KO cells, cells were pre-treated with beta-carotene for 4 hours prior to the addition of 15 and 17.5 mM of SI for 18 hours. SI exposure (15 and 17.5 mM) caused a significant reduction of cell viability in ARPE-19 BIEC5 KO cells. Cell viability was significantly maintained by both concentrations of beta-carotene in the presence of 17.5 mM of SI when compared to WT cells.

These results indicate the likelihood of fewer oxidative intermediates being formed in the oxidation-reduction reaction of beta-carotene. As discussed previously by Kalariya et al. (2008), carotenoids can produce cytotoxic aldehyde intermediates in the reduction of the compound (Kalariya et al. 2008). These cytotoxic aldehyde intermediates are known to be toxic to ARPE-19 cells, reduce cell viability, and increase oxidative stress (Kalariya et al. 2008). Although there is no literature reporting the effect of beta-carotene in ARPE-19 KO cell lines, results from this investigation suggest the

need for further research and understanding on the pathways in which antioxidants confer protection on ARPE-19 cells.

5.10 Intracellular ROS Levels of ARPE-19 BIRC5 KO Cells

5.10.1 ARPE-19 BIRC5 KO – Lutein – CMH2-DCFDHA Assay

While the direct link between BIRC5 and lutein in relation to intracellular ROS has not been explicitly addressed in the literature, the role of BIRC5 in cellular processes and diseases suggests its potential interaction with lutein. Lutein has been shown to be a strong antioxidant across a variety of *in vitro* and *in vivo* models, as well as clinical trials of AMD (Bian et al. 2012b; "The Age-Related Eye Disease Study (AREDS)" 1999; Chew et al. 2014).

In order to investigate levels of intracellular ROS within ARPE-19 BIRC5 KO cells pre-treated with lutein for 4 hours then exposed to SI for 18 hours, a CMH2-DCFDHA assay was performed. Pre-treatment with lutein illustrated a significant reduction in intracellular ROS compared to the non-treated SI controls. SI exposure at both concentrations caused an increase of oxidative stress in ARPE-19 BIRC5 KO cells. Lutein treatment decreased oxidative stress in the KO cells which was similar to the results found in ARPE-19 WT cells. These similarities indicate that lutein is still offering protection to the cells, even though the BIRC5 pathway is not functioning suggesting that the decrease in oxidative stress may be independent of the BIRC5 pathway.

5.10.2 ARPE-19 BIRC5 KO – Zeaxanthin – CMH2-DCFDHA Assay

Treatment with zeaxanthin illustrated a significant increase in intracellular ROS when compared to the non-treated SI controls. SI exposure at both concentrations caused a significant increase of oxidative stress in the ARPE-19 BIRC5 KO cells. Pre-treatment with zeaxanthin at both concentrations increased oxidative stress in ARPE-19 BIRC5 KO cells in the presence of both concentrations of SI. The trends observed in the ARPE-19 BIRC5 KO cells are opposite to the trends in the results observed in ARPE-19 WT cells. This increase indicates the possibility of toxic byproducts or CDA's increasing oxidation within the cells as reported by Kalariya et al. 2008. Moreover, there is a possibility of zeaxanthin working somewhere downstream of the BIRC5 pathway to offer antioxidant protection within the cell.

The increase in ROS with zeaxanthin treatments aligns with the cell viability data in the same BIRC5 KO cells. These results indicate zeaxanthin could be utilizing the BIRC5 pathway as a mechanism of antioxidant protection through regulation of apoptosis and detoxification of ROS. Although no literature currently explains any linkage between BIRC5 and zeaxanthin, there seems to be a strong correlation between the two, indicating that impairment of the pathway has strong implications for cell functionality. Given the absence of direct literature on the topic, further dedicated research is necessary to explore the potential connection between this antioxidant's uptake and the BIRC5 pathway.

5.10.3 ARPE-19 BIRC5 KO – Beta-Carotene – CMH2-DCFDHA Assay

Pre-treatment with beta-carotene illustrated a significant reduction in intracellular ROS compared to the non-treated SI controls. SI exposure caused an increase of oxidative stress at both concentrations. Pre-treatment with beta-carotene in the presence of SI decreased oxidative stress in the BIRC5 KO cells. The trends observed in the CMH2-DCFDHA assay on ARPE-19 BIRC5 KO cells follow similar trends to the results found in WT cells. These similarities indicate that beta-carotene is still able to offer protection, even without the BIRC5 pathway functioning. This suggests that beta-carotene can confer protection through an alternate pathway or mechanism of protection. A recent study highlighted that carotenoids, including beta-carotene, can inhibit the downstream production of inflammatory cytokines by suppressing the NF-κB pathway (Huang et al. 2018). Beta-carotene could also be functioning as an antioxidant under stress conditions to scavenge free radicals that damage cellular organelles and not functioning through a specific pathway.

There are no direct studies available that specifically address the relationship between beta-carotene and the BIRC5 pathway. Given the absence of direct literature on the topic, further dedicated research is necessary to explore the potential connection between beta-carotene and the BIRC5 pathway.

5.11 Protein Expression of ARPE-19 BIRC5 KO Via Western Blot

In order to investigate markers of antioxidants (MnSOD and catalase)and cellular apoptosis (caspase-3), Western blots were performed on cellular lysates prepared from ARPE-19 BIRC5 KO cells.

5.11.1 Lutein – ARPE-19 BIRC5 KO – Western Blot

Pre-treatments of lutein were investigated on ARPE-19 BIRC5 KO cells with respect to protein expression of MnSOD, catalase, and caspase-3. Protein expressions of MnSOD, catalase, and caspase-3 remained unchanged when pre-treated with lutein (10 and 25 μM) prior to exposure to SI (15 and 17.5 mM). Although the results of the Western blots illustrate no significant differences, studies have investigated the effects of lutein on the BIRC5 gene with *in vivo* brain injury models (Foster et al. 2017). Foster et al. (2017) noted that lutein altered the expression of several apoptosis related genes, including BIRC5 (Foster et al. 2017). Although no studies have investigated lutein on an ARPE-19 KO model, results showing altered gene expression could be a hint to lutein's pathway of protection, through BIRC5. With the KO cell model of BIRC5, if the gene is inactive or reduced, lutein has no mechanism to confer protection, hence, the lack of significance in the results. Furthermore, the lack of studies on lutein's involvement in the BIRC5 pathway highlight the importance of further investigation as an antioxidative compound and further understanding of the involvement between mechanism of protection and BIRC5.

5.11.2 Zeaxanthin – ARPE-19 BIRC5 – Western Blot

Protein expression levels of MnSOD and catalase remained unchanged when pre-treated with zeaxanthin (2.5 and 10 μM) prior to exposure to SI (15 and 17.5 mM). Caspase-3 displayed a significant decrease in expression levels when comparing a pre-treatment with 2.5 μM of zeaxanthin, prior to exposure to 17.5 mM of SI. This decrease indicates a reduction in cellular apoptosis. Although no studies have directly investigated ARPE-19 BIRC5 KO cells or damaging effects of SI, studies have noted the protection of antioxidant compounds on models of H_2O_2 induced cellular damage. Notably, Du et al. (2018), Ma et al. (2021) and Chiang et al. (2020) reported similar decreases in caspase-3 levels when pre-treated with an antioxidant compound prior to exposure to H_2O_2 (Du et al. 2018b; Chiang et al. 2020; N. Ma et al. 2021). These results and that reported by other investigators suggest that zeaxanthin confers protection to ARPE-19 cells through the anti-apoptotic pathway by which caspase-3 activation is inhibited and consequently downregulates cellular death.

5.11.3 Beta-Carotene – ARPE-19 BIRC5 – Western Blot

Catalase remained unchanged when pre-treated with beta-carotene (5 and 20 μM) prior to exposure to SI (15 and 17.5 mM). Protein expression levels of MnSOD, when pre-treated with 5 μM of beta-carotene, also illustrated no significant change.

However, when pre-treated with 20 μM of beta-carotene, there was a decrease in MnSOD protein expression at 15 mM of SI, and an increase in MnSOD protein expression levels at 17.5 mM of SI. Caspase-3 protein expression levels illustrated a significant reduction when pre-treated with 5 μM of beta-carotene prior to exposure to 15 mM SI. Conversely, when pre-treated with 20 μM of beta-carotene and then exposed to 17.5 mM of SI, a significant increase in caspase-3 levels were observed. While the literature has not documented protein expressions changes in ARPE-19 BIRC5 KO cells in response to beta-carotene, there is one notable study that has looked at the protein response of MnSOD, catalase, and caspase-3 on cells pre-treated with beta-carotene prior to exposure to a stressor. In a study with HUVEC cells, BIRC5 was found to have no significant change in the gene expression of MnSOD, catalase, and caspase-3 when treated with beta-carotene at increasing dosages (Dembinska-Kiec et al. 2005). The results from the study by Dembinska-Kiec et al. (2005) support the possible lack of interaction between beta-carotene and the BIRC5 pathway, resulting in the non-significant changes found in the ARPE-19 WT cell experiments. Further studies should verify these findings; however, it provides a possible explanation to the results seen.

A lack of research on the involvement of the BIRC5 pathway and eye diseases provides possibility of future targets of treatments for AMD. From this study it is evident that antioxidants may modulate the BIRC5 pathway to provide cytoprotective effects on ARPE-19 cells in a SI induced RPE damage model of AMD. Through the use

of ARPE-19 BIRC5 KO cells it was found significant difference in results between WT and BIRC5 KO cells. These significant differences can hint at the possibility of the BIRC5 pathways involvement in protective mechanisms of the antioxidants in ARPE-19 cells. Further research and investigation into the mechanisms of the BIRC5 pathway on ARPE-19 cells need to be investigated, however this study provides promise into the possibility of new targets for RPE cell protection.

5.12 SIRT-1 Knockout – ARPE-19 cell line

SIRT-1 is a protein that has been the center of recent research due to its involvement in numerous biological pathways, with a strong connection to longevity and cell survival. SIRT-1 has been implicated in ocular diseases, such as AMD, by means of reducing oxidative stress and ROS within the eyes (Zhou, Luo, and Zhang 2018b). Multiple SIRT-1 polymorphisms have been proposed as a possible increased risk of development of wet AMD (Kaikaryte et al. 2022). Due to the lack of research on ARPE-19 CRISPR Cas-9 Knockout Cells, SIRT-1 was selected as a possible pathway in which antioxidant compounds may modulate an antioxidant response, conferring a cellular protective response.

An ARPE-19 CRIPSR Cas 9 Knockout cell line of the SIRT-1 gene was created by Synthego Biotech. Further testing using PCR amplification and restriction enzyme

digest were completed to verify the success of the KO. Results from this testing found that in the cell pool, over 50% have successful KOs of SIRT-1.

It is hypothesised that if the antioxidant compound is utilizing the SIRT-1 pathway for its antioxidant response, in a KO model, the results will exhibit significant reductions in cell viability compared to the WT controls tested earlier. The specific aim of this study is to investigate how a KO of the SIRT-1 gene plays a role in cytoprotective effects of SI on ARPE-19 cells with relation to cell viability, ROS accumulation, and antioxidant ability.

a. Investigate cell viability of selected compounds alone and in the presence of SI on ARPE-19 SIRT-1 KO cells
b. Investigate ROS levels of selected compounds alone and in the presence of SI on ARPE-19 SIRT-1 KO cells
c. Determine the protein expression of antioxidant enzymes (MnSOD and catalase) and cell death markers (caspase-3) with protein blotting techniques on ARPE-19 SIRT-1 KO cells.

5.13 Cell Viability Assay – SIRT-1 KO ARPE-19 Cells – MTS Assay

5.13.1 ARPE-19 SIRT-1 KO – Lutein - MTS Assay

SI treatments of 15 and 17.5 mM caused a significant reduction of cell viability. The 4-hour pre-treatments with lutein alone (10 and 25 μM) caused a significant

maintenance of cellular viability in ARPE-19 SIRT-1 KO cells when compared to the SI treated cells alone.

The trends observed in the cell viability assay on ARPE-19 SIRT-1 KO cells follow similar trends to those found in the ARPE-19 WT experiments with lutein. These results indicate that lutein's cytoprotective role is likely not conferring protection through the SIRT-1 pathway. In a recent study, lutein was found to upregulate SIRT-1 in ARPE-19 cells, interfering with the hyperglycemia-induced RPE senescence model (Hwang et al. 2018). In the SIRT-1 KO model of ARPE-19 cells, a decrease in cellular protection from the pre-treatments of lutein prior to SI exposure was not evident. These results indicate that lutein may be modulating multiple pathways to confer protection from oxidative stress or that it could be inducing further downstream signaling molecules that are offering protection. SIRT-1 is an important pathway to investigate due to its unique cytoprotective properties and ability to detoxify ROS.

5.13.2 ARPE-19 SIRT-1 KO – Zeaxanthin – MTS Assay

SI exposure of ARPE-19 SIRT-1 KO cells caused a significant reduction in cell viability with both 15 and 17.5 mM concentrations. Treatment with zeaxanthin alone was found to cause a significant increase in cellular viability in ARPE-19 SIRT-1 KO cells. When pre-treated with both concentrations of zeaxanthin then exposed to SI, cell viability was significantly maintained when compared to cells exposed to SI alone. The

trends observed in the results of the cell viability assay of zeaxanthin on ARPE-19 SIRT-1 KO cells follow similar trends to those seen in the ARPE-19 WT experiments with zeaxanthin. This indicates that zeaxanthin is likely not conferring protection to the cells through the SIRT-1 pathway.

Even though zeaxanthin is an isomer of lutein, studies have not investigated the activation of the SIRT-1 pathway by zeaxanthin specifically. In a study by Sahin et al. (2019), lutein and zeaxanthin treatments were investigated through an *in vivo* model under intense light conditions. In their study, Sahin et al. (2019) found that zeaxanthin was able to offer improved antioxidant capacity under the conditions but they found a decrease in nuclear factor-kappa B (NF-kB) (Sahin et al. 2019). NF-kB is transcription factor that is generally active in many illnesses and has been linked to inflammation and a variety of diseases (T. Liu et al. 2017). Although not reported in ocular tissues, SIRT-1 has been found to modulate the activation of the NF-kB pathways in liver fibrosis (Lee et al. 2019). This modulation could explain the findings of NF-kB downregulation via lutein and zeaxanthin treatment in Sahin et al. (2019), indicating that zeaxanthin could play a role in SIRT-1 modulation in AMD, offering a pathway of interest for development of new treatments. These results, in combination with the findings in this study, can be the starting point for further investigations into the exact mechanism of SIRT-1 and how antioxidants, including zeaxanthin, could be utilized to module many diseases, including AMD.

5.13.3 ARPE-19 SIRT-1 KO – Beta-Carotene - MTS

Beta-carotene is a precursor to vitamin A and has been extensively studied for its role as a potential antioxidant in eye diseases, however, no current study investigates the role of SIRT-1 on beta-carotene's antioxidant potential on ocular function. In lung tissues, SIRT-1 levels have been found to be reduced in smokers, where the oxidative by-products of cigarettes have caused a reduction in SIRT-1 levels (Rajendrasozhan et al. 2008). Iskander et al. (2013) were able to conclude that beta-cryptoxathin, an oxygenated form of beta-carotene, was able to restore the nicotine suppresses levels of SIRT-1 when compared to the control group (Iskandar et al. 2013). Results from Iskander et al. (2013) on a lung tissue model suggest the possibility that more active forms of beta-carotene could offer more potential therapeutics in AMD with future studies on ocular tissue.

In this study, SI treatments (15 and 17.5 mM) caused a significant reduction of cell viability. A 5 μM treatment of beta-carotene prior to exposure to 17.5 mM of SI caused a significant maintenance of cell viability when compared to SI exposed cells alone. Results from the cell viability assay with beta-carotene on ARPE-19 SIRT-1 KO cells are different from ARPE-19 WT cells. Any cellular protection of WT cells offered by beta-carotene with the 5 μM pre-treatment was not observed after exposure to 15 mM of SI. At the higher dosage of SI (17.5 mM), 5μM of beta-carotene conferred protection on

the ARPE-19 cells increasing cell viability when compared to the control. Both treatments of beta-carotene offered little to no significant protection on the ARPE-19 SIRT-1 KO cells, indicating the possibility of beta-carotene conferring an antioxidant response through the SIRT-1 pathway. Although there is a lack of literature linking beta-carotene and SIRT-1, the interaction between the two in protecting against oxidative stress further emphasizes the significance of beta-carotene in maintaining cellular health and combating oxidative damage.

5.14 Intracellular ROS Levels of ARPE-19 SIRT-1 KO Cells

5.14.1 ARPE-19 SIRT-1 KO – Lutein – CMH2-DCFDHA Assay

In order to investigate levels of intracellular ROS within ARPE-19 SIRT1 KO cells, cells were pre-treated with lutein for 4 hours then exposed to SI for 18 hours, after which a CMH2-DCFDHA assay was performed. Results from the pre-treatment with lutein alone illustrated a significant reduction in intracellular ROS when compared to the non-treated SI controls. SI exposure caused an increase of oxidative stress in the ARPE-19 SIRT-1 KO cells. When pre-treated with 10 μM of lutein and exposed to 17.5 mM of SI, intracellular ROS was significantly reduced. When pre-treated with 25 μM of lutein and exposed to both concentrations of SI, intracellular ROS was significantly reduced.

Comparing these results of oxidative stress from ARPE-19 SIRT-1 KO cells with the WT cells, it became apparent that they followed a similar trend. These similarities indicate the unlikelihood of lutein working independently through the SIRT-1 pathway. Chae et al. (2018) also confirmed similar findings to the oxidative stress results where lutein (5, 10, and 20 μM) was able to mitigate the oxidative damages caused by H_2O_2, and this was associated with increased expression of SIRT-1 (Chae, Park, and Park 2018). Given the known link that the SIRT-1 pathway plays in AMD progression, and the results from our study, more investigations into the involvement and mechanisms in SIRT-1 activation should be conducted.

5.14.2 ARPE-19 SIRT-1 KO – Zeaxanthin – CMH2-DCFDHA Assay

Zeaxanthin treatment alone resulted in a significant reduction in intracellular ROS when compared to the non-treated SI controls at both concentrations of zeaxanthin. SI caused an increase in oxidative stress at both concentrations. When pre-treated with zeaxanthin at 2.5 μM then exposed to SI, intracellular ROS was reduced at 17.5 mM. When pre-treated with 10 μM of zeaxanthin then exposed to SI, intracellular ROS was decreased at 17.5 mM.

Oxidative stress results with zeaxanthin on ARPE-19 SIRT-1 KO cells were different from the corresponding results in ARPE-19 WT cells**.** In the SIRT-1 KO model, zeaxanthin offered no protective effects in reducing the intracellular ROS levels on 15 mM of SI exposure. At higher concentrations of SI, ROS levels were reduced

significantly. The lack of protection at lower levels may indicate that zeaxanthin plays a potential role in the SIRT-1 pathway. Although not previously documented in the literature, it is known that zeaxanthin's analog lutein, is able to modulate SIRT-1 expression (Chae, Park, and Park 2018). Future studies should investigate the role of zeaxanthin on SIRT-1 expression with ARPE-19 cells.

5.14.3 ARPE-19 SIRT-1 KO – Beta-Carotene – CMH2-DCFDHA Assay

Beta-carotene treatment alone caused a significant reduction in intracellular ROS when compared to the non-treated SI controls at 17.5 mM of SI. SI caused an increase of oxidative stress at both concentrations. When pre-treated with beta-carotene at 5 μM and then exposed to SI, intracellular ROS was significantly reduced at 15 mM. When pre-treated with 20 μM of beta-carotene then exposed to SI, intracellular ROS was significantly decreased at both 15 and 17.5 mM.

Results from the CMH2-DCFDHA assay with beta-carotene on ARPE-19 SIRT-1 KO cells indicate a deviation from the corresponding WT experiment investigating intracellular ROS. In the SIRT-1 KO model, beta-carotene illustrated a protective effect in reducing the intracellular ROS levels on 15 mM of SI exposure, whereas in the WT

model, no reduction of ROS was observed. Findings from these assays indicate the need to further divulge in the mechanistic pathways of antioxidant compounds and oxidative stress within ARPE-19 cells. The lack of literature on the involvement of beta-carotene on the SIRT-1 pathway does not allow for more conclusions to be drawn from these findings at this time.

5.15 Protein Analysis of ARPE-19 SIRT-1 Via Western Blot

In order to investigate markers of antioxidants and cellular apoptosis, Western blots were performed on cellular lysates prepared from ARPE-19 SIRT-1 KO cells.

5.15.1 Lutein – ARPE-19 SIRT-1 KO – Western Blot

Catalase levels were significantly reduced when pre-treated with 10 μM of lutein prior to exposure to 15 and 17.5 mM of SI, when compared to the controls. Conversely, when pre-treated with 25 μM of lutein and exposed to 15 mM of SI, Catalase protein expression levels were found to increase. Results from the protein expressions that were found for Catalase differ from what has been previously reported in literature. Chang et al. (2021) found decreases in Catalase levels when exposed to SI and increases in

Catalase levels when pre-treated with an antioxidant prior to exposure to SI (Y.-Y. Chang et al. 2021). Lutein has been also been previously investigated in RPE cells and found to upregulate SIRT-1, conferring cellular protection (Hwang et al. 2018). The deviation from the published results indicates an agreement with previous studies of modulation of lutein's protective effect through the SIRT-1 pathways within the ARPE-19 cells. Knockout of the SIRT-1 pathway resulted in deviation from the results observed in the ARPE-19 WT cell line protein expression with pre-treatments of lutein.

5.15.2 Zeaxanthin – ARPE-19 SIRT-1 – Western Blot

The protein expressions of MnSOD, Catalase, and Caspase-3 had no significant changes when pre-treated with zeaxanthin (2.5 and 10 μM) prior to being exposed to SI (15 and 17.5 mM). These results indicate the likelihood that the SIRT-1 pathway is not involved in the cellular protective effects of zeaxanthin. Although zeaxanthin is an analog of lutein and has been found to play a role in SIRT-1 modulation, zeaxanthin has not been documented to provide protection through the same pathways. These gaps in studies require further information and experimentation to understand the links between zeaxanthin and SIRT-1 pathways.

5.15.3 Beta-Carotene – ARPE-19 SIRT-1 – Western Blot

No significant changes were found in MnSOD or catalase protein expression levels when comparing pre-treated to non-pre-treated ARPE-19 SIRT-1 KO cells. Caspase-3 protein expression levels remained widely unchanged. One exception was found, however, with the pre-treatment with 5 μM of beta-carotene and exposure to 17.5 mM of SI, which resulted in a decrease in Caspas-3 levels compared to the control. Results from this experiment indicate that beta-carotene assisted in reducing the Caspase-3 levels in the cell, and in turn, a reduction of overall cellular apoptosis signalling. These results are similar to the findings of the ARPE-19 WT cell treatment conditions, suggesting that beta-carotene is likely not offering cellular protection through the SIRT-1 pathway. Chang et al. (2021) reported similar findings; SI causes an increase in Caspase-3 protein expression levels, whereas pre-treatment with an antioxidant resulted in a decrease of Caspase-3 levels when compared to the control.

Although some studies indicate the involvement of SIRT-1 in ocular diseases, more research needs to be performed in order to fully understand the mechanistic link between the two. From this study, it is noted that SIRT-1 has a strong possibility to function as a protective pathway for antioxidant supplementation in SI induced RPE cell damage. Through the use of ARPE-19 SIRT-1 KO cells it was found that a significant difference in results between WT and SIRT-1 KO cells. These significant differences can hint at the possibility of the SIRT-1 pathways involvement in protective mechanisms of the antioxidants in ARPE-19 cells. Further research and investigation into the

mechanisms of the SIRT-1 pathway on ARPE-19 cells need to be investigated, however this study provides promise into the possibility using SIRT-1 as a target for RPE cell protection.

Chapter 6: Conclusions & Future Directions

6.1 Conclusion

In conclusion, AMD poses a significant concern to the aging population globally, with oxidative stress being a key player in its pathogenesis. This study focused on exploring the potential of groups of antioxidant compounds, carotenoids (lutein, zeaxanthin, and beta-carotene), stilbenoids (resveratrol and pterostilbene), tannins (chebulagic acid and punicalagin), or iridoid glycoside (loganin) as mediators in oxidative stress in retinal cells. Blue light irradiation and chemical stressors were utilized initially for the induction of oxidative stress within ARPE-19 cells. Blue light exposure was omitted from further studies due to the unreliability of results following exposure due to a heat build-up under the blue light panel. SI was selected as the main stressor and was optimized and used as a repeatable inducer of intracellular oxidative stress causing a decreased cell viability. Each antioxidant compound was tested in multiple concentrations alone to determine toxicity on the cells as well as in combination with SI to determine if there was a protective effect. From the testing, lutein, zeaxanthin, and beta-carotene were selected as the main compounds to continue investigation on as they had the most promising effect on cell viability.

CRISPR Cas-9 knockout cells were utilized to further investigate the link between these compounds and the antioxidant response pathway they are associated with. BIRC5 and SIRT-1 knockout ARPE-19 cells were employed to elucidate the association between the compounds and the antioxidant response pathways.

Results indicated that lutein and zeaxanthin exhibited promising cytoprotective properties by enhancing cell viability and reducing intracellular ROS levels in wild-type ARPE-19 cells. However, beta-carotene showed conflicting outcomes, necessitating further investigation at lower concentrations. Beta-carotene's lack of cellular protection may be attributed to the formation of cytotoxic CDA's which can cause significant decreases to cellular viability.

When the BIRC5 pathway was knocked down, zeaxanthin showed a decrease in maintaining cell viability and an increase in intracellular ROS, indicating that the BIRC5 pathway may be important for zeaxanthin to render its antioxidant capabilities. More investigations into the role of the BIRC5 pathway and zeaxanthin protection needs to be conducted in order to fully determine if zeaxanthin activates the BIRC5 pathway. These results from this study provide a baseline for future studies and establish a possible link that has not been identified.

No major changes in trend were found between wild-type cells and SIRT-1 knockdown cells, indicating the compounds' cytoprotection likely is not involved in key factors downstream of the SIRT-1 pathway response. This study underscores the necessity for future investigations into the precise mechanisms through which these compounds confer cellular protection, as highlighted by the results from the Western blot analyses of both wild-type and knockout cells.

6.2 Future Directions

Future studies should include further investigations into the BIRC5 pathway in terms of further understanding the link between BIRC5 and zeaxanthin. Further investigation into other caspase molecules such as caspase 1, caspase 8 and caspase 9 should be investigated due to their involvement in the BIRC5 cellular response. By further investigating the cell death pathways with respect to apoptosis and necroptosis more valuable indicators of cellular protection may be uncovered. Future work with the SIRT-1 knockdown cell line should revolve around the investigation of non-enzymatic antioxidant responses such as reduced glutathione and glutathione disulfide.

Future pathways of interest in future work include the BCL-2 cell death pathway and the KU70 DNA repair pathway. Both pathways may hold a significant role in the oxidative stress response these various compounds with respect to mitigating oxidative stress increase in a SI induced *in vitro* model of AMD. By further understanding the involvement of oxidative stress pathways in ARPE-19 cells, future supplements can be developed to further target pathways of interest and involvement in the development and progression of AMD.

Future experiments should investigate each of the 8 compounds independently and optimize both concentrations and exposure time points in order to determine if there is a possible protective effect. There are thousands of possible other antioxidant

compounds to investigate and others may have more pronounced cytoprotective effects at maintaining cellular viability.

Chapter 7: Limitations and Delimitations

7.1 Limitations

The ARPE-19 cell line has been utilized in various experiments to investigate the effects of oxidative stress inducers such as hydrogen peroxide, blue light, and SI. However, as with all models used in research, it is important to consider the limitations associated with using ARPE-19 cells in research, and specifically in oxidative stress testing (Kaczara, Sarna, and Burke 2010).

One of the primary limitations of this study is the immortalized nature of ARPE-19 cells. Immortalized cell lines may not fully represent the characteristics of primary cells, which could lead to differences in responses to treatments and stressors, in this experiments case, oxidative stress. Additionally, using ARPE-19 cell lines may only partially capture the complexity of the environment within the eye, which includes interactions with neighboring cells and structures. This limitation impacts the translation of findings from ARPE-19 cell studies to *in vivo* situations and this has been acknowledged by other investigators as well (Trakkides et al. 2019). Furthermore, the response of ARPE-19 cells to oxidative stress may be influenced by various factors such as the expression of complement proteins and receptors, and the activation of multiple signaling pathways (Trakkides et al. 2019). These factors may not fully mirror the natural conditions in the retina, potentially limiting the comparison of findings from this study in ARPE-19 cell studies to the complex *in vivo* environment.

Additionally, the use of ARPE-19 CRISPR Cas-9 knockout cells as cell pools is a limitation; however, due to constraints of cost and timing on the ability to get complete knockout clones, cell pools were the only option at the time. For future experiments, pure clones of knockouts will be created limiting the possibility of WT cell deviation in experimental results.

In conclusion, while ARPE-19 and knockout cells have been valuable in studying oxidative stress in RPE cells, it is essential to acknowledge the limitations associated with their use. Careful consideration of these limitations is essential for the accurate interpretation and translation of findings from ARPE-19 cell studies.

7.2 Delimitations

While utilizing an immortalized cell line has limitations that are not able to be fully removed while continuing *in vitro* studies, experimental design attempted to mitigate all other limitations to the best of our abilities. The ARPE-19 cell line may not fully capture the complexity of the *in vivo* environment of the retina, by utilizing a cell line involved in the progression of AMD allows for the closest environment for the condition rather than an unrelated cell line. ARPE-19 cells are a commonly studied cell line for early studies of eye conditions prior to moving into an *in vivo* model.

While oxidative stress is a particularly complex pathway to investigate due to its vast involvement in various biological pathways, our model of inducing oxidative stress changed from blue light exposure to a chemically induced SI model to mitigate variability in the stress response. SI induced ARPE-19 cell damage is an established and reproducible *in vitro* model that allows for an in-depth analysis of the mechanism of bioactive compounds.

Rather than targeting the apoptosis (BIRC5) and cell survival (SIRT1) markers at the protein or RNA level or modulating their function temporarily, this experiment used gene editing technology of CRSIPR-Cas9 that allows permanent disruption of the encoding genes at the DNA level. This minimizes variability in studies that involve long term exposure to any oxidant or antioxidant compounds. Although not a perfect model as a knockout cell pool, utilization of pools with high knockout cell scores and analysis

of knockout cells allowed for mitigation of wildtype takeover of the pools. Through these channels, it was attempted to reduce the number of limitations within the knockout cell pool model.

Chapter 8: References

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