Role of Phenolic compounds on Blue Light Induced Retinal Pigment Cell Damage:

An *in vitro* study

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Abstract

Age-related macular degeneration (AMD) is a progressive eye disease that affects the macula, causing blurred central vision making it difficult for diagnosed patients to see fine details and have trouble with everyday tasks. Recent studies have suggested that blue light is able to cause damage to eyes that progresses to AMD. Exposure of blue light to our eyes is a growing concern in our everyday lives due to the prevalence of computers, phones, and tablets. Blue light is responsible for cell damage, cell death, and oxidative stress, all of which can lead to vision loss. Resveratrol and pterostilbene are polyphenols found in the skin of various fruits including grapes and blueberries and have been well studied for their antioxidant properties. The aim of this study was to investigate the biological activity of resveratrol and pterostilbene on cell viability (MTS assay), cell death markers (Caspase-3/7 activity, Caspase-3 protein expression and Propidium Iodide assay), antioxidant potential (catalase and MnSOD), and the levels of oxidative stress (4HNE), in retinal pigment ARPE-19 cells exposed to blue light. ARPE-19 cells were incubated with a pre-treatment of 50µM and 100µM of resveratrol or 10µM and 250µM of pterostilbene for 4 hours followed by the exposure of blue light (475nm) for 12 hours. Blue light exposure on ARPE-19 cells resulted in a 56% reduction (p<0.0001) in cell viability along with an increase in caspase 3/7 activation, and a 50% increase (p<0.0001) in cell necrosis. This was accompanied by an increase in protein adduct formation of 4HNE and protein expression of antioxidants such as, catalase,

and MnSOD. Resveratrol treatment of 50μM was able to maintain cell viability by 37% ($p < 0.05$), and significantly ($p < 0.05$) reduced the caspase 3/7 fluorescence activity when compared to control cells exposed to blue light. Cellular necrosis was also significantly reduced ($p < 0.05$) by both 50μM and 100μM treatments of resveratrol. In conclusion, the damaging effects of blue light was mitigated by treatments of resveratrol in ARPE-19 cells. Pterostilbene treatments of 10 and 25μM were unable to maintain cellular viability across both MTS assay and caspase 3/7 fluorescence assay. Both concentrations showed opposite results when compared to the 50 and 100μM treatments of resveratrol.

Lay Summary

This research project was part of the human sciences, specifically in regard to the study of Age-Related Macular Degeneration (AMD). It set out to investigate two naturally occurring polyphenols from grapes and berries, resveratrol and pterostilbene, and their role in offering protection against damage caused by blue light and slowing down the progression of blue light induced AMD. By analyzing the results from various assays, it was concluded that blue light is able to cause significant damage to eye cells. Resveratrol was able to attenuate damages caused by blue light whereas pterostilbene was less effective. Future studies are directed towards investigating the role of these phenolic compounds in combination with currently used AMD treatments to study its synergistic effects.

Acknowledgments

Chapter 1: Literature Review

1.1 The Eye

The human eye has many parts which work together towards the central goal of completing vision. The front portion of the eye is made up of the cornea, iris, pupil, and lens which work together to help focus the light entering the eye onto the back portion of the eye, the retina. When light enters the eye, the cornea bends the rays in order for them to pass through the circular pupil. The iris opens and closes to regulate the amount of light entering the eye. The light then passes through the lens which is able to further bend and change the shape in order to focus them on the retina (Figure 1.1). The retina is a thin layer of tissue that contains millions of light sensing nerve cells called rods and cones (Figure 1.2).
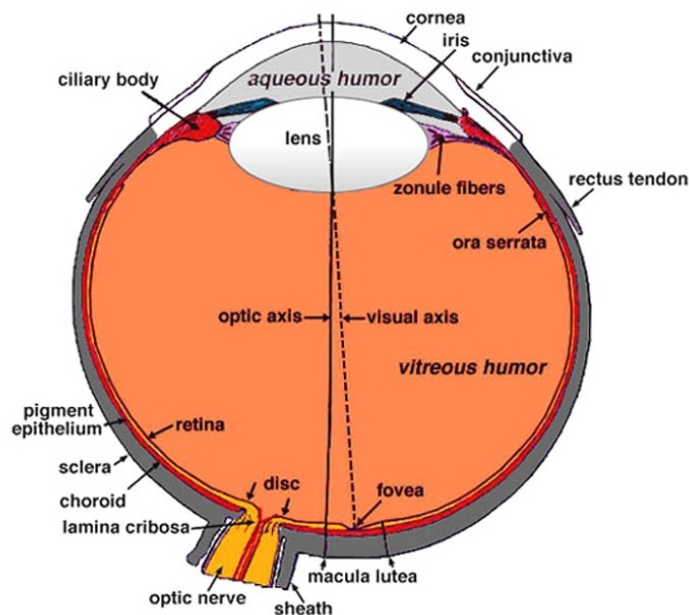
Figure 1.1. Anatomy of the Human Eye: The basic structure of the human eye. Figure from Kolb, H. (2007).
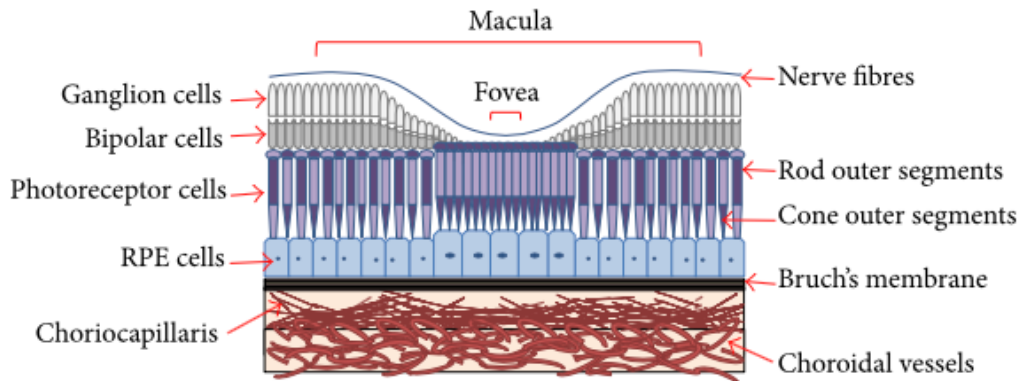
Figure 1.2: Layers of the Human Retina Human retinal layers shown in relation to the macula of the eye. Figure adapted from Al Gwairi, O., et al. 2016.

Photoreceptors are specialized cells within the retina that respond to light, three types of photo receptors are known, rods, cones, and photosensitive retinal ganglion cells (pRGC). Rods are located outside the macula and extend to the outer edge of the retina. Rods allow for peripheral vision as well as assist in motion detection and low light vision. Cones are concentrated at the center of the retina in the macula. Cones allow for vision in bright conditions while also detecting color and fine details.

Posterior to the photoreceptors there is a very thin layer of cells called the Retinal Pigment Epithelial (RPE) cells, which are part of the blood-ocular barrier. RPE cells function to (1) transport nutrients, ions, vitamins, and water, (2) absorb light and protect against photooxidation, (3) convert trans-retinal into 11-cis-retinal, which is essential for the vision cycle, (4) phagocytosis shed of photoreceptor membrane, and (5)

to secrete essential factors for structural integrity of the retina (Crabb, J. W., et al. 2002, Jager, R. D. et al. 2008, Simó, R., 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 functions to separate the retina from the choriocapillaris and also is a vital transport membrane of nutrients and metabolic waste from the RPE cells and photoreceptors (Strauss, O. 2005). Oxygen, electrolytes, nutrients and growth factors destined for RPE cells pass through the choriocapillaris 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, A. et al. 1999, Curcio, C. A. et al. 2013).

In higher mammals, the center of the vision field contains a high concentration of photoreceptors, known as the macula. The macula is the functional center of the eye and is the area where light is focused by the structures in the front portion of the eye, the cornea and lens. In general, the macula takes the picture which is then sent through the optic nerve to the brain where vision can be completed. The overall structure of the macula is important to have good vision, any small disturbance or increased fluid can cause deterioration in vision (Jager, R. D. et al.2008). The macula contains the densest concentration of photoreceptors within the retina which is responsible for the ability to see fine details, read, and recognize faces (Jager, R. D. et al. 2008).

1.2 Macular Dysfunction in Dry and Wet Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a progressive eye condition that affects the macula, causing blurred central vision making it difficult for diagnosed patients to see fine details and have trouble with everyday tasks such as reading and driving (Moutray, T. et al. 2011, Colijn, J. M., et al. 2017). The first change that usually occurs is the formation of drusen between the RPE cells and Bruch's Membrane. Drusen deposits are acellular polymorphous debris and are observed during a funduscopic eye examination as pale, yellowish regions in both the macula and the retina (Crabb, J. W. et al. 2002, Jager, R. D. et al. 2008, Abdelsalam, A. et al. 1999). Drusen formation can range from one or two deposits to several hundred, increasing amounts of deposits correlates with slow loss in vision acuity (Bressler, N. M. et al. 1988, Jager, R. D. et al.2008). There are presently two types of drusen that can be detected in the macula. Hard drusen are typically small and are distinctly separated from one another (Tomi, A. et al. 2014). They typically do not cause serious distortion of vision in small numbers due to their size and separated distribution (Tomi, A. et al. 2014). Soft drusen are typically larger in size and can be harder to distinguish due to the less clearly defined borders (Tomi, A. et al. 2014). Soft drusen deposits can increase some patients' risk for developing AMD. Both hard and soft drusen develop as a natural process with aging, its exact relationship is not fully understood, however, having many soft drusen deposits is a common symptom of dry AMD (Bressler, N. M., et al. 1988).

In contrast to the slow progressive changes in dry AMD, wet or exudative AMD

is a rapid changing process. Wet AMD develops when there is the formation of blood

vessels from the choroid layer into the macula, called choroidal neovascularization

(Noble, J. et al. 2010, Bressler, N. M., et al. 1988). These abnormal blood vessels can

easily break and leak fluid and blood into the layers of the retina (Noble, J. et al. 2010,

Bressler, N. M., et al. 1988). Fluid buildup can collect between the RPE layer and Bruch's

membrane causing a bump in the RPE cell (Figure 1.3), altering the shape which leads

to photoreceptor damage and severe vision loss. Figure 1.3 depicts the structural

changes that occur in patients diagnosed with AMD.
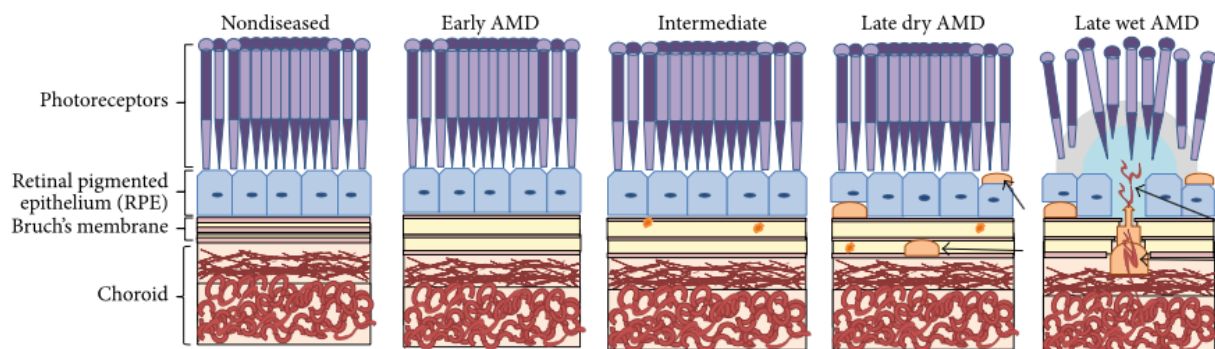
Figure 1.3. Structural Changes During AMD Progression. A normal retina can be seen
on far left. Early AMD shows thickening of Bruch's membrane. Intermediate AMD
shows small drusen deposits forming. Late Dry AMD shows small and large drusen
deposits effecting Bruch's Membrane as well as the RPE cells. 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.

The onset of wet AMD is much more rapid than the dry form; however, it is typical for dry AMD to be a precursor to wet AMD (Ambati, J., et al. 2012). Although it is typical for dry AMD to occur prior to the wet form, both forms of AMD are not mutually exclusive. Figure 1.4 shows the differences seen between wet and dry forms of AMD clinically (Acharya, U. R., et al. 2016)
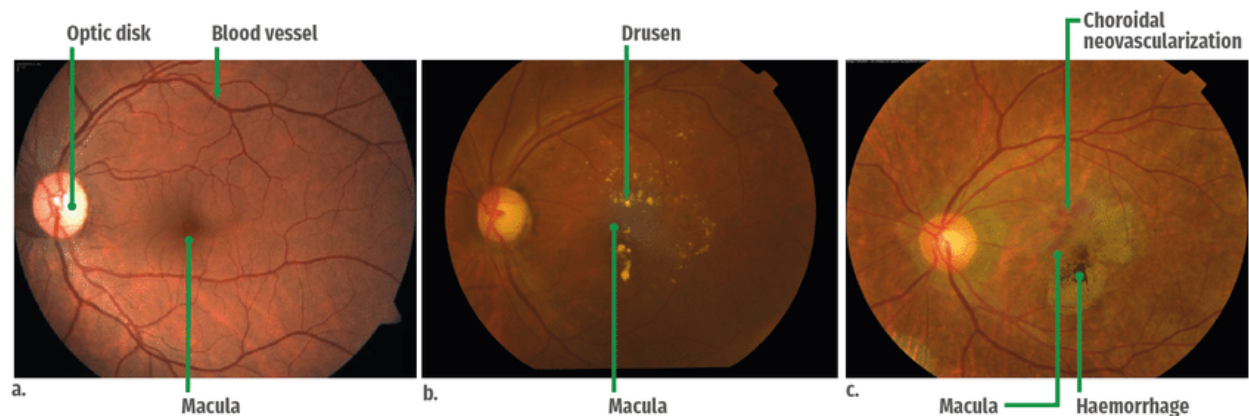
Figure 1.4: Types of AMD a. Normal image of eye b. Dry form of AMD showing drusen formation c. Wet form of AMD showing vascularization of the choroidal layer. Image from Acharya, U. R., et al. (2016)

Vascular endothelial growth factor A (VEGF-A) is a key signaling molecule in wet AMD (Noble, J., et al. 2010). When cells are deprived of oxygen, they increase their production of VEGF-A. VEGF-A is a protein that induces vascular proliferation and migration of endothelial cells from preexisting vessels. In AMD, these blood vessels form and can leak fluid into the retina causing interference with the retinal function resulting in vision distortedness which may develop within a few days to a few weeks (Noble, J., et al. 2010, Bressler, N. M., et al. 1988). VEGF-A is able to bind to either of the two VEGF receptors, VEGFR-1 and VEGFR-2 (Shibuya, M. 2011). Both receptors are

closely related and share common ligands. VEGFR-1 is a kinase-impaired RTK whereas VEGFR-2 is a highly active kinase. Despite their differences, both receptors are required for development and angiogenesis.

AMD is the leading cause of blindness among persons of European-descent over the age of 50 and accounts for a total of 8.7% of worldwide blindness (Colijn, J. M., et al. 2017). The prevalence of AMD is expected to increase by more than 50% in the next 10-15 years (Colijn, J. M., et al. 2017, Friedman, D. S., et al. 2004, Bressler, N. M., et al. 1988). Some of the major risk factors that can predispose an individual to AMD are; smoking, obesity, high dietary intake of vegetable fats, and a low dietary intake of antioxidants (Jager, R. D. et al. 2008).

1.3 Smoking and AMD

The most significant risk factor for developing AMD after the age of 50 is smoking. Studies in the United States, Australia, France and the Netherlands have demonstrated a 2.5- to threefold increase in the risk of both wet and dry AMD in smokers (Mares, J. A., et al. 2017, Chader, G. J., et al. 2013, Montezuma, S. R., et al. 2007). Smoking causes vasoconstriction to the blood vessels which reduces the supply of blood and oxygen to the eyes (Frayser, R., et al. 1964). Smoking also introduces many free radicals into the body, these cause irreversible cellular damage and depletes the levels of antioxidants available to combat free radicals (Cheng, A. C., et al. 2000). Oxidative

damage and reduction of local antioxidative enzymes in RPE cells can also decrease

macular pigment density, which serves as a filter for short-wavelength light (Bonilha, V.

L. 2008). Cigarette smoke contains many toxic compounds, ocular exposure to these

compounds can cause oxidative damage, vascular changes, and inflammation leading

to the pathogenic cascade of AMD (Cheng, A. C., et al. 2000).

Genetic factors that contribute to the aging of the retina are still being studied

through microarray technology. Changes in retina gene expression patterns are found

to be greatly linked to oxidative stress and energy metabolism responses (Montezuma,

S. R., et al. 2007). Certain ethnic groups such as the Caucasian populations have been

found to exhibit higher frequencies of the disease than others (Kenney, M. C., et al.

2013). There are a variety of other studies being conducted currently to try and narrow

down if AMD has a genetic link within the population. AMD is most likely

multifactorial disease which is typically caused by a combination of many genetic

variants and environmental factors as discussed above like smoking and diet

(Ratnapriya, R., et al. 2013).

1.4 Treatments for Dry AMD

Current treatments for AMD are dependent on the stage of the disease. Dry

AMD is commonly treated with modifiable lifestyle changes. Generally, nutritional

therapy with a balanced healthy diet with a high vitamin intake and antioxidants

support the cells of the macula. If the disease progresses to a further advanced form of dry AMD, supplements are commonly prescribed to increase the concentration of vitamins and minerals known to support cell health (Bandello, F., et al. 2017). Two studies AREDS and AREDS 2 (Age Relates Eye Disorder Studies) illustrated promising results that dietary supplements were able to slow down the progression of AMD (Chew, E. Y., et al. 2012). AREDS was a randomized clinical trial with over 4000 patients. These patients received placebo or treatment of dietary supplements containing antioxidants, zinc oxide, or a combination of both. 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% (Age-Related Eye Disease Study Research Group. 2003). AREDS2 looked at more patients and a different formulation of supplements. Patients were divided into different groups and given either the original AREDS formula 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 are correlated with a reduction in risk of developing AMD (Chakravarthy, U., et al. 2013).

1.5 Treatments for Wet AMD

Since its discovery in the early 1970s, AMD has been a challenging disease to treat or cure. Even today in 2020 there is yet to be a cure for AMD and current treatments only decrease the rate of disease progression. Originally, laser photocoagulation was thought to be a possible treatment for wet AMD (Burgess, D. B., et al. 1993). Although studies showed promising results in extrafoveal lesions, parafoveal lesions showed less favorable results (Burgess, D. B., et al. 1993). One notable study, The Macular Photocoagulation Study was halted prior to completion due to many of the patients in the treatment group having a decrease in visual acuity (Macular Photocoagulation Study Group. 1991). Today, photocoagulation is rarely used in the treatment of AMD due to numerous issues including; vision loss, increased recurrence rate, and limited visual improvement (Al-Zamil, W. M., et al. 2017).

In the late 1990s photodynamic therapy (PDT) was introduced as a possible treatment, and approved by the US food and Drug Administration (FDA) for ophthalmic use (Bressler, N. M. 1999, Al-Zamil, W. M., et al. 2017). PDT worked through the use of intravenously dosed photosensitizer Verteporfin which was later activated by a 689nm red laser. Once activated, the dye forms reactive free radicals that cause blood vessels to clot, preventing the further spread (Al-Zamil, W. M., et al. 2017). PDT was used worldwide for a brief period, however, due to some unpredictable results, PDT led to the recurrence of AMD and the need for multiple retreatments (Al-

Zamil, W. M., et al. 2017). Following PDT treatment, blood vessels would re-canalize in a short time period leading to recurrence and requiring more treatment. Currently, PDT is rarely used in treatment of AMD due to expensive cost of equipment and the use of more favorable and effective treatments available today.

Macugen (Pegaptanib) was the first anti-VEGF drug approved by the FDA in 2004 for the treatment of wet AMD. Macugen was the first molecular approach used to combat the formation of new blood vessels within the eye. Macugen is an RNA aptamer that binds specifically to the VEGF-165 isomer with a very high binding affinity. One study, VEGF Inhibition Study in Ocular Neovascularization-1 (VISION-1), treated patients with Macugen (0.3, 1.0, or 3.0 mg intravitreal) every 6 weeks for 48 weeks (Gragoudas, E. S., et al. 2004). Following the trial, patients who received the 0.3mg treatment remained the same or gained visual acuity compared to the patients who received the aptamer control (Gragoudas, E. S., et al. 2004, Al-Zamil, W. M., et al. 2017). Since this study, newer and more effective anti-VEGF treatments have come to market, have replaced Macugen.

Avastin (Bevacizumab) was briefly used off-label in treatment of AMD due to its effectiveness over Macugen and its low cost for treatment of eyes (Raftery, J., et al. 2007). Avastin which is a mouse monoclonal antibody was humanized via site-directed mutagenesis of a human antibody framework. This new humanized antibody binds to VEGF with the same affinity as the original human antibody (Ferrara, N., et al. 2004).

Avastin was designed to be used in the treatments of metastatic colorectal cancer, however, the anti-VEGF properties of Avastin was used to help fuel the development of Lucentis specifically for patients suffering with AMD. Avastin is still used to treat MAD today as an off-label application; due to its low cost.

Lucentis (Ranibizumab) was the second anti-VEGF drug that was approved by the FDA in 2006 for the treatment of wet AMD. Lucentis is a recombinant humanized IgG1 Monoclonal Fab fragment, which can inhibit all biologically active isoforms of VEGF-A (Bakri, S. J., et al. 2007). Lucentis is created using the same parent antibody as Bevacizumab (Avastin). Both Lucentis and Avastin work by blocking the binding of VEGF which prevents the growth of new blood vessels in the eye. One notable study, which funded by The National Eye Institute, Comparison of AMD Treatments Trials (CATT), compared Lucentis and Avastin for treatment of wet AMD. Over the 24 month course of the trial, there was no difference between the two (Martin, D. F., et al. 2012, Kim, B. J., et al. 2014, Al-Zamil, W. M., et al. 2017). Other notable studies suggest a difference in effectiveness when comparing Avastin to Lucentis when treating wet AMD. Lucentis remains within the eye much longer and therefore fewer treatments of intraocular injections would be needed to control the disease (Chakravarthy, U., et al. 2012).

The most recent anti-VEGF drug approved by the FDA for the treatment of wet AMD is Eylea (Aflibercept). Eylea 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

(Browning, D. J., et al. 2012, Stewart, M. W. 2012, Al-Zamil, W. M., et al. 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

(Browning, D. J., et al. 2012, Al-Zamil, W. M., et al. 2017). One notable study, VEGF

Trap-Eye: Investigation of Efficacy and Safety in Wet AMD (VIEW), compared

treatments of Aflibercept to Ranibizumab (Heier, J. S., et al. 2012). It showed that

Aflibercept was superior to Ranibizumab (Heier, J. S., et al. 2012, Al-Zamil, W. M., et al.

2017). The increased effectiveness of Aflibercept is most likely accounted for by its

higher binding affinity for VEGF when compared to Ranibizumab (Papadopoulos, N.,

et al. 2012).

1.6 Oxidative Stress

AMD, like many diseases, may be caused by a combination of oxidative stress

and environmental or genetic factors. Oxidative stress may be defined as an excess of

reactive oxygen species (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, H. U.,

et al. 2000). Chronic inflammation and excess ROS within cells have been shown to lead

to tissue damage and ultimately cell death (Ray, P. D., et al. 2012; Khaper, N., et al, 2010). Antioxidant enzymes within the body help to control ROS within the body namely: superoxide dismutase, catalase, and glutathione peroxidase (Poljsak, B., et al. 2014). These enzymes convert ROS molecules into unreactive or inert molecules that the body can metabolize. Superoxide dismutase (SOD) converts superoxide (O_2^-) radical into oxygen (O_2) and hydrogen peroxide (H_2O_2) (Oberley, L. W., et al. 1979). Catalase in turn transforms H_2O_2 into water and oxygen (Aebi, H. 1974). Glutathione peroxidase reduces H_2O_2 and lipid peroxides into water and lipid alcohols. These in turn can be removed from the body (Wendel, A. 1981). If the concentrations of these enzymes are insufficient to breakdown ROS, excess ROS ultimately leads to cell damage and cell death. Overproduction of ROS within cells can lead to lipid peroxidation (Zhong, H., et al. 2015). Lipid peroxidation can be measured through the lipid peroxidation byproduct 4-hydroxynonenal (4HNE) (Zhong, H., et al. 2015). Figure 1.5 illustrates how the negative effects of oxidative stress are counteracted in the body by naturally occurring antioxidants.
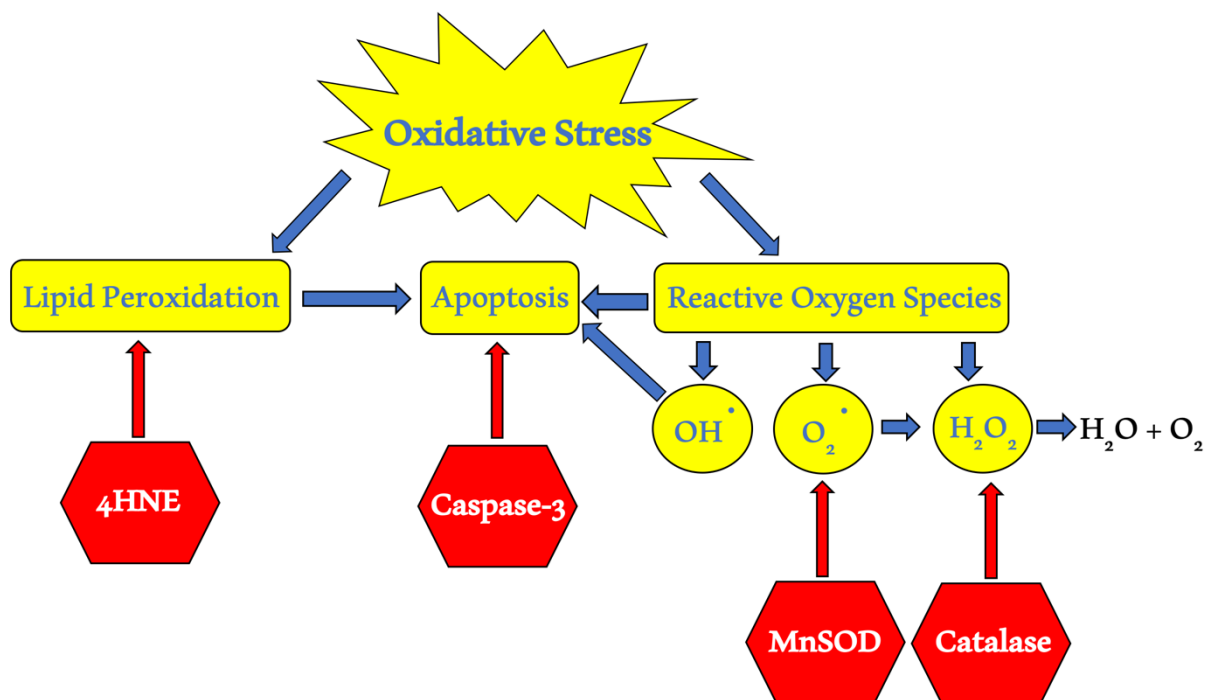
Figure 1.5: The Oxidative Stress Response. Negative effects of oxidative stress are counteracted in the body by naturally occurring antioxidants MnSOD, and catalase. These antioxidants convert ROS into unreactive or inert molecules the body can metabolize. Caspase-3 plays a crucial role in apoptosis signaling during oxidative stress. 4HNE is an indicator of lipid peroxidation.

1.7 Antioxidants

Antioxidants are produced naturally within the body; however, antioxidants can also be taken as supplements in order to boost the body's natural defense to these molecules. Antioxidants are substances that slow down the damage caused by free radicals (Halliwell, B. 1996). A common antioxidant is Vitamin C. Natural antioxidants are found commonly in plants and fruits. These can be categorized into flavonoids, tannins, phenols and lignans. Antioxidants can act in many different ways within the body. They can function to attenuate inflammation, enzyme inhibition and inhibit cell

proliferation. Antioxidants have the potential to slow down aging within cells and do

this by working together with the intracellular reductant network (Rice-Evans, C. 2001).

The chemical structure ultimately determines the metabolism and uptake for each

antioxidant. (Rice-Evans, C. 2001).

1.8 Stilbenes

Naturally occurring bi-phenol compounds in plants are one of many biologically

active polyphenolic compounds produced by plants. Stilbenes are of small molecular

weight and are naturally occurring compounds produced by plants. They are

commonly used in aromatherapy products and dietary supplements (Roupe, K. A., et

al. 2006). In plants, stilbenes are produced as a defense mechanism to environmental

threats like parasite infestation, fungal infection, or excessive UV exposure (Roupe, K.

A., et al. 2006). Stilbenes exist in both *trans-* and *cis-* isomers, however, in nature the

trans- isomer is more biologically active (Roupe, K. A., et al. 2006). Stilbenes are known

for their strong antioxidant and therapeutic properties, two commonly studied stilbenes

are resveratrol and pterostilbene (Shen, T., et al. 2009). Figure 1.6 shows the structures

of isomers of both resveratrol and pterostilbene (Shin, H. J., et al. 2020).
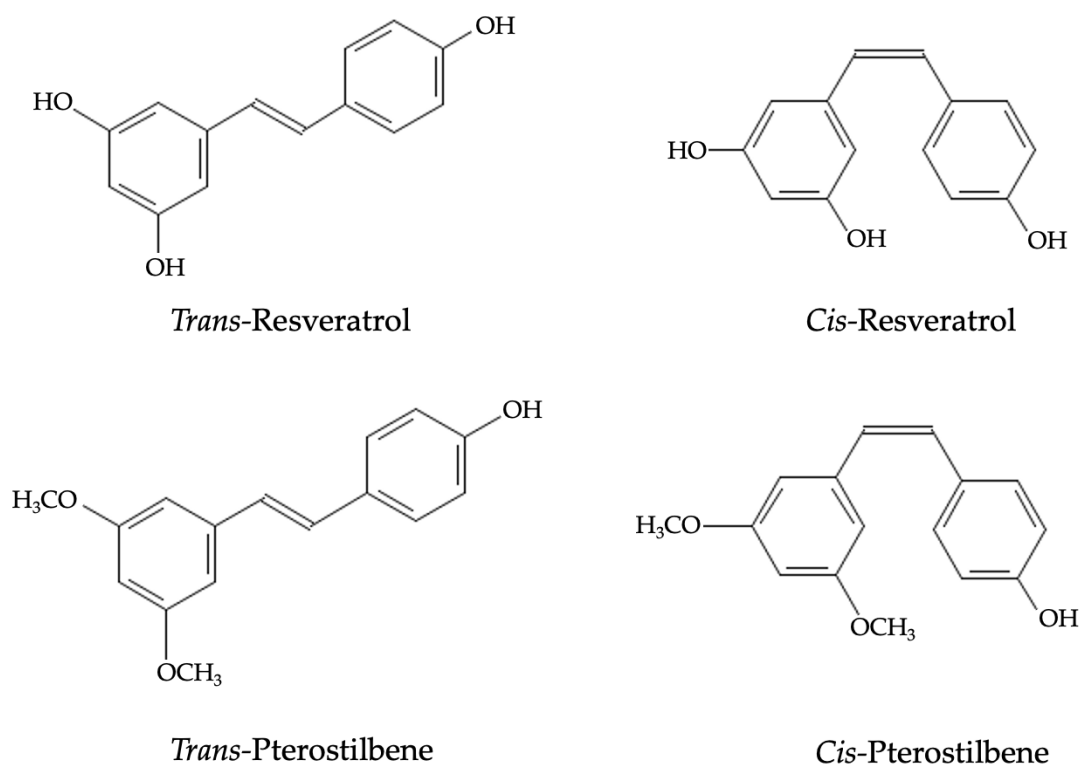
Figure 1.6. Molecular structures of resveratrol and pterostilbene. *Trans*-resveratrol (trans-3,5,4' trihydroxy- stilbene) has three hydroxy groups and a molecular weight of 228.25g/mol, whereas *Trans*-pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene) has two methoxy groups and one hydroxy group and a molecular weight of 256.29g/mol. Both compounds have *Trans-* and *Cis-* isomers.

1.9 Resveratrol

Resveratrol (trans-3, 5, 4'trihydroxy- stilbene) is a polyphenol produced by a variety of

plants in response to injury or fungal attack (Frémont, L. 2000). It was first identified in

the 1940s from the roots of the White Hellebore (Takaoka, M. 1940, Silva, P., et al. 2019)

and shortly after in large quantities in Japanese Knotweed (Nonomura, S., et al. 1963,

Silva, P., et al. 2019). In Japanese and Chinese traditional medicine, resveratrol is used to treat conditions like skin inflammation, fungal diseases, and diseases of the heart, liver, and blood vessels (Bradamante, S., et al. 2004). Resveratrol exists in both cis- and trans-isomers, however the trans- isomer is most commonly studied due to its prevalence in nature and its biological activity (Frémont, L. 2000, Burns, J., et al. 2002). Resveratrol is found specifically in the skins of some fruits such as grapes, blueberries, mulberries, cranberries, as well as certain teas (Frémont, L. 2000, Burns, J., et al. 2002, Wenzel, E., et al. 2005). Grapes, specifically grape skin, has a very high concentration of resveratrol and red wine has been noted to be the most concentrated food source for resveratrol in the human diet (Silva, P., et al. 2019).

Resveratrol is found in high concentrations in red wine and low concentrations in white wine, the overall concentration will range depending on a variety of factors including type of grape, geographic origin, type of wine, and growth practices (Frémont, L. 2000). The concentration of resveratrol in wine is mostly determined by the fermentation time with grape skins (Jeandet, P., et al. 1991, Frémont, L. 2000). Higher concentrations of resveratrol are therefore found in red wine as opposed to white wine due, to longer fermentation time.

Resveratrol has a variety of therapeutic properties including antioxidative, anticarcinogenic, cardioprotective, anti-inflammatory, and anti-aging (Burns, J., et al. 2002, Baur, J. A., et al. 2006). Tamaki, N., et al. (2014), hypothesized that resveratrol

reduces oxidative stress through the sirtuin 1 (SIRT1), AMP-activated protein kinase (AMPK), and nuclear factor E2-related factor 2 (NRF2) defense pathways. Upon activation of these pathways, an increased level of resistance to oxidative stress and protection against inflammation takes place (Tamaki, N., et al. 2014). Resveratrol in red wine has been suspected to be a cardio-protective nutritional supplement. Resveratrol can provide protection to the cells of the cardiac muscle by minimizing cardiomyocyte apoptosis (Vidavalur, R., et al. 2006). Kalantari, H., et al. (2010) have extensively reviewed the many beneficial physiological effects of resveratrol and their results can be found summarized in Table 2.1.

Table 1.1 Therapeutic Uses of resveratrol. Highlighted findings from the review conducted by Kalantari, H., et al. 2010.

Property	Summary of Relevant Information

Anti-oxidative	• Resveratrol can scavenge free radicals (antioxidant properties) due to its favorable structure.
	  • *in vitro*- Indicate RES is poor antioxidant properties
	  • *in vivo*- Indicates potent antioxidant properties

Cellular Protection	• Protection of heart, brain, and kidney cells • Increases Apoptosis and reduces tumor growth
Anti-carcinogenic	• Resveratrol blocks the multistep process of carcinogenesis at various stages: it blocks carcinogen activation by inhibiting aryl hydrocarbon-induced CYP1A1 expression and activity, and suppresses tumor initiation, promotion and progression.
Cardioprotective	• Resveratrol can be used as a treatment for various cardiovascular disorders; myocardial ischemia reperfusion injury, congestive heart failure, coronary arterial atherosclerosis, and chemically induced cardiotoxicity.
Anti-inflammatory	• Protection of cardiovascular muscle comes from its ability to minimize inflammation. • Suppressed aberrant expression of tissue factor and cytokines in vascular cells.
Anti-aging	• A 2006 study showing resveratrol made mice live longer when given resveratrol supplements. • Tests on RPE cells show that resveratrol can reduce oxidative stress and hyperproliferation of RPE. • resveratrol generates a survival signal by inducing activation of Akt, and inhibiting P38MAPK as well as increasing DNA binding of NF-kB. • Mechanism is not fully known, suggestions that resveratrol activates SIRT1 and PGC-1α and improves functioning of mitochondria.

1.10 Resveratrol and AMD

Resveratrol has been studied in ARPE-19 cells for its protective ability in damage caused by H_2O_2. King, R. E., et al. (2005) showed that 50μM and 100μM treatments of resveratrol were able to significantly reduce the proliferation of ARPE-19 cells by 10% for 50μM treatments and 25% for 100μM treatments. From their studies, resveratrol was also able to inhibit the H_2O_2-induced cellular oxidation and provide protection for ARPE-19 cells (King, R. E., et al. 2005). These results suggest that in diseases like AMD where oxidative stress is thought to be a strong contributing factor, resveratrol may be used as a treatment in reducing oxidative stress and minimizing the progression of AMD. Two notable studies AREDS and AREDS 2 (Age Relates Eye Disorder Studies) recently illustrated promising results that dietary supplements were able to slow down the progression of AMD (Chew, E. Y., et al. 2012).

Resveratrol has been studied to investigate its effects on VEGF expression for cancers and other diseases. Numerous studies have shown that resveratrol has a regulatory negative effect on angiogenesis in many diseases including cancer (Khan, A. A., et al. 2010, Cullberg, K. B., et al. 2013, Wu, H., et al.2018). Studies showing resveratrol mediated reduction of angiogenesis suggests that this is caused by overall reduction in glycolysis. This may be mediated by the PI3K/Akt and mTOR signaling pathways (Faber, A. C., et al. 2006, Wu, H., 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.

1.11 Pterostilbene

Pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene) is a naturally occurring di-methylated derivative of resveratrol primarily found in blueberries (Roupe, K. A., et al. 2006, McFadden, D. 2013). The concentration of pterostilbene in blueberries is dependent on the species and origin but generally ranges from 99ng/gram to 520ng/gram of fruit (McFadden, D. 2013). Pterostilbene has been well studied and has shown promising results as a strong antioxidant (McFadden, D. 2013, Li, Y. R., et al. 2018), protectant against cardiovascular disease (Li, Y. R., et al. 2018), and many types of cancer (McCormack, D., et al. 2012).

Although pterostilbene's mechanism of action in treating many of these diseases is not fully understood, it is suggested that ROS plays a strong role (McFadden, D. 2013). Pterostilbene is able to increase the antioxidant concentrations to help fight diseases where ROS is involved, and it is also able to induce cell apoptosis in various types of cancers (McFadden, D. 2013). A recent animal study has suggested that pterostilbene has a much higher bioavailability at 80% compared to resveratrol at 20% (McCormack, D., et al. 2012). Pterostilbene may have higher bioavailability in

mammals than resveratrol due to the addition of two methyl groups (Figure 1.6) which

improves its lipophilic nature and oral absorption (McCormack, D., et al. 2012).

In an animal study by Wang, B., et al. (2016), pterostilbene showed significant

results when used to minimize oxidative stress in the brains of mice. Along with

minimizing oxidative stress, pterostilbene was able to reduce neuronal cell apoptosis

(Wang, B., et al. 2016). These results indicate strong antioxidant capacity and suggest

promising results when investigating diseases where oxidative stress plays a role.

Therefore, pterostilbene, like resveratrol, may be an important player in the treatment of

AMD due to its ability to scavenge ROS and reduce overall cellular oxidative stress.

1.12 Blue Light

Excessive blue light can lead to the accumulation of oxidants and ultimately lead

to cell death and vision loss. A study by Taylor, H. R., et al. (1990) has shown that blue

light can cause cellular damage within the eye similar to those seen in patients with

AMD. Blue light is a growing concern in our everyday lives, due to the number of hours

we spend in front of screens, and is characterized by the 400 to 495nm wavelengths on

the visible light spectrum. The short wavelength increases the ability to penetrate to the

eye, leading to damage to the RPE layer (Taylor, H. R., et al. 1990). The retina is at risk

for oxidative stress as it has a 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 450nm

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, a metabolic waste 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, A. R., et al. 2010).

Blue light has been studied in cell lines, specifically ARPE-19 cells. ARPE-19 is a

spontaneously arising RPE cell line derived in 1986 from the normal eyes of a 19-year-

old male who died from head trauma in a motor vehicle accident (ATCC 2020).

Brandstetter, C., et al. (2015) showed that exposing cells to blue light caused significant

damage to ARPE-19 cells and increased apoptosis. Marshall, J. C. A., et al. (2006)

showed that exposing human uveal melanoma cells to 3 hours of blue light per day for

4 days increased the proliferation of cells when compared to control cells that were not

exposed to any blue light.

Blue light exposure, smoking, and high fat diets all play crucial roles in the

progression of AMD. In AMD, Retinal Pigment Epithelial (RPE) cells are directly

involved with the deterioration of vision as the disease progresses. In both wet and dry

AMD, it is believed that ROS is a key player in the origin and progression of AMD.

Although resveratrol and pterostilbene have shown promising results in studies

relating to AMD and across many other areas of research (ie. anti-cancer, anti-aging, and anti-tumor), their precise mechanism of action is not fully understood. This study explores the potential benefits of resveratrol and pterostilbene as nutritional supplements in patients with AMD. This will be accomplished through the use of *in vitro* studies involving retinal cell exposed to blue light with reproducible conditions in order to better understand the effects of resveratrol and pterostilbene.

Chapter 2: Rationale

2.1 Rationale

Age-related macular degeneration (AMD) is a common progressive eye condition that is the leading cause of visual impairment in adults over the age of 50 worldwide (Moutray, T. et al. 2011). AMD is characterized by acute or chronic vision loss (Jager, R. D. et al.2008).

In AMD, RPE cells are directly involved with the deterioration of vision as the disease progresses. In both wet and dry AMD, it is believed that ROS is a key player in the origin and progression of AMD. Although resveratrol and pterostilbene have shown promising results in studies relating to AMD and across many other areas of research (ie. anti-cancer, anti-aging, anti-tumor), their mechanism is not fully understood. This study is a primary step in understanding resveratrol and pterostilbene and possible beneficial effects in patients with AMD. This will be accomplished through the use of *in vitro* studies with reproducible conditions.

2.2 Hypothesis

Resveratrol and pterostilbene, due to their similar structure and strong antioxidant capacity, will attenuates oxidative stress-mediated damage in cells exposed to blue light by upregulating the endogenous antioxidants.

2.3 Specific Aims

1. Optimize the cell culture model of blue light exposure to ARPE-19 cells.

2. Determine the effects of resveratrol and pterostilbene on cell proliferation.

3. Investigate the effects of resveratrol and pterostilbene on oxidative stress and cell

death in ARPE cells exposed to blue light.

Chapter 3: Methods

3.1 Cell Culture Methods

Human retinal pigment epithelial (RPE) cell line, ARPE-19, were obtained from the American Type Culture Collection (Manassas, VA, USA). ARPE-19 cells were grown 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). At first, ARPE-19 cells were grown to 80% confluency and 70% of cells were frozen under liquid nitrogen. These were defined as passage 1 or 2. A single vial of passage 1 or 2 cells was used to start each set of experiments. All ARPE-19 cells were cultured at 37°C with 5% CO2 and grown to approximately 95% confluency prior to splitting or performing experiments. All experiments were performed within 15 passages of each other between passage 3 and passage 18.

3.2 Preparation of Resveratrol and Pterostilbene Stock Solutions

Resveratrol (Millipore Sigma, Oakville, ON, CA) 5mM stock solution was prepared in distilled water. Varying concentrations of resveratrol were prepared by diluting 5mM stock solutions into serum and antibiotic-free DMEM:F12 media.

Pterostilbene (TCI America, Portland, OR, USA) 5mM stock solution was prepared in dimethylsulfoxide (DMSO). Different concentrations of pterostilbene were prepared by diluting 5mM stock solutions into serum and antibiotic-free DMEM:F12 media. Final dilutions of pterostilbene which were added to the cells were created in a way that the concentration of DMSO was less than 1%.

Cells treated with resveratrol or pterostilbene were incubated at standard cell culture conditions for 12 hours. For experiments using pterostilbene, appropriate controls were carried out with dilutions of DMSO (vehicle control).

3.3 Preparation of ARPE-19 Cells for Each Experiment

ARPE-19 cells were seeded with DMEM:F12 into two 96 well plates with 5×10^5 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 treatment. 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.

3.3.1 Resveratrol Treatment

5mM stock concentrations of resveratrol was diluted to 2.5mM with DMEM:F12 media. Appropriate amounts of freshly diluted stock solutions were added to each treatment well. Vehicle control (DMSO) and positive control (300μM hydrogen peroxide) were also added to designated wells on each plate. Following the addition of resveratrol, each plate was placed in an incubator at standard conditions, for 4 hours prior to the addition of blue light. One plate was then subjected to blue light treatment, while the identical plate was kept in the dark under the same conditions.

3.3.2 Pterostilbene Treatment

5mM stock concentrations of pterostilbene was diluted 500μM with DMEM:F12 media. Appropriate amounts of freshly diluted stock solution were added to each treatment well. Vehicle control (DMSO) and positive control (300μM hydrogen peroxide) were also added to designated wells on each plate. Hydrogen peroxide was chosen as a positive control due to its use as a standard oxidant in cell culture models. Following the addition of each treatment, each plate was placed in an incubator at standard conditions, for 4 hours prior to the addition of blue light. One plate was then subjected to blue light treatment, while the identical plate was kept in the dark under the same conditions.

3.4 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 470nm (Figure 3.2). 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 spectra. 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 mechanical timer in order to ensure all exposure times were

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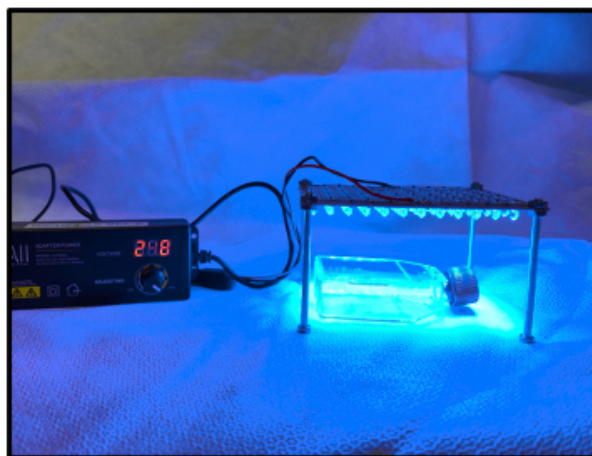
Figure 3.1 Blue Light Manifold on T-25 Flask. The blue light manifold supported on
top of a T-25 flask to show how it is used in the tissue culture incubator. The LED panel
is connected to a power supply and mechanical timer. These work together to ensure
that the LED panel receives the required voltage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

3.5 Cell Viability Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was originally used to measure 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 by adding the desired concentration of treatment for 4 hours followed by the appropriate 12-hour exposure to blue light. Following the blue light exposure, $20\mu L$ of MTT reagent was added directly to each well and incubated for 4 hours. Following incubation, media was aspirated from the wells and $50\mu L$ of DMSO was added. The plate was covered with tinfoil and placed on a shaker for 10 minutes. Following shaking, the absorbance was read using a BioTek Cytation 5 96-well plate reader (BioTek, Winooski, VT, USA) at 490 and 650nm. Difference in absorbance was used as a quantitative assessment of viability.

Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc, USA) was used to assess 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 by adding the desired concentration of treatment for 4 hours followed by the appropriate 12-hour exposure to blue light. Following the blue light exposure, $10\mu L$ 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 4 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 wells that contained media and MTS reagent. Values were expressed as a relative percentage of the controls.

3.6 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 were made and images were collected following the manufacturer's protocol. The CellEvent Caspase 3/7 Green Detection Reagent was diluted to 2μM 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 465nm 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 Preparation of Total Cellular Protein Lysate

In order to collect sufficient quantities of total cellular protein, ARPE-19 cells were seeded in T-25 flasks at a 1:3 ratio. ARPE-19 cells were treated with resveratrol and pterostilbene under both control (darkness) and treatment (blue light exposure) conditions for 12 hours prior to cell collection 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 25mM Tris (pH 7.5), 150mM NaCl, 1% Triton X-100, 2mM EDTA, 1% protease inhibitor cocktail, 1% sodium orthovanadate, and 4% sodium fluoride. Lysis was performed using a sterile Qiagen 5mm stainless-steel bead (Qiagen, Toronto, ON, CA) then homogenized using the Qiagen TissueLyser (Qiagen, Toronto, ON, CA) run at 20Hz 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 DC protein assay kit (Bio-Rad Laboratories, Mississauga, ON, CA).

3.8 Western Blot Analysis

Cellular lysates were diluted to a final concentration of 1µg/µL using dH_2O and

4x SDS reducing loading dye. 30µg of each protein was loaded into an SDS-PAGE gel

(7-15% dependent on the size of the protein of interest) and subjected to electrophoresis.

Following completion, the gel was transferred to a nitrocellulose membrane (GE

Healthcare, CA) electrophoretically using a Trans-Blot apparatus (Bio-Rad Laboratories,

Mississauga, ON, CA). The membrane was blocked using 5% nonfat milk in Tris-

buffered saline (TBS) (20mM Tris, 137mM NaCl) for 1 hour at room temperature.

Following blocking, membranes were incubated overnight at 4°C with the following

antibodies: 4-HNE (1:1000) (Cell Signaling, CA), Caspase 3/7 (1:1000) (Cell Signaling,

CA), catalase (1:1000) (Abcam, USA), and MnSOD (1:1000) (Millipore, CA). Membranes

were washed using 1xTBST then incubated for two hours using an anti-rabbit

secondary antibody (1:1000) (R&D Systems, USA) at room temperature.

Following incubation, membranes were again washed and proteins if 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.

Membranes were washed in ECL solution for 5 minutes prior to detection.

Chemiluminescent immunoblots were detected by using Chemidoc imager (Bio-Rad

Laboratories, Mississauga, ON, CA) in protein gel mode. Figure 3.2 summarizes the

major steps used in visualizing proteins through a western blot.
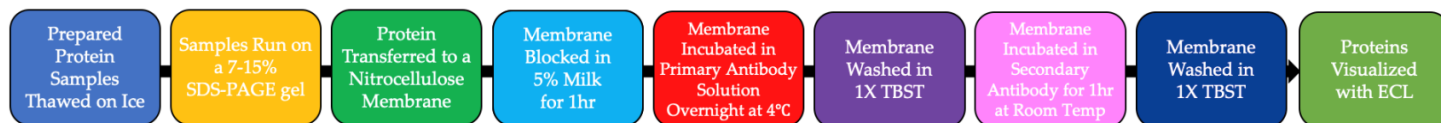
Figure 3.2 Summary of Western Blot Protocol. Visualization of cellular protein was performed utilizing western blots as described above.

3.9 Necrosis Assay

To ensure adequate cell density, ARPE-19 cells were seeded in a 6-well plate

prior to treatment. Following treatment and exposure to blue light, cells were removed

by trypsinization and collected by centrifugation at 500xg for 5 minutes. The cellular

pellets were washed twice using 1x PBS. Finally, the cells were resuspended in 400μL of

PBS, followed by the addition of 2μL of Propidium Iodide (PI) solution (Sigma-Aldrich,

Oakville, ON, Canada). ARPE-19 cells that received no compound were used as a

control from both the dark plate and the blue light exposed plate, H_2O_2 was used as a

positive control. The percentage of positive (apoptotic) cells was determined using flow

cytometry (BDFACS Calibur Flow Cytometer).

Figure 3.3 summarizes the major assays preparation for all the various

experiments performed in this project.
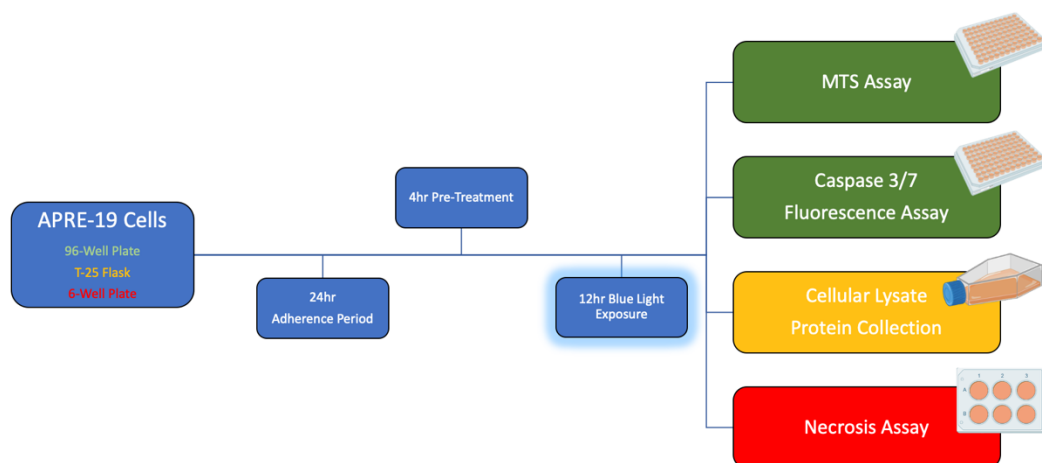
Figure 3.3 Treatment Summary ARPE-19 cells were plated in either 96-well, 6-well, or T-25 culture vessels as indicated and allowed to adhere for 24 hours. They then received 4 hours of pre-treatment of either resveratrol or pterostilbene. After 4 hours, the blue light was turned on and allowed the cells were exposed for 12 hours. Subsequently, assays were carried out as described in the methods section and as shown in the figure.

3.10 Statistical Analysis

The optimized data corresponds to four (n=4) independent experiments with the

exception of the western blots (n=1). All statistical analyses were performed using

Graph Pad Prism 8 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.

Chapter 4: Results

4.1 Optimization

Prior to testing the effects of blue light, resveratrol, and pterostilbene on ARPE-19 cells, the experimental system needed to be optimized. The variables to be considered were concentration of the compounds (resveratrol and pterostilbene), duration of pre-treatment, duration of treatment, and duration of blue light exposure. Due to a paucity of detailed information in previous literature, it was essential to create an experimental system that was reproducible. Moreover, due to conflicting results in previous studies, the dose-dependent effects of resveratrol and pterostilbene on ARPE-19 cells needed to be determined. Figure 4.1 shows the parameters that were optimized prior to commencement of the experiments.
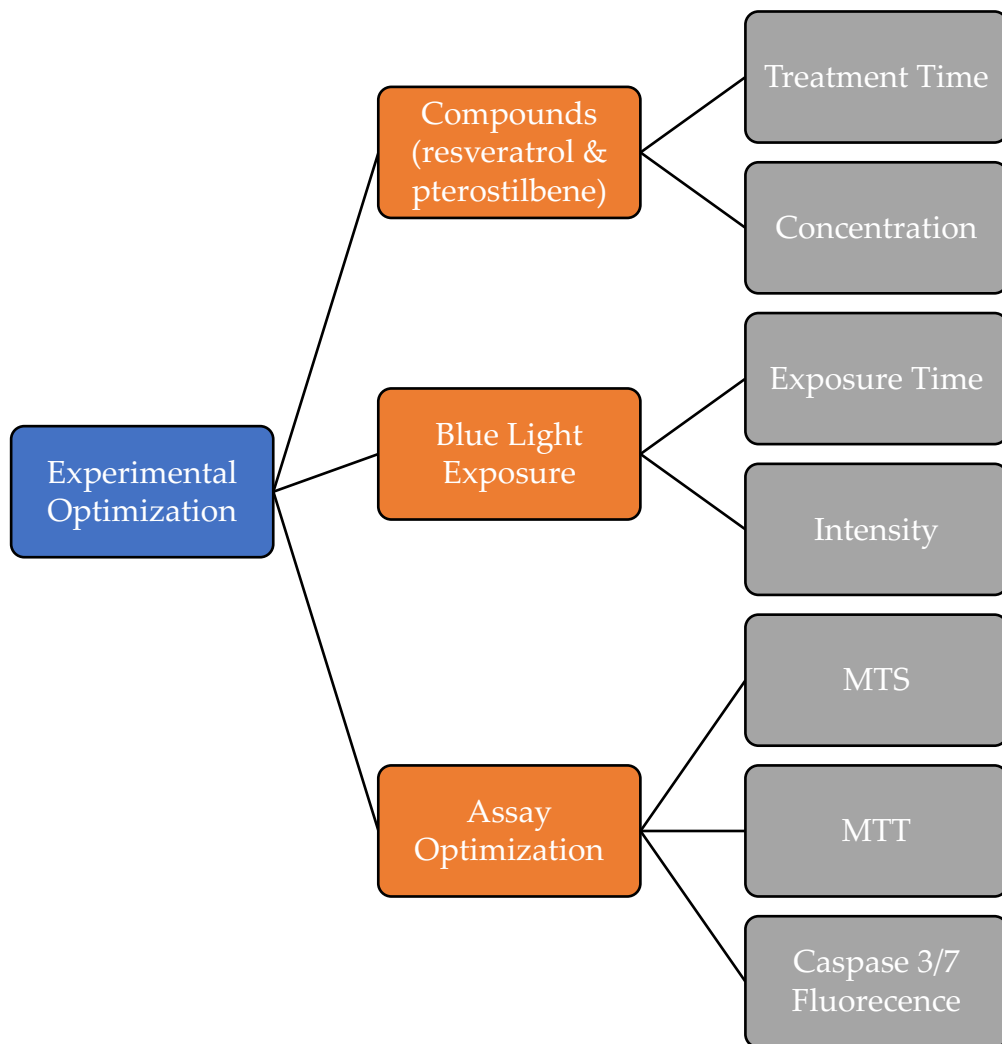
Figure 4.1. Optimization Steps. This figure outlines the major steps of optimization that had to be completed prior to beginning experiments.

4.2 Compound Optimization

4.2.1 Compound Optimization – resveratrol

      To test the dose-dependent effects of resveratrol on ARPE-19 cells, cells were

plated at $3x10^4$ cells per well in a 96-well plate. Treatments of 50μM, 100μM, and 200μM

of resveratrol were added to appropriate wells. Following treatment, cell viability

assays were performed and values were plotted on a bar graph verses control cells

which received no treatment at all. Figure 4.2 represents the results from the

optimization of resveratrol. Results from the viability assay of 50μM, 100μM, and

200μM of resveratrol after 12 hours of treatment showed that all three concentrations

had significant reductions ($p < 0.050$) in cell viability on ARPE-19 cells. A slight reduction

of cell viability was expected on control untreated cells in culture due to a lack of ROS

present. However, resveratrol concentrations of 50μM and 100μM were selected for

future experiments for their minor effect on cell viability.
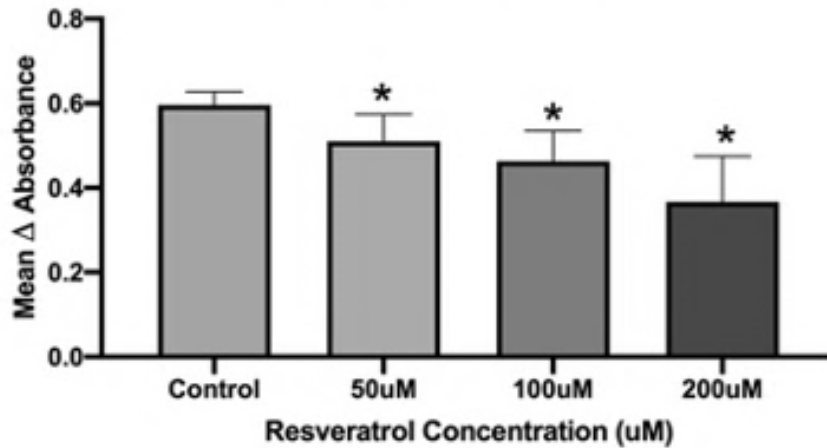
Figure 4.2. Resveratrol optimization. Effects of resveratrol alone on ARPE-19 cells. Cells were treated with 50μM, 100μM, and 200μM of resveratrol for 12 hours before media was removed and MTT reagent was added. Absorbance values were obtained using a BioTek Plate reader using KC4 acquisition software. (n=7, * denotes significant difference from control cells $p < 0.050$)

4.2.2 Compound Optimization – pterostilbene

      To test the dose-dependent effects of pterostilbene on ARPE-19 cells, cells were plated at $3x10^4$ cells per well in a 96-well plate. Treatments of 10μM, 25μM, 50μM, 75μM, 100μM, and 125μM of pterostilbene was added to appropriate wells. Following treatment, cell viability assay were performed and values were plotted on a bar graph. Figure 4.3 represents the results from the optimization of pterostilbene. Results from the viability assay of 10μM, 25μM, 50μM, 75μM, 100μM, and 125μM of pterostilbene after 12 hours of treatment showed that all concentrations, with the exception of 10μM and 125μM, showed a significant increase ($p<0.050$) in cell proliferation. Following these results, 10μM and 25μM concentrations of pterostilbene were used in all future experiments. These were selected to conform with amounts used in previous studies, and to account for the higher bioavailability of pterostilbene when compared to resveratrol.
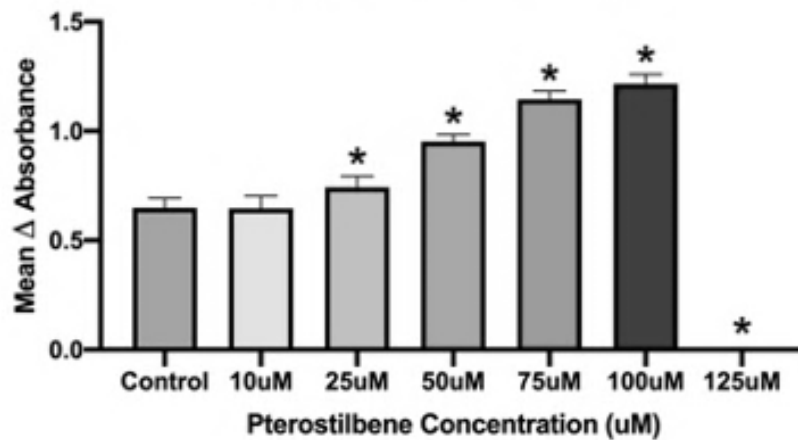
Figure 4.3. Pterostilbene optimization. Effects of pterostilbene alone on ARPE-19 cells. Cells were treated with 10μM, 25μM, 50μM, 75μM, 100μM, and 125μM for 12 hours before media was removed and MTT reagent was added. Absorbance values were obtained using a BioTek Plate reader using KC4 acquisition software. (n=4, * denotes significant difference from control cells $p < 0.050$)

4.3 Blue Light Optimization

There is a lack of detailed information on in previous studies on exposing cells to blue light. It was important to design a blue light device that was able to not only provide reproducible results but also be able to be employed in a tissue culture incubator at 37°C and 5% CO_2 injection. The prototype of the blue light apparatus is shown in Figure 4.4. This apparatus consisted of a small breadboard with 12 LED lights connected to a 9-volt battery. The LED lights were then supported on top of a 96-well plate using a specially designed hood which was 3D printed out of ABS plastic to be the match the plate. Using this manifold, exact placement over the wells was possible.
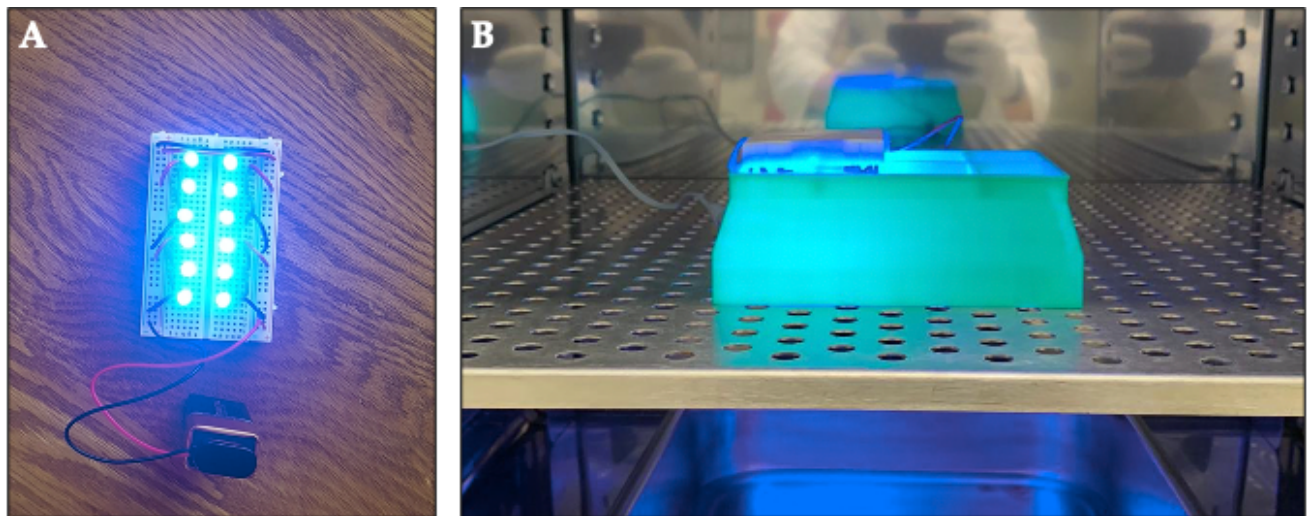
Figure 4.4 A&B. Blue Light Apparatus 1. A: Twelve LED's connected on a breadboard to 9 volts of power. B: LED panel supported on top of a 96-well plate in the tissue culture incubator.

We discovered, upon testing that the first Blue light apparatus prototype had problems due to the battery power supply. There was the possibility of voltage drop from the 9-volt battery over the course of the exposure time. As the voltage to the LED dropped, the intensity of light would reduce therefore altering exposure to blue light over the course of the experiment. Overall duration of full intensity would also be dependent on the starting capacity of the battery, storage conditions, and brand of battery. A 9-volt power supply therefore used to provide constant power. The power supply was also placed outside the tissue culture incubator to prevent the exposure of cells to excess radiant heat. By connecting the power supply outside the incubator, any radiant heat generated from the device would not affect the temperature within the tissue culture incubator.

To test the effects of blue light on ARPE-19 cells, cells were plated at 3×10^4 cells per well in a 96-well plate and allowed to adhere for 24 hours. Following adherence, the blue light apparatus was placed on top of the 96-well plate and turned on for 24 hours. Following the blue light treatment, media was removed and cell viability assays were performed using MTT assay. Values were plotted on a bar graph. Control cells in a second plate which received no light treatment at all. Figure 4.5 represents the results from the optimization of this experiment.
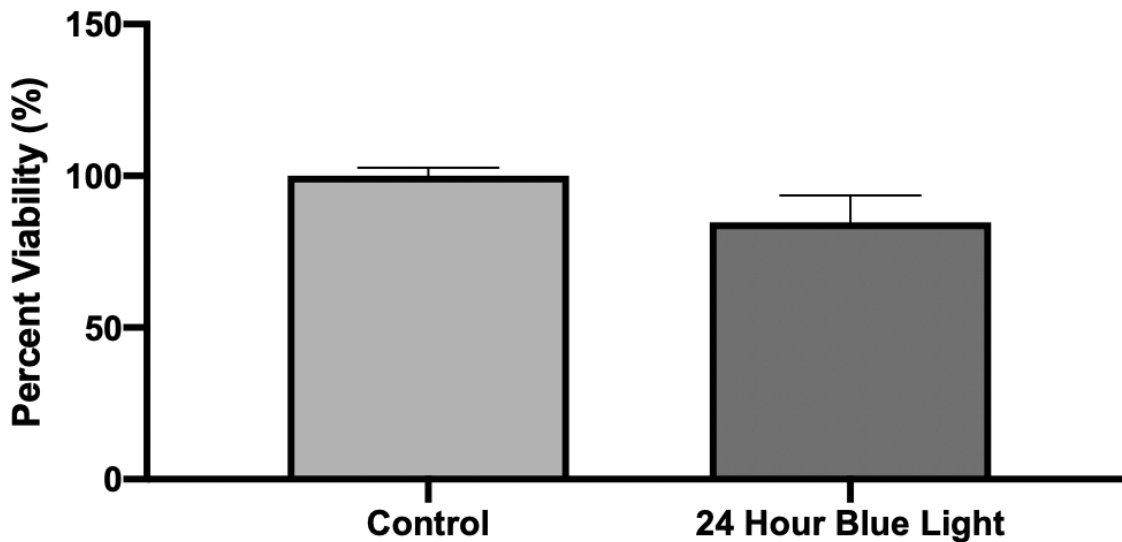
Figure 4.5. Blue Light Optimization. Effects of blue light alone on ARPE-19 cells. Cells were treated with blue light for 24 hours before media was removed and MTT reagent was added. Absorbance values were obtained using a BioTek Plate reader using KC4 acquisition software. (n=3)

This experiment showed blue light exposure reduced viability of the cells. At this juncture a second prototype was built which would allow an entire 96 well plate to be tested with blue light. This would allow for twice as many treatment conditions to be conducted at once. By adding the second LED panel the voltage from the power supply was doubled to maintain the same intensity. A second blue light exposure experiment was then performed to ensure similar results were seen. The new dual-panel blue light apparatus was placed on top of the 96-well plate and turned on for 24 hours. Results from this assay illustrated that all the cells that were exposed to 24 hours of blue light were dead. These results were unexpected as the second experiment was performed in

the same manner as the first, with the additional blue light panel being the only alteration to the manifold.

A third experiment was performed, reducing the blue light exposure time to 12 hours to determine if this made a difference on the amount of cells that survived. The dual-panel blue light apparatus was placed on top of the 96-well plate and turned on for 12 hours. Results from this MTT assay showed that all the cells exposed to 12 hours of blue light were also dead. We expected cells that were exposed for a shorter duration would be more viable, however this was not the case.

A benchtop experiment was performed with a lab grade fridge/freezer thermometer that was placed under the blue light apparatus where maximum and minimum values were reset. The lights were left on for 12 hours and the temperature was monitored over the course of the experiment. From this benchtop experiment, we determined that the temperature (at the cell level) increased by 15°C. At this juncture, we concluded that the heat from the blue light manifold might be killing the cells. The increase in temperature was caused by an increase in voltage, due to the additional LED panel. Another attempt to vacate some of the air from around the cells during treatment the LED panels, by suspending the breadboards and LEDs above the plate, failed and the cells completely died. Following this discovery, a second prototype of the blue light device was created.

The second prototype consists of a high-quality fiberglass printed circuit board

(PCB) that has 72 LED's soldered in parallel. In order to ensure the LEDs did not get too

much voltage and prematurely burn out, 150 Ohm resistors were added. These LEDs

and resistor combinations were then soldered onto the computer board and connected

in series with a positive and negative lead, which was then connected to a high-quality

power supply with an adjustable voltage output. The voltage that was calculated based

on the LED manufacturer's guidelines to ensure a desired output wavelength of 470nm.

The LED panel was supported on top of the 96-well tissue culture plate by machine

screws and nuts that allowed the panel to be suspended at the same height as the first

prototype, but also to ensure that no air was trapped on top or around the culture plate.

The second prototype of the blue light apparatus is shown in Figure 4.6.
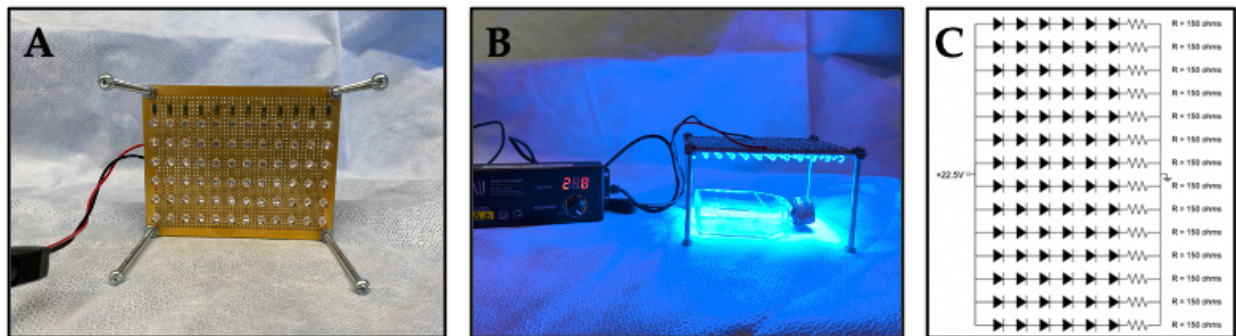
Figure 4.6 A,B,&C. Blue Light Apparatus 2. A: 72 LED's connected on a computer board with power leads to supply 22.5 volts of power. B: LED panel supported on top of a tissue culture flask to show how the open sides allow for constant airflow around the tissue culture vessel in the tissue culture incubator. 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.

Following the completion of the second prototype, an analysis of temperature change was carried out. The benchtop temperature test showed no change in temperature over the course of 12 hours. Cells were exposed to blue light for 12 hours using the second prototype. Results from this exposure can be seen in Figure 4.7. This showed a 56% reduction in viable cells after exposure to blue light.
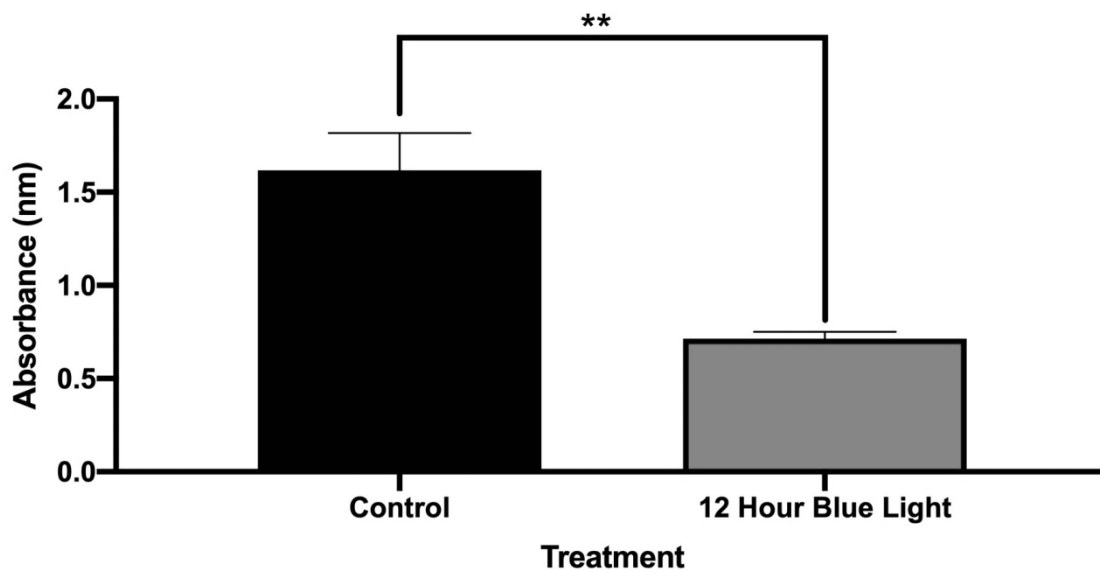
Figure 4.7. Blue Light Optimization for prototype 2. Effects of blue light alone on ARPE-19 cells. Cells were treated with blue light for 12 hours before media was removed and MTT reagent was added. Absorbance values were obtained using a BioTek Plate reader using KC4 acquisition software. **$p<0.0001$

These tests confirmed that the second prototype of the blue light apparatus worked well on cells and could be used for future experiments. A second blue light manifold was constructed to allow twice as many tissue culture vessels to be exposed to blue light at once. Further tests showed that the two units of the second prototype were identical.

4.4 Assay Optimization

At the start of the project, the MTT assay was used as a method of measuring cell viability. The MTT assay was compared to the more modern MTS assay and showed different results. We suspected that the results from the MTT assay were different that the MTS assay due to the removal of media post treatment. When removing media with vacuum, any cells that may be dead or dying might be removed from the bottom of the vessel unexpectedly. The small size of the wells on a 96-well plate may actually emphasize the pressure the cells are experiencing and cause even more to dislodge. The MTT assay was therefore phased out and all further experiments were conducted using the MTS assay other studies have suggested it is the more superior method of measuring cell viability due to its one step simplicity with no removal of media.

4.5 Optimized Results

4.5.1 MTS Assay: resveratrol, pterostilbene and Blue Light

The MTS assay was used to measure cell viability across different treatments on ARPE-19 cells. Resveratrol 50 & 100μM and pterostilbene 10 & 25μM was added to appropriate wells. Cells were pre-incubated with resveratrol and pterostilbene for 4 hours before the blue light apparatus was turned on and cells were exposed to blue light for 12 hours. Figure 4.8 represent the results from the MTS assay testing the protective role of resveratrol and pterostilbene on ARPE-19 cells exposed to blue light

for 12 hours. Resveratrol (50µM) significantly maintained cell viability by 37% when compared to control cells exposed to blue light. H_2O_2 was used as a positive control for both control and blue light treatments. Pterostilbene showed no significant changes in cell viability for either condition, rather, the number of viable cells when treated with 25µM of pterostilbene was less than the control.
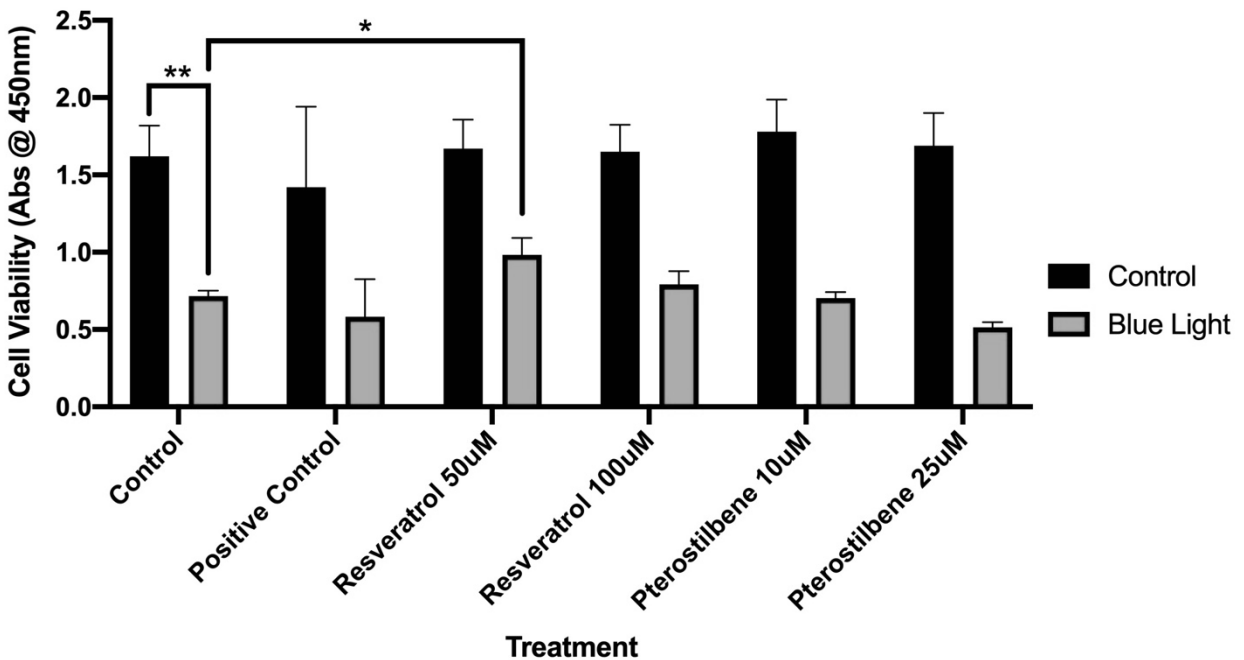
Figure 4.8. MTS Assay. Cell Viability using MTS assay. Cells were pre-treated for 4 hours with resveratrol 50µM & 100µM and pterostilbene 10µM & 25µM, followed by 12 hours of exposure to either blue light or kept in the dark. MTS reagent was used to assess cell viability. *p<0.05, **p<0.0001

4.5.2 Caspase 3/7 Fluorescence Assay: resveratrol, pterostilbene and Blue Light

Caspase 3/7 is an important protein which is produced during cellular apoptosis. It was expected that cells exposed to blue light would have a higher fluorescence signal than its corresponding cells that were kept in the dark, indicating an increase in Caspase 3/7 production. A Caspase 3/7 Fluorescence assay was used to measure cell death across different treatments on ARPE-19 cells. To test the effects of resveratrol and pterostilbene on ARPE-19 cells Caspase 3/7 activation, resveratrol (50 & 100µM) and pterostilbene (10 & 25µM) were added to appropriate wells. Cells were pre-incubated with treatments, then exposed to blue light for 12 hours before media was removed and Caspase 3/7 Fluorescence detection reagent was added and incubated for 30 min before beginning the fluorescence readings. Along with fluorescence measurements, cell images were taken across all treatments to allow for a visual representation of Caspase 3/7 in each treatment. Following the assay, values were plotted on a bar graph verses control cells which received no treatment. Figure 4.9 represents the results from the Caspase 3/7 assay testing the protective role of resveratrol and pterostilbene on ARPE-19 cells exposed to blue light for 12 hours. Figure 4.10 shows the images that were taken under each treatment condition. As shown with MTS cell viability, resveratrol treatment at 50µM significantly ($p < 0.05$) reduced the fluorescence activity in cells exposed to blue light indicating that there is a reduction in cellular apoptosis. Pterostilbene treatment of 25µM showed an increase in fluorescence activity meaning that there was more cellular

apoptosis than that in cells that were exposed to blue light only. One possibility could

be due to the structural changes occurring when pterostilbene is exposed to blue light.

These changes could be making the compound more cytotoxic resulting in more cell

death.
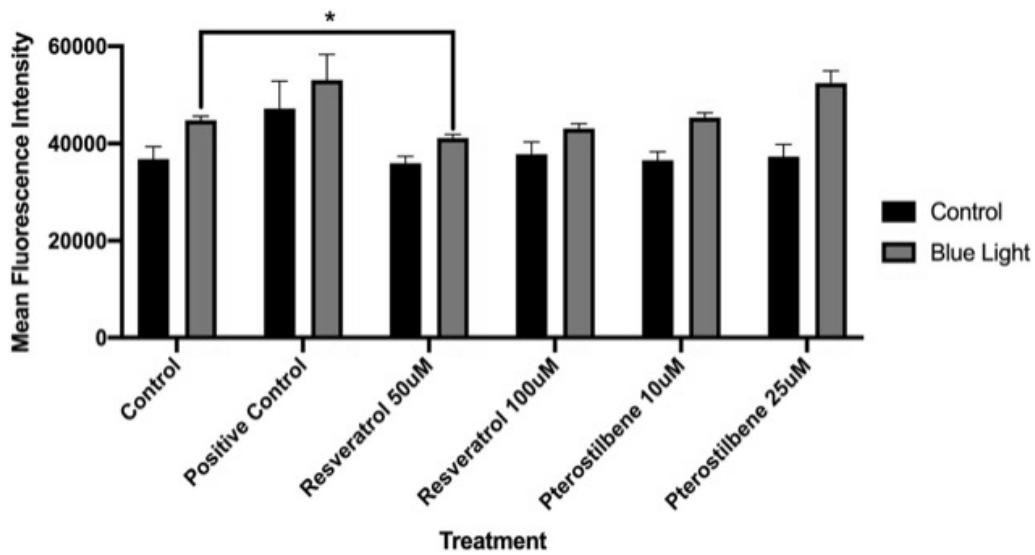
Figure 4.9. Caspase 3/7 Fluorescence Assay. Caspase 3/7 activation using Caspase 3/7 fluorescence assay. Cells were pre-treated for 4 hours with resveratrol 50μM & 100μM and pterostilbene 10μM & 25μM, followed by 12 hours of exposure to either blue light or kept in the dark. *p<0.05

Control (Dark)

| Control | Positive Control | Resveratrol 50uM | Resveratrol 100uM | Pterostilbene 10uM | Pterostilbene 25uM |

Blue Light

Figure 4.10. Cell Images from Caspase 3/7 Assay. Caspase 3/7 activation using Caspase 3/7 fluorescence assay. Cells were pre-treated for 4 hours with resveratrol 50μM & 100μM and pterostilbene 10μM & 25μM, followed by 12 hours of exposure to either blue light or kept in the dark.

4.5.3 Propidium Iodide Assay: resveratrol, pterostilbene and Blue Light

Propidium Iodide (PI) staining was used to measure cell death across different treatments on ARPE-19 cells. Resveratrol (50 & 100μM) and pterostilbene (10 & 25μM) was added to appropriate wells. Cells were pre-incubated with treatments, then exposed to blue light for 12 hours before media was removed in order to perform Propidium Iodide staining and objective measurement using flow cytometry. Figure 4.11 represents the results from the Propidium Iodide staining bar graphs testing the protective role of resveratrol and pterostilbene on ARPE-19 cells exposed to blue light for 12 hours. Resveratrol at 50μM and 100μM both significantly reduced the mean fluorescence intensity, indicating a decrease in cell death. Pterostilbene showed large increases in fluorescence intensity, indicating that more cell death took place. This could

be due to the changes occurring when pterostilbene is exposed to blue light. These changes could be making the compound more cytotoxic resulting in more cell death. Figure 4.12 shows the histogram output via the flow cytometer following the reads indicating FSC-H fluorescence versus cell count. Population A indicates cells that have membrane damage. In the control panel, darkness, the only treatment that presents itself is the H_2O_2 positive control group. In the blue light panel, all treatment groups are present in population A. This indicates that blue light is inflicting the same damages to ARPE-19 cells as H_2O_2 exposure. Population B is normal unaffected cells, which can be seen as slightly reduced when compared to the control cells.
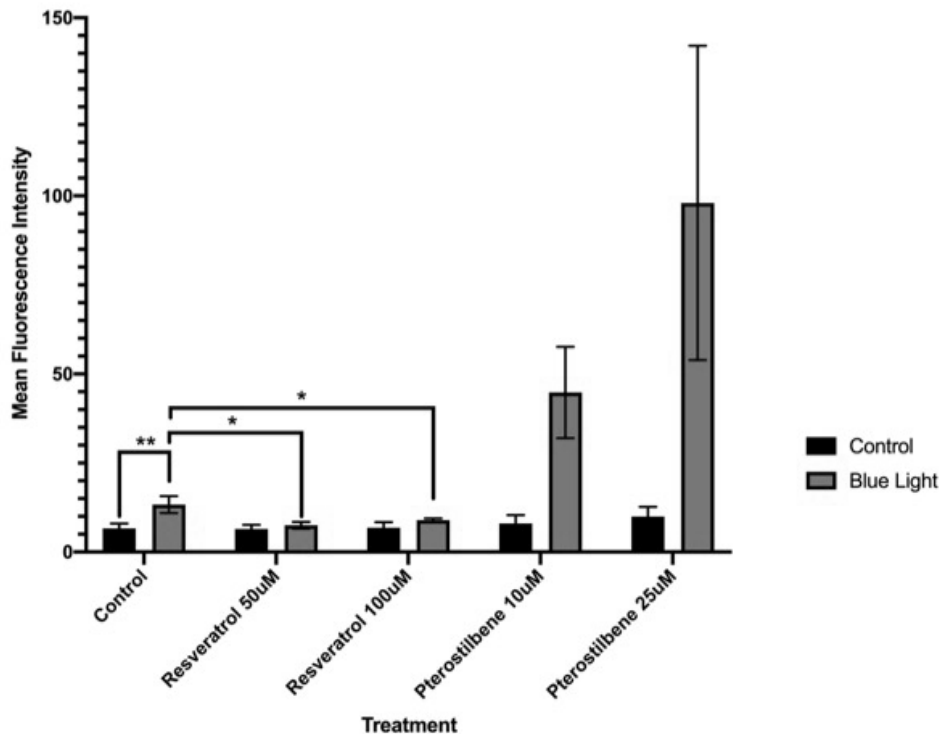
Figure 4.11. Propidium Iodide Cell Death Assay. Cells were pre-treated for 4 hours with resveratrol 50μM & 100μM and pterostilbene 10μM & 25μM, followed by 12 hours of exposure to either blue light or kept in the dark. *$p<0.05$, **$p<0.0001$
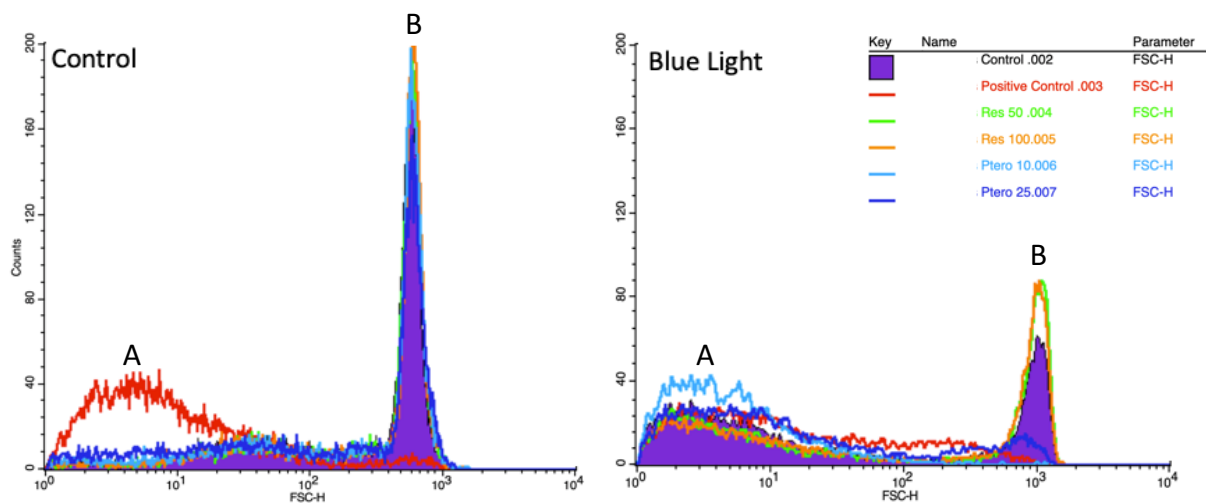
Figure 4.12. Propidium Iodide Cell Death Assay Histograms. Representative histogram indicating FSC-H fluorescence versus cell count. Cells were pre-treated for 4 hours with resveratrol 50μM & 100μM and pterostilbene 10μM & 25μM, followed by 12 hours of exposure to either blue light or kept in the dark.
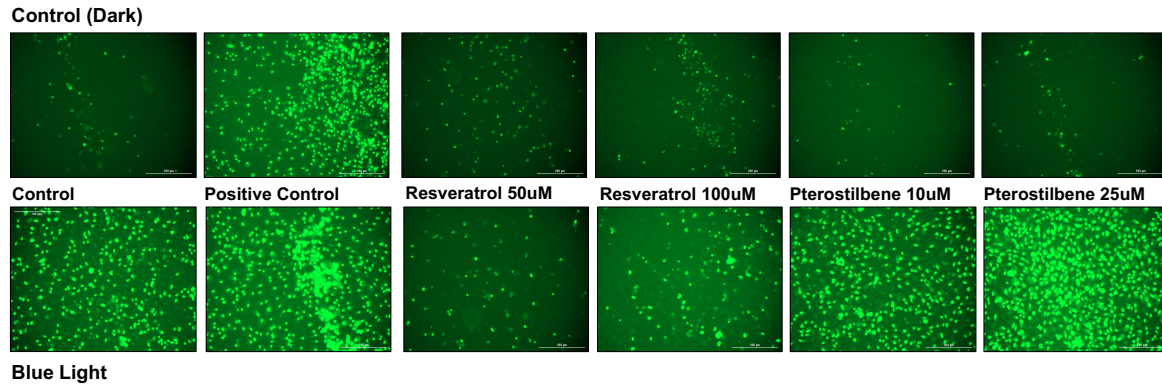
4.5.4 Effect of resveratrol, pterostilbene and Blue Light on 4HNE

Immunoblotting was used to measure the oxidative stress marker, 4HNE. 4HNE is a product of lipid peroxidation, which occurs when free radicals steal electrons from lipids in cell membrane. 4HNE protein adduct content was significantly increased in ARPE-19 cells exposed to blue light for 12 hours. Resveratrol was seen to minimize the increase of 4HNE within ARPE-19 cells exposed to 12 hours of blue light. Results from the positive control show similar results on reducing 4HNE content, indicating the need for more testing. Pterostilbene was shown to result in higher 4HNE concentrations

when exposed to blue light, again, suggesting it was becoming more cytotoxic to the

cells. The results are summarized in Figure 4.13.

Figure 4.13. Effect of Blue light, resveratrol and pterostilbene on 4HNE protein expression. Protein expression of 4HNE in control, positive control, resveratrol (50µM & 100µM) and pterostilbene (10µM & 25µM) treatments. Cells were pre-treated for 4 hours with resveratrol 50µM or 100µM and pterostilbene 10µM or 25µM, followed by 12 hours of exposure to either blue light or kept in the dark. Protein expression was determined using western blot and expressed as a ratio to β-actin (n=1).

4.5.5 Effect of resveratrol, pterostilbene and Blue Light on catalase

Immunoblotting was used to investigate antioxidant enzyme catalase. Catalase is an important enzyme that is responsible for the decomposition of H_2O_2 within cells. When H_2O_2 accumulates in the cell it leads to increases in oxidative stress and can lead to cell death. An increase in catalase concentration signifies that there is an increase in oxidative stress within the cell. Catalase content was significantly decreased in ARPE-19 cells exposed to blue light for 12 hours. Resveratrol (50μM) was seen to minimize the increase of catalase within ARPE-19 cells exposed to 12 hours of blue light. Pterostilbene was shown to result in slightly higher catalase concentrations, compared to resveratrol, when exposed to blue light. The results are summarized in Figure 4.14. From these preliminary results we suspect that resveratrol is able to minimize cellular damage on ARPE-19 cells caused by blue light and accumulation of oxidative stress.

Figure 4.14. Effect of Blue light, resveratrol and pterostilbene on catalase protein expression. Protein expression of catalase in control, positive control, resveratrol (50μM & 100μM) and pterostilbene (10μM & 25μM) treatment. Cells were pre-treated for 4 hours with resveratrol 50μM or 100μM and pterostilbene 10μM or 25μM, followed by 12 hours of exposure to either blue light or kept in the dark. Protein expression was determined using western blot and expressed as a ratio to β-actin (n=1).

4.5.6 Effect of resveratrol, pterostilbene and Blue Light on MnSOD

Immunoblotting was used to investigate antioxidant protein marker MnSOD. MnSOD concentration increases with increased oxidative stress and ROS within cells. MnSOD assists in the breakdown of O_2^- radicals. MnSOD content was increased in ARPE-19 cells exposed to blue light for 12 hours. The 50 and 100μM treatment of resveratrol was seen to minimize the increase of MnSOD within ARPE-19 cells exposed to 12 hours of blue light. Pterostilbene illustrated similar MnSOD concentrations as resveratrol when exposed to blue light. The results are summarized in Figure 4.15 and Table 4.. Reduction in MnSOD concentrations suggests the overall reduction of oxidative stress in the cells. Resveratrol has shown in these preliminary results to act as an antioxidant and provide protection to the ARPE-19 cells when exposed to blue light.
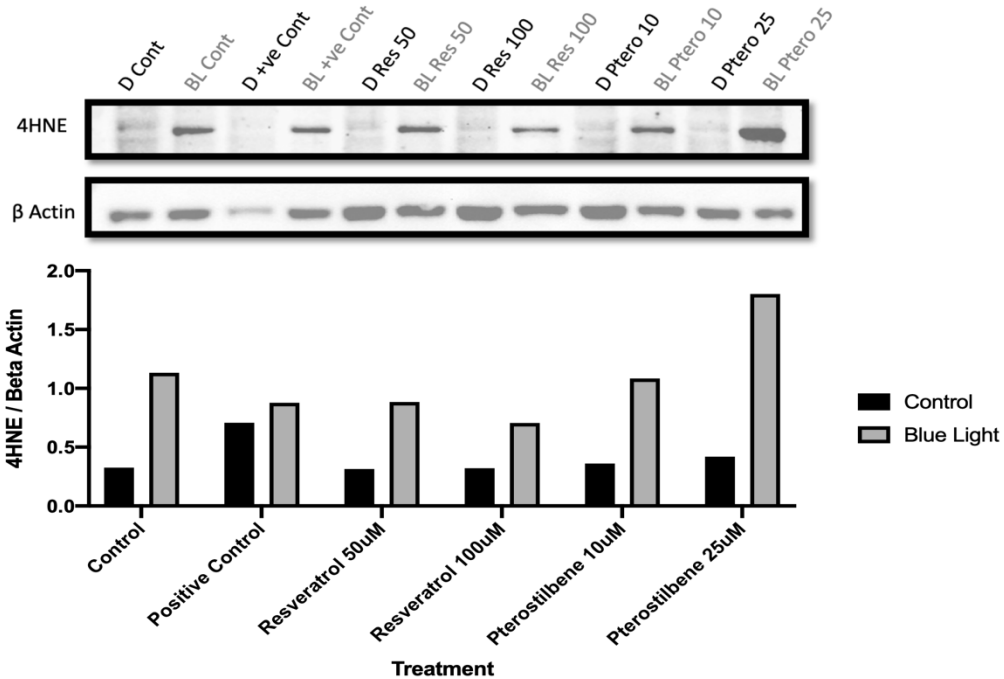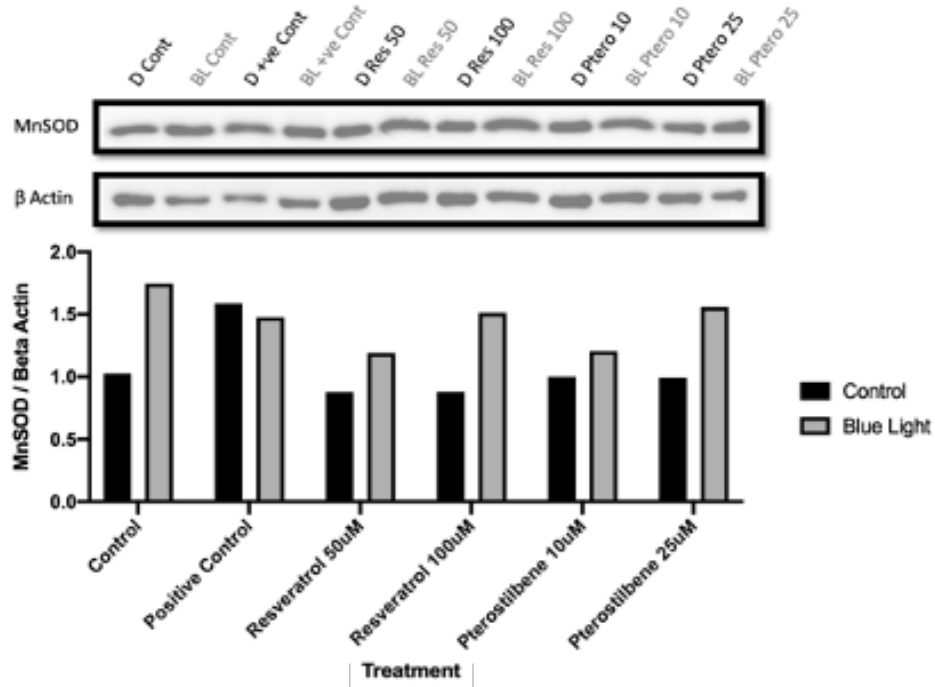
Figure 4.15. Effect of Blue light, resveratrol and pterostilbene on MnSOD protein expression. Protein expression of MnSOD in control, positive control, resveratrol (50μM & 100μM) and pterostilbene (10μM & 25μM) treatments. Cells were pre-treated for 4 hours with resveratrol 50μM or 100μM and pterostilbene 10μM or 25μM, followed by 12 hours of exposure to either blue light or kept in the dark. Protein expression was determined using western blot and expressed as a ratio to β-actin (n=1).

4.5.7 Effect of resveratrol, pterostilbene and Blue Light on Caspase-3

Immunoblotting was used to investigate apoptotic protein marker Caspase-3.

Caspase-3 is a protein which is expressed during cellular apoptosis. Increase in

Caspase-3 concentration would signify an increase in cellular damage and death.

Caspase-3 content was increased in ARPE-19 cells exposed to blue light for 12 hours.

The 50 and 100μM treatment of resveratrol was seen to minimize the concentration of

Caspase-3 within ARPE-19 cells exposed to 12 hours of blue light when compared to

control cells. Pterostilbene was shown to result in slightly higher Caspase-3

concentrations, compared to resveratrol, when exposed to blue light. Results are

summarized in Figure 4.16. Reduction in the Caspase-3 concentration by both

resveratrol and pterostilbene suggest that they in fact have a protective role against blue

light induced damage. These preliminary results show promising results for these

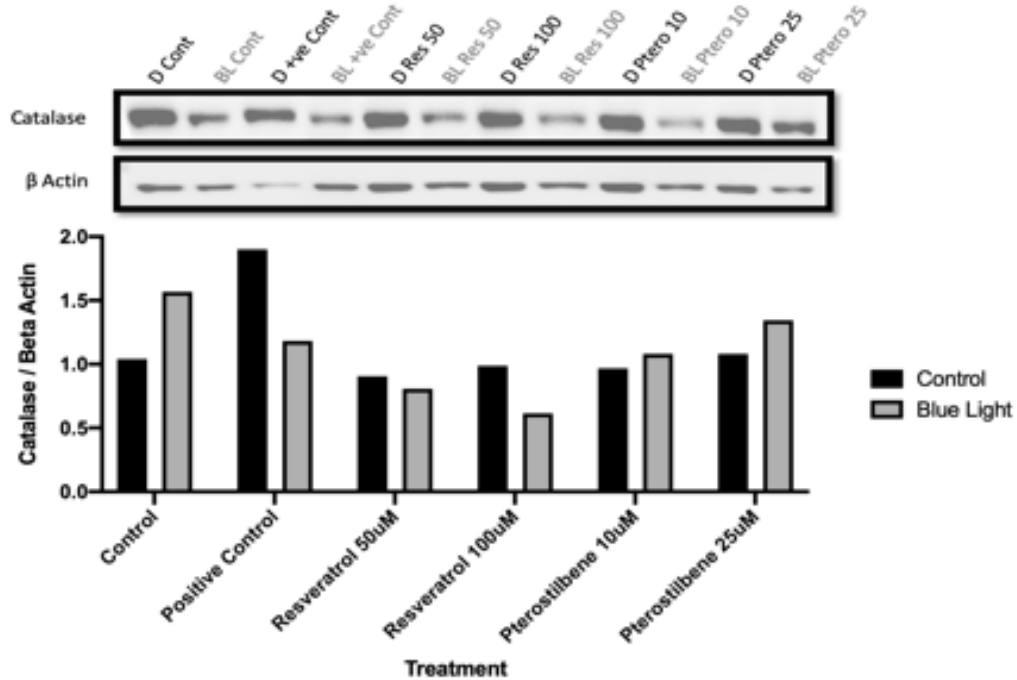compounds for protection of cells with an increased oxidative stress level.
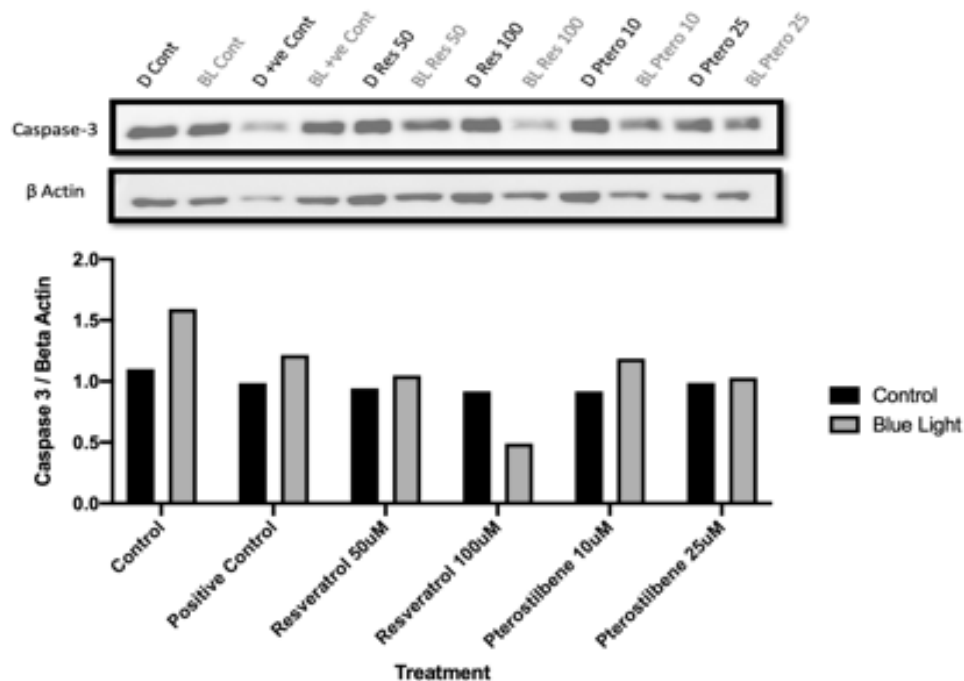
Figure 4.16. Effect of Blue light, resveratrol and pterostilbene on Caspase-3 protein expression. Protein expression of Caspase-3 in control, positive control, resveratrol (50µM & 100µM) and pterostilbene (10µM & 25µM) treatments. Cells were pre-treated for 4 hours with resveratrol 50µM or 100µM and pterostilbene 10µM or 25µM, followed by 12 hours of exposure to either blue light or kept in the dark. Protein expression was determined using western blot and expressed as a ratio to β-actin (n=1).

Chapter 5: Discussion

Chapter 5:

5.1 Discussion

Age-Related Macular Degeneration (AMD) is a progressive eye disease. AMD causes a blurred central vision making it difficult for diagnosed patients to see fine details and perform everyday tasks such as reading and driving. This condition affects a large portion of the population over the age of 50 (Moutray, T., et al. 2011., Colijn, J. M., et al. 2017).

Resveratrol has been studied for its variety of therapeutic properties including antioxidative, anti-inflammatory, antitumorigenic, anticarcinogenic, antiaging, and cardioprotective (Burns, J., et al. 2002, Baur, J. A., et al. 2006). Many studies suggest that resveratrol possesses strong antioxidant potential and is able to mitigate the damaging effects of ROS (Sinclair, D. A. 2006, Wu, H., et al.2018). Resveratrol has been studied extensively in cardiac modes of oxidative stress. Within the cardiac model systems resveratrol is able to inhibit cell death through the AMP-Activated Kinase (AMPK) pathway (Hwang, J. T., et al. 2008). Resveratrol has been studied in ARPE-19 cells for its ability to attenuate damage caused by H_2O_2. King, R. E., et al. (2005) showed that 50μM and 100μM treatments of resveratrol were able to significantly reduce the proliferation of ARPE-19 cells by 10% for 50μM treatments and 25% for 100μM treatments.

An analog of resveratrol, pterostilbene, has also been studied and has shown promising results for reducing oxidative stress and acting as a strong antioxidant

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(McFadden, D. 2013, Li, Y. R., et al. 2018), being cardioprotective (Li, Y. R., et al. 2018), and possessing antitumor properties (McCormack, D., et al. 2012). The use of pterostilbene to treat AMD has not been investigated and future studies need to be done in order to determine its place as an adjuvant treatment for AMD alongside the established medications.

Resveratrol based supplements have been used to treat wet (exudative) AMD in patients that had either refused or failed anti-VEGF injections for AMD. Longevinex was a supplement consisting 100mg of resveratrol designed to be used to promote health and longevity of organs of the body which are exposed to higher levels of oxidative stress and DNA damage (Richer, S., et al. 2013). Results from these supplements showed an improvement of visual function and macular thickness (Richer, S., et al. 2013). This study was limited by the number of patients enrolled, but shows promising results for using resveratrol in treatments of both wet and dry AMD.

5.2 Development of the Experimental Model

Taylor, H. R., et al. (1990) showed that blue light can cause similar cell damage within the eye similar to that seen in AMD. Damage caused by blue light in the eye can lead to the accumulation of oxidative stress molecules and ultimately lead to cell death. In our study, blue light was delivered to cells in culture vessels by a specially designed apparatus that was supported on top of the cells in the tissue culture incubator. Two

prototypes of the apparatus existed over the course of this experiment. Prototype one (Figure 4.4) was designed to run off a 9-volt battery, however, over the course of our experiment the battery would lose capacity and the LED lights would not be emitting full-spectrum wavelengths and intensities. This problem was solved by connecting the LED panel to a 9-volt power supply which supplies a constant voltage of 9-volts to ensure no voltage drop will occur. The blue light delivered was able to cause a noticeable decrease in cell viability. Issues with the first prototype occurred when a second panel was added and more voltage was required. With the addition of a second panel too much heat was generated, leading to compete cell death.

The second prototype of the blue light apparatus was built using a PCB computer board that had LEDs and resistors soldered directly to it, which allowed for exact permanent positioning of all the parts (Figure 4.6). To ensure proper airflow and heat dissipation around the cells, this panel was supported on top of the cell culture flask by machine screws which allowed for all sides to be open, rather than having the entire area above the cells closed off. The panel was supplied powered by a power supply which was kept external to the tissue culture incubator to ensure no extra heat was introduced into the system. In a study by Brandstetter, C., et al. (2015) blue light caused significant damage to ARPE-19 cells and increased apoptosis. The second prototype of the blue light apparatus was designed to ensure that the surface of the cells in the tissue culture vessel received an even coverage of blue light over the course of the

experiment. The panel was able to cause a very strong decrease in cell viability as seen in Figure 4.7. ARPE-19 cells exposed to blue light for 12 hours showed a 56% reduction measured through MTS assay when comparing cells exposed to blue light for 12 hours to cells that were kept in a controlled setting (dark) for the same period of time. This significant ($p<0.0001$) reduction in cell viability shows how dangerous blue light is on retinal cells. Following successful optimization of the blue light apparatus, testing on cells with our two compounds could begin.

MTS assay was used to assess cell viability. The MTS assay works by using a water-soluble tetrazolium salt, which is reduced inside the cell by dehydrogenase activities to give an orange-color formazan dye, which is soluble in the tissue culture media. The amount of the formazan dye, measured through absorbance readings, is directly proportional to the number of living cells. When compared to control cells, resveratrol was shown to have the ability to maintain cell viability by 43% ($p<0.0001$) in ARPE-19 cells that were exposed to blue light for 12 hours when compared to the blue light control (Figure 4.8). As suggested by Burns, J., et al. (2002) resveratrol's antioxidant capacity may have promising results in diseases which are easily progressed by oxidative stress. Pterostilbene showed less promising results in the cell viability assay. Results were expected to be better than resveratrol due to its higher bioavailability within cells, however, that was not the case. When exposed to blue light, pterostilbene decreased cell viability when compared to the control cells (Figure 4.8).

Although not verified, a possibility of this reduction in viability could be due to a side reaction occurring and pterostilbene being broken down into compounds that are toxic to cells. One possibility causing the decrease in cell viability when pterostilbene is exposed to blue light is the methyl group acting as a leaving group. This leaving group would be able to bind to DNA within the cell causing DNA methylation. Methylation of the cell's DNA would cause changes in function and ultimately could lead to cell death. In order to test this theory, further testing on the compound would have to be performed. Future testing could include High-Performance Liquid Chromatography (HPLC) which can be used to separate and identify components in a mixture. By using HPLC on the pterostilbene sample, we would be able to see any changes in structure that have occurred when exposed to blue light.

Caspase 3/7 Fluorescence Detection reagent was used to monitor apoptosis within the cells exposed to blue light. Caspase 3/7 is a common apoptotic enzyme released by cells undergoing cell death. The green detection reagent works by having a four amino acid peptide bound to a nucleic acid binding dye. When Caspase 3/7 is present within the cell this activates the dye causing it to cleave the peptide, which allows it to be detected through fluorescence microscopy. From the fluorescence values obtained, a 50μM treatment of resveratrol significantly ($p<0.05$) reduced the Caspase 3/7 activation in cells exposed to blue light when compared to control cells under the same conditions (Figure 4.9). Mean fluorescence intensity was reduced from 44762 to

41148 by the 50μM treatment of resveratrol. In a recent study by Ogawa, K., et al. (2014) Caspase 3/7 activity was decreased by polyphenols extracted from berries and fruit after being exposed to blue light for 6 hours (Ogawa, K., et al. 2014). In this study, pterostilbene again showed negative effects in protecting APRE-19 cells from blue light damage when compared to control cells of the same treatment. The fluorescence microscopy images (Figure 4.10) depict the trends observed in the fluorescence intensity (Figure 4.9) where green cells were positive for Caspase 3/7.

Blue light exposure for 12 hours in ARPE-19 cells resulted in higher cell death and/or necrosis measured via flow cytometry. Necrosis is characterized by permeability changes in the mitochondrial membrane resulting in the breaking of the outer membrane releasing proteases into the cytosol (Ormerod, M. G., et al. 1993). Propidium Iodide (PI) is a membrane-impermeable dye that is only able to enter cells when membrane damage has occurred (Ormerod, M. G., et al. 1993). In this study, PI positive cells were increased when ARPE-19 cells were exposed to blue light for 12 hours, however, both 50μM and 100μM treatments of resveratrol were able to attenuate the damages caused by blue light when compared to the control cells (Figure 4.11). When exposed to blue light, pterostilbene exponentially increased the PI positive cells when compared to the control cells (Figure 4.11). Again, this could be due to a side reaction occurring and pterostilbene being broken down by the blue light wavelength into compounds that are toxic to cells. Representative histograms indicating FSC-H

fluorescence versus cell count (Figure 4.12) show a definitive shift in peaks when comparing the control (dark) to treatment (blue light) conditions.

Overproduction of ROS within cells can lead to oxidative damage in cells including lipid peroxidation (Zhong, H., et al. 2015). Lipid peroxidation occurs when free radicals steal electrons from lipids in cell membranes. Lipid peroxidation can be measured through quantification of the lipid peroxidation product 4HNE (Zhong, H., et al. 2015). Measurement of 4HNE is quantified using protein adduct which are formed during times of oxidative stress within cells. In a study by Brandstetter, C., et al. (2015) 4HNE protein adduct was increased in ARPE-19 cells exposed to blue light for 6 hours. In cells exposed to blue light, an increase in 4HNE production can be observed (Figure 4.13). Resveratrol treatments of 50µM and 100µM showed noticeable decreases in 4HNE protein adduct formation when compared to control cells of the same condition. Again, as seen in the previous assays, pterostilbene treatments of 10µM and 25µM showed increases in 4HNE when compared to the control cells of the same condition. These preliminary western blots (n=1) suggest that resveratrol is able to reduce oxidative stress in blue light conditions.

Antioxidants can scavenge damaging ROS molecules within cells in order to achieve a cellular redox balance. In this study, we investigated the protein expression of catalase, which promotes the conversion of H_2O_2 into H_2O and O_2. In a recent study by Arumugam, B., et al. (2019) ARPE-19 cells exposed to H_2O_2 saw a significant increase in

catalase concentration when compared to untreated cells. In our study, ARPE-19 cells that were exposed to blue light for 12 hours showed an increase in catalase concentration (Figure 4.14). Both concentrations of resveratrol was able to reduce the catalase concentrations when compared to control cells that were just exposed to blue light. Pterostilbene showed no improvement when comparing the catalase concentrations between control (dark) and treatment (blue light). This preliminary western blot (n=1) suggests that resveratrol is able to reduce oxidative stress in blue light conditions by acting as an antioxidant.

We investigated the protein expression of MnSOD, which catalyzes the reduction of $O_2^{\bullet-}$ to H_2O_2 and O_2. In a recent study by Arumugam, B., et al. (2019) ARPE-19 cells exposed to H_2O_2 saw a significant increase in MnSOD concentrations when compared to untreated cells. In our study, ARPE-19 cells that were exposed to blue light for 12 hours showed an increase in MnSOD concentration (Figure 4.15). Cells that were treated with resveratrol showed a reduction in MnSOD concentrations when compared to the control cells. Pterostilbene showed no improvement when comparing the MnSOD concentrations between control (dark) and treatment (blue light). This preliminary western blot (n=1) suggests that resveratrol, again, is able to reduce oxidative stress in blue light conditions

Caspase-3 is a common apoptotic protein released by cells undergoing cell death. In an early study by Sparrow, J. R., et al. (2001), blue light caused an increase in

Caspase-3 activity in ARPE-19 cells. In this study, Caspase-3 concentrations were seen to increase in ARPE-19 cells exposed blue light for 12 hours. Treatments of resveratrol were shown to minimize the concentrations of Caspase-3 when comparing the blue light treated cells to the control cells (Figure 4.16). Pterostilbene showed no improvement when comparing the Caspase-3 concentrations between control (dark) and treatment (blue light). This preliminary western blot (n=1) again suggests that resveratrol is able to reduce the early stages of apoptosis of cells in blue light conditions.

5.3 Clinical Significance of this Project

Age-Related Macular Degeneration (AMD) is a progressive eye disease that causes a blurred central vision (Moutray, T., et al. 2011., Colijn, J. M., et al. 2017). AMD is the leading cause of blindness in Caucasian Americans over the age of 50. According to the Eye Disease Prevalence Group Study 54.4% of blindness in Americans is caused by AMD. As the aging population increases the number of AMD cases is projected to rise, resulting in more blind patients. Certain populations are at higher risk of getting AMD (Kenney, M. C., et al. 2013). Smoking introduces many free radicals into the body and can causes serious damage and increase the chance of getting AMD (Cheng, A. C., et al. 2000). Cigarettes causes vasoconstriction to the blood vessels which cuts down the supply of blood and oxygen to the eyes (Frayser, R., et al. 1964). The retina is at risk for oxidative stress as it has a high proportion of polyunsaturated fatty acids, high oxygen

consumption, and chronic exposure to visible and ultraviolet light spectrums. It is predicted that AMD is easily progressed by oxidative stress within the eyes, this study aimed to investigate oxidative stress caused by blue light, a growing concern in AMD progression.

Two forms of AMD are commonly diagnosed in clinical settings, they are wet and dry AMD (Jager, R. D. et al.2008, Ambati, J., et al. 2012). Dry AMD is characterized by the formation of small yellow deposits in the macula called drusen. Wet AMD develops when there is the formation of blood vessels from the choroid layer into the macula, called choroidal neovascularization. These abnormal blood vessels can easily break and leak fluid and blood into the layers of the retina (Noble, J., et al. 2010, Bressler, N. M., et al. 1988). Fluid buildup can collect between the RPE layer and Bruch's membrane causing structural changes in the RPE cell layer, altering the shape which leads to photoreceptor damage and severe vision loss.

The dry form of AMD is usually treated with nutritional supplements, ensuring a balanced healthy diet with a high vitamin intake and antioxidants support the cells of the macula. Supplements are commonly prescribed to increase the concentration of vitamins and minerals known to support cell health (Bandello, F., et al. 2017). (Age-Related Eye Disease Study Research Group. 2003).

The benefits of resveratrol are well documented in previous studies for numerous conditions. When investigating blue light damage resveratrol shows

promising results at minimizing the effects of oxidative stress on ARPE-19 cells.

Although these results are preliminary findings, the evidence suggests resveratrol may

have a strong role in the treatment of AMD. Pterostilbene failed to show positive results

in our study. It's cell damaging properties after being exposed to blue light suggest the

possibility of a side reaction occurring and future experiments need to be performed.

From this study we were able to propose a model for how resveratrol acts on ARPE-19

cells under blue light conditions (Figure 5.1). Future experiments into the cytotoxic

properties of pterostilbene following blue light exposure will be performed. By

exposing the compound to blue light prior to treating the cells will determine if it is the

compound specifically, or a product of chemical reactions that are occurring.
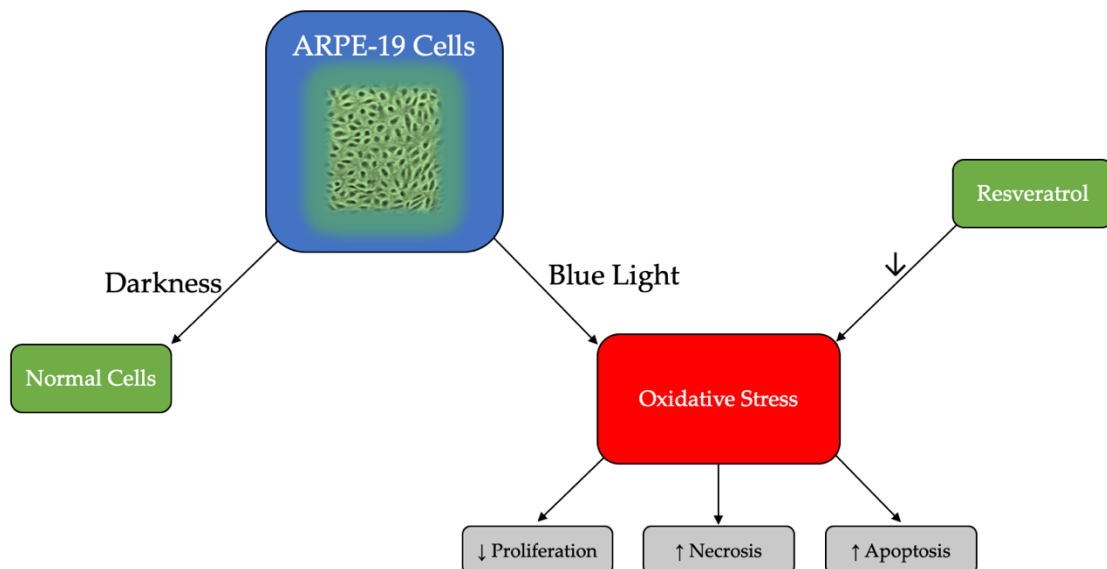
Figure 5.1 Model of resveratrol: Blue light induces oxidative on ARPE-19 cells. Oxidative stress decreases proliferation, and increases necrosis and apoptosis.

Resveratrol acts to reduce oxidative stress on ARPE-19 cells that are exposed to blue light.

5.4 Limitations

This study was performed using an *in vitro* model of blue light induced-cell damage and the results should be extrapolated accordingly. Preliminary results of cell survival and oxidative stress measuring protein expression show promising results, however, due to the ongoing COVID-19 pandemic, further studies could not be completed at this time.

5.5 Delimitations

To minimize the challenges of using an *in vitro* model, human ARPE-19 cells were chosen in an attempt to match real world outcomes of this project. By performing *in vitro* studies, results from assays can be directly correlated to individual changes in compound concentration or procedural changes rather than many contributing external factors with *in vivo* models.

Chapter 6: Conclusion

Conclusion

Blue light can cause damage to the eyes and can lead to conditions like AMD. AMD is likely caused by a combination of oxidative stress, environmental factors, and genetic factors which can lead to cell damage, death, and ultimately vision loss. Resveratrol is an antioxidant present in grapes and blueberries that has been studied in other conditions where oxidative stress plays a role in disease progression. In this study, we investigated the role of resveratrol and its analog pterostilbene on cell viability (MTS), apoptosis markers (Caspase-3/7 assay), membrane integrity (PI), antioxidant signaling (catalase and MnSOD), reactive oxygen species (4HNE), and apoptosis signaling (Caspase-3). The results of the *in vitro* studies demonstrated that ARPE-19 cells were susceptible to damages caused by exposure to blue light for 12 hours, as shown by decreases in cell viability and increases in membrane damage assays. Treatment with resveratrol mitigated the detrimental effects of blue light on ARPE-19 cells, suggesting a possible role for resveratrol as a protective agent in the prevention of damage to the eyes by blue light.

Chapter 7: Future Studies

7.1 Future studies

Future studies aim to investigate the possibility of resveratrol being used in combination with current anti-VEGF drugs used clinically. By testing combination treatments of resveratrol and anti-VEGF drugs, a possible increase in effectiveness may be seen when treating patients with wet AMD. A secondary method of investigating the effects of resveratrol would be to perform the same tests outlined in this project on a secondary cell line. Lastly, future studies aim to investigate the mechanistic pathway of resveratrol is to look at gene knock out cells. It is suspected that resveratrol acts through either SIRT1, Survivin, BCL-2, or Ku-70 gene pathways. By creating ARPE-19 cell knockouts of each and performing the same tests that was covered in this project, it is expected that resveratrol would have little to no cellular protection on the knockout it works through.

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Appendix One:

List of Abbreviations:

AMD – Age-Related Macular Degeneration

AMPK – AMP-Activated Protein Kinase

CCK-8 – Cell Counting Kit-8

DMEM:F12 – Dulbecco's Modified Eagle Medium F:12

DMSO – Dimethylsulfoxide

ECL – Enhanced Chemiluminescence

FBS – Fetal Bovine Serum

FDA – Federal Drug Administration

H$_2$O – Water

H$_2$O$_2$ – Hydrogen Peroxide

LED – Light Emitting Diode

MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

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

Nrf2 – Nuclear factor E2-Related Factor 2

O$_2$ – Oxygen

O$_2^-$ - Superoxide Radical

PBS – Phosphate Buffered Saline

PDT – Photodynamic Therapy

PI – Propidium Iodide

ROS – Reactive Oxygen Species

RPE – Retinal Pigment Epithelium

SEM – Standard Error of the Mean

Sirt 1 – Sirtuin 1

VEGF – Vascular Endothelial Growth Factor

VEGF-A – Vascular Endothelial Growth Factor A