INVESTIGATION OF ANTI-ANGIOGENIC EFFECTS OF 3,4 DIHYDROXYPHENYL ETHANOL IN MACULAR DEGENERATION

by

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ENGLISH ABSTRACT

Age Related Macular Degeneration (AMD) is the most common cause of vision loss among the elderly in developed countries. It occurs primarily in individuals over the age of 50. Currently, 1.75 million people in the US suffer from the advanced form of AMD. AMD is characterised as an abnormality of retinal pigment epithelium (RPE) and/or choroid leading to photoreceptor degeneration of central retina (macula). There are two forms of AMD: Dry AMD (characterized by the build-up of drusen between the choroid and RPE layer resulting in RPE and photoreceptor cell death) and Wet AMD (characterized by abnormal blood vessel growth from the choroid into the retinal pigment epithelium). Current pharmacotherapies in AMD include anti-angiogenics (anti-VEGF) such as Macugen, Avastin, and Lucentis. Thus current research is focusing on trying to form combination therapies (such as anti-VEGF and other agents) to achieve better clinical efficacy. We will be investigating the compound 3,4 dihydroxyphenyl ethanol (DPE), which is a polyphenol present in virgin olive oil known to have antioxidant, anti-angiogenic, anti-inflammatory, and antithrombotic properties. Previous studies have investigated DPE and its ability to prevent cardiovascular diseases and treat different types of cancer. We believe that DPE can reduce angiogenic signalling in the macula. Our objective is to assess the potential utility of DPE as a therapeutic agent in combination with anti-VEGF drugs. ARPE-19 cells were treated with 0.25 mg/ml bevacizumab to study the effects of bevacizumab on the secretion of pro-angiogenic cytokines. The cells were then treated with 100μM DPE for 24 hours in culture in both normoxic and $CoCl_2$-simulated hypoxic conditions. RPE cells were also treated with the combination of DPE and bevacizumab in order to determine the effectiveness of the combination therapy on RPE cells. Media was then harvested after 24 hours for sandwich ELISA-based angiogenesis arrays. The secretion of the following 10 pro-angiogenic cytokines

was measured: Angiogenin, ANG-2, EGF, bFGF, HB-EGF, PDGF-BB, Leptin, PlGF, HGF, and VEGF-A. The secretion of three (Angiogenin, ANG-2, and EGF) was increased following treatment with bevacizumab, however only Angiogenin was significant. Angiogenin and VEGF-A were secreted under normoxia, and significantly increased under $CoCl_2$-simulated hypoxia, whereas ANG-2, HB-EGF, and PlGF were increased under hypoxia. Following treatment with DPE, levels of Angiogenin and VEGF-A were significantly reduced under normoxia, whereas secretion of all 5 secreted cytokines were significantly decreased under hypoxia. The combination of DPE and bevacizumab significantly reduced the secretion of Angiogenin under both normoxic and hypoxic conditions compared to bevacizumab alone. Considering the implications of angiogenesis in AMD, these studies could provide the framework for future studies to further investigate a potential therapeutic role for DPE. DPE may reduce the secretion of pro-angiogenic cytokines, such as Angiogenin, that are up-regulated following treatment with bevacizumab as a possible compensatory mechanism. Therefore, the combination of DPE and bevacizumab may represent a valuable therapeutic option for the wet form of AMD.

FRENCH ABSTRACT

La dégénérescence maculaire liée à l'âge (DMLA) est la cause la plus fréquente de la perte de la vision chez les personnes âgées dans les pays développés. Il survient principalement chez les personnes âgées de plus de 50 ans. Actuellement, 1,75 millions de personnes aux États-Unis souffrent de la forme avancée de la DMLA. La DMLA se caractérise par une anomalie de l'épithélium pigmentaire rétinien (EPR) et / ou de la choroïde, conduisant à la dégénérescence des photorécepteurs de la rétine centrale (macula). Il existe deux formes de DMLA: la DMLA de type sèche (caractérisée par l'accumulation de petites taches blanches sous la rétine (drusen) entre la choroïde et l'EPR résultant en la mort des cellules photoréceptrices) et la DMLA de type humide (caractérisée par une croissance anormale de néovaisseaux choroïdiens (NVC) sous l'épithélium pigmentaire de la rétine). Les pharmacothérapies actuelles pour traiter la DMLA comprennent les anti-angiogéniques (anti-VEGF), tels que Macugen, Avastin et Lucentis. Ainsi la recherche actuelle se concentre à essayer de former des combinaisons thérapeutiques (tels que des agents anti-VEGF et d'autres) pour parvenir à une meilleure efficacité clinique. Nous examinerons le 3,4 dihydroxyphenyl ethanol (DPE), qui est un polyphénol présent dans l'huile d'olive vierge connu pour avoir des propriétés antioxydantes, anti-angiogéniques, anti-inflammatoire, et des propriétés antithrombotiques. Des études antérieures sur le DPE ont démontré sa capacité à prévenir les maladies cardio-vasculaires et traiter les différents types de cancer. Nous croyons que le DPE peut réduire la signalisation angiogénique dans la macula. Notre objectif est d'évaluer la potentielle utilité de DPE comme agent thérapeutique en combinaison avec des médicaments anti-VEGF. Les cellules ARPE-19 ont été traitées avec 0,25 mg/ml de bevacizumab pour étudier les effets du bevacizumab sur la sécrétion de cytokines pro-angiogéniques. Ces cellules ont ensuite été traitées avec 100µM de DPE en culture pendant 24

heures à la fois dans des conditions normoxiques et des conditions simulé hypoxiques ($CoCl_2$). Les cellules de l'EPR ont également été traitées avec la combinaison de DPE et de bevacizumab en vue de déterminer l'efficacité de la thérapie avec cette combinaison sur les cellules de l'EPR. Le milieu de culture a ensuite été récolté après 24 heures pour proceder au sandwich ELISA pour tester l'angiogenèse. La sécrétion des 10 suivants cytokines pro-angiogéniques a été mesurée: Angiogenin, ANG-2, EGF, bFGF, HB-EGF, PDGF-BB, Leptin, PlGF, HGF, and VEGF-A. La sécrétion de trois d'entre eux (Angiogenin, ANG-2, et EGF) a été augmentée à la suite du traitement par bevacizumab, mais seulement celle de l'Angiogenin a été significative. L'Angiogenin et le VEGF-A ont été sécrétés sous normoxie, et ont considérablement augmenté en vertu de l'hypoxie simulée par $CoCl_2$, alors que le ANG-2, le HB-EGF et le PlGF ont été augmentée en vertu de l'hypoxie. Après le traitement avec DPE, les niveaux de Angiogenin et le VEGF-A ont été considérablement réduits en normoxie, tandis que la sécrétion de toutes les 5 cytokines sécrétées a été significativement diminuée sous l'hypoxie. La combinaison de la DPE et du bevacizumab a considérablement réduit la sécrétion de l'Angiogenin dans des conditions à la fois normoxiques et hypoxiques en comparaison avec le bevacizumab utilisé seul. Considérant les implications de l'angiogenèse dans la DMLA, ces études pourraient servir de base pour de futures études pour pousuivre les recherches sur le rôle thérapeutique potentiel du DPE. Le DPE peut réduire la sécrétion de cytokines pro-angiogéniques, comme celle de l'Angiogenin, qui augmentent après un traitement par le bevacizumab comme un possible mécanisme compensatoire. Par conséquent, la combinaison du DPE et du bevacizumab peut représenter une option thérapeutique valable pour la forme humide de la DMLA.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AMD	Age-Related Macular Degeneration
ANG-2	Angiogenin-2
CFH	Complement Factor H
CNV	Choroidal Neovascularization
DPE	3,4 Dihydroxyphenyl Ethanol
EGF	Epidermal Growth Factor
bFGF	Basic Fibroblast Growth Factor
HGF	Hepatocyte Growth Factor
HB-EGF	Heparin-Binding EGF-like Growth Factor
HIF	Hypoxia Inducible Factor
HUVEC	Human Vascular Endothelial Cells
PDGF-BB	Platelet Derive Growth Factor
PlGF	Placental Growth Factor
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
TLR3	Toll-Like Receptor 3
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
PEDF	Pigment Epithelium Derived Factor

LIST OF FIGURES AND TABLES

Chapter 1

INTRODUCTION

1.1 Ocular Anatomy

The human eye is the organ that is responsible for the sense of vision. When light enters the eye, it passes through a clear layer at the front and center of the eye called the cornea. The cornea is involved in focusing the light, and is located just anterior to the iris. The iris is the colored tissue, responsible for controlling the amount of light entering the eye through the central opening called the pupil. Light then passes through the posterior chamber, which is filled with a nourishing substance called the aqueous humor, towards the lens. The lens is a clear structure surrounded by a ring of muscular tissue called the ciliary body. Together the lens and the ciliary body control the fine focus of light entering the eye. Light then passes through the vitreous cavity which is filled with a gel-like substance called the vitreous humor. At this point, the image is both inverted and reversed as it hits the neurosensory retina. The neurosensory retina is composed of 7 different cell types: cone, rod, ganglion, amacrine, horizontal, bipolar and glial cells. Rods are important in dim-light situations and are present in a higher concentration in the periphery of the retina. Cones help perceive color, and require more light to do so. They are at the highest concentration in the macula, which is the center of the retina, involved in color vision, reading, and central fine visual acuity. Together, these cells convert the light signals into nerve signals and send them to the brain via the optic nerve. The layer of cells directly behind the retina is called the retinal pigment epithelium (RPE), responsible for various functions such as retinal maintenance. Posterior to the RPE is the choroid, which is a layer of blood vessels that nourishes both the RPE and the outer retina. The choroid is separated from the RPE by a thin

membrane called Bruch's membrane. Finally, located posterior to the choroid, the sclera is a white, tough tissue that surrounds the eye, is continuous with the cornea, and gives the eye its shape.

1.2 Overview of Age-related Macular Degeneration

Age-related macular degeneration (AMD) is currently the leading cause of blindness in developed countries, affecting individuals over the age of 50 years old. Currently, two million Canadians are affected by AMD, with 17,100 new cases diagnosed annually [1]. AMD is a degenerative disease that affects the macular area of the retina, causing photoreceptor degeneration and consequential blindness. Early AMD is characterized by the presence of drusen accumulation in the macula in addition to hyper- or hypo-pigmentation of the RPE, whereas advanced AMD is characterized either by geographic atrophy, or choroidal neovascularization (CNV) [2]. The wet form accounts for the most severe visual deficits. CNV occurs due to aberrant growth of blood vessels from the choroid (the network of blood vessels that nourishes the retina), which penetrates Bruch's membrane and becomes leaky in the sub-RPE and subretinal space. This can result in a retinal detachment, photoreceptor death, pigment remodeling, RPE tears, or lipid exudation, ultimately causing irreparable damage and loss of vision [3]. Over 250,000 Canadians and 1.75 million Americans are affected by this form of the disease [1, 4]. Although 80-90% of AMD cases are of the dry form, over 90% of individuals with severe vision loss have developed CNV [2]. Therefore, there is an increasing amount of research being dedicated to studying the neovascular form of the disease. Although the molecular targets and pathways involved in the development of AMD are currently unknown, one molecule that has received a lot of attention due to its suspected involvement in AMD is vascular endothelial growth factor (VEGF). VEGF is a pro-angiogenic cytokine involved in blood vessel stimulation, and has been the focus of many studies to date. However, many compensatory angiogenic pathways exist to compensate for the inhibition of angiogenic

signalling [5, 6]. Therefore there is still a need to identify other pathways and players in the pathogenesis of AMD, in order to target multiple mediators in the disease.

Clinical Diagnosis

The two forms of AMD (dry AMD and wet AMD) present themselves differently in terms of their physical manifestation. Dry AMD is classified as RPE atrophy and consequential photoreceptor cell death in overlying areas. There is also a sub-retinal build-up of hard deposits called drusen, which are made up of lipids and proteins. These deposits are either located within Bruch's membrane, or between Bruch's membrane and the overlying RPE. In the wet form of AMD, there is an aberrant growth of blood vessels from the choroid that penetrate Bruch's membrane and become leaky in the subretinal space in the macular area. These fluids cause photoreceptor cell death, contributing to the onset of AMD.

The initial diagnosis can be attained through fundoscopy. However, the subtype of AMD is important for determining the prognosis of the disease as well as how the patient will respond to various forms of treatment. Therefore, either of the following two tests can be performed in order to determine the leakage patterns of the neovascularization: Fluorescein Angiography (FA) and Indocyanine green angiography (ICG). Although these tests might be similar, they are able to help determine whether the patient has the occult or classic subtype of choroidal neovascularization. During FA, a green die is administered intravenously, allowed to circulate through the ocular blood supply, and then photographed so that a trained individual can identify the abnormal blood patterns, as well as any leakage that may be occurring. ICG follows the same principles as FA. According to a study performed by Giovanni et al, ICG does not offer

any additional benefits other than to visualize the seat and extension of the neovascularization in the eye [7].

Risk Factors

There are several risk factors that are associated with the development of AMD. The most important risk factor for AMD is age. Although AMD occurs in individuals over the age of 50, as age increases, so does the prevalence of the disease. Individuals over the age of 90 have a 8-10 fold increased risk of developing AMD [8]. Individuals of advanced age show changes in Bruch's membrane as well as increases in RPE autofluorescence and residual body content, although with a large variation between individuals [9].

In addition, the prevalence of AMD is significantly higher in the Caucasian population compared to African Americans [10-12]. Consistent with this finding, Caucasian individuals with lighter colored irises have a higher prevalence of AMD compared to individuals with darker colored irises [13]. Women are also thought to be at an increased risk of developing AMD, however this may be due to the fact that they tend to live longer, and there may be differences in health-seeking behaviour between the two sexes [8]. Although there is evidence that AMD may be associated with a positive family history, this is difficult to study due to the fact that AMD is a disease of individuals of advanced age, and therefore surviving parents and family trees are hard to find in order to get an accurate family history.

AMD is a complex and highly multifactorial disease. It is generally accepted that the disease manifests due to the interaction of both genetic and environmental factors [14]. Oxidative damage is a process that occurs in the photoreceptors of the retina due to the presence of light and oxygen. Consequentially, free-radicals are formed within the retina which are highly

reactive and toxic [15]. The RPE and retina are areas of high metabolic activity and oxygen consumption; therefore they are readily affected by oxidative damage, resulting in RPE apoptosis and impaired retinal vascular endothelial function [16]. Cigarette smoke is a major source of oxidative damage in the retina, resulting in a build-up of reactive oxygen species (ROS). There is an extremely strong association between smoking and AMD. It is also one of the most important environmental risk factors for AMD. Smoking can cause photoreceptor death and degeneration. In addition, smoking can reduce choroidal blood flow to the affected areas, promoting a hypoxic environment and causing ischemia [17]. Recently, researchers have been investigating the possibility of smokers developing AMD due to genetic risk factors, further implicating the interplay between genetic and environmental factors in the manifestation of the disease. Several studies have identified a gene in the 10q26 region to be associated with an increased risk for the development of AMD in individuals that smoke [18]. Due to the apparent effects of smoking on the development of AMD, one study investigated the effects of nicotine on choroidal neovascularization in an AMD mouse model. This study demonstrated an increase in size and vascularity of choroidal blood vessels in response to orally administered nicotine, at an increased rate in older mice compared to younger mice [19].

Inflammation has also been implicated in the progression of AMD [20]. In 2005, a variant of the Complement Factor H (CFH) gene was discovered to be strongly associated with the development of AMD. Alleles on chromosomes 1q and 10q of CFH were implicated in the development of AMD [21-24]. Specifically, the 1q32 region was associated with increasing susceptibly to AMD, as well as Y402H, a common coding variant that significantly increases an individual's risk for developing AMD [22, 25]. There have also been studies identifying other polymorphisms in CFH that have a higher association with AMD [26]. Toll-Like Receptor 3

(TLR3) has been implicated in AMD as well. A variant of TLR3 was found to protect against geographic atrophy in AMD [27]. In addition, according to a study performed in our laboratory, TLR3 expression was observed in the RPE cells of human CNV membranes, while no staining was seen in control eyes, illustrating a potential role for TLR3 in AMD [28]. Therefore not only has TLR3 been found in association with geographic atrophy, but it has been implicated in CNV as well, illustrating TLR3's potential role in advanced AMD.

Lastly, systemic issues such as cardiovascular disease (atherosclerosis and hypertension), obesity (high dietary fat intake and high levels of cholesterol), and diabetes are all factors that may contribute to the development of AMD [29].

Animal Models

Animal models are important means of studying various diseases. They provide more of an insight of how specific diseases will affect a living organism, and what sort of treatments can be considered for the disease, as well as their side effects. There were no effective animal models for the dry form of AMD until 2008, when Hollyfield and colleagues published a paper in Nature Medicine describing a mouse model that presented with drusen build up with increasing age, and retinal cell degeneration [30]. The mice were immunized with mouse serum albumin adducted with carboxyethylpyrrole, which resulted in the accumulation of drusen below the RPE as well as lesions in the RPE, changes that are characteristic of dry AMD. This mouse model presented as the first dependable animal model for dry AMD, and gave researchers a method of studying dry AMD in a living organism.

There are several animal models for the wet form of AMD. An animal model for wet AMD was first described in 1982, where argon laser photocoagulation was used to induce

subretinal neovascularization in a rhesus monkey model by causing breaks in Bruch's membrane [31]. This technique has been modified for other animal models such as in rats [32-35], mice [36-38], and pigs [39, 40], however it has been met with much skepticism due to the fact that these animal models mimic the consequence of AMD (neovascularization) rather than the cause. However, since the exact etiology of the disease is not yet known, researchers must make use of this sort of animal model to study wet AMD until the pathogenesis of disease is better understood.

Transgenic animals have also been used as an animal model for wet CNV. One study looked at the role of basic fibroblast growth factor (FGF2) in CNV in mice with the targeted disruption of the FGF2 gene [36]. Another study introduced a coupled retinal pigment epithelium promoter with a murine $VEGF_{164}$ cDNA into the genome of albino mice in order to study how the overexpression of VEGF by the RPE affects CNV [41]. A rhodopsin/VEGF transgenic mouse model has also been studied with respect to VEGF expression, as well as a transgenic mouse expressing prokineticin 1 (hPK1), a mitogen that was thought to induce CNV, in the retina with a rhodopsin promoter [42, 43].

Lastly, researchers are able to inject various compounds into the subretinal space of animal models in order to study CNV [44-47]. Spilsbury et al. injected a recombinant adenovirus vector expressing the rat $VEGF_{164}$ cDNA into the rat's subretinal space in order to study and influence CNV [48]. Therefore, there are various animal models that can be used and manipulated in order to study the wet form of AMD, however they only allow for the study of the consequential CNV of wet AMD rather than the cause. An improved animal model will only be possible once more is known about the pathogenesis of AMD.

1.3 Hypoxia in AMD

Hypoxia and ischemia have been shown to be associated with the onset and development of AMD. Tissue edema due to CNV, retinal elevation, drusen, and other factors associated with AMD can affect the delivery of oxygen from the choroid to the macula, resulting in a hypoxic environment [49]. Vascular Endothelial Growth Factor (VEGF) is a pro-angiogenic cytokine that has been shown to increase in retinal pericytes, RPE cells, and retinal endothelial cells under hypoxia [49, 50]. If these cells are in a hypoxic environment throughout the pathogenesis of AMD, this can lead to the increase of VEGF expression and secretion, which promotes the aberrant growth of choroidal blood vessels and encourages the progression of the neovascular form of AMD.

Hypoxia-inducible-factor alpha (HIF-1α) is a protein that has been found in subretinal CNV membranes, as well as the endothelium and macrophages. Its expression and stabilization has been shown to increase under hypoxic conditions, which in turn regulates the expression of VEGF [51]. In addition, the expression of both HIF-1α and HIF-2α has been found in human choroidal neovascular membranes [3]. Therefore, the study of RPE cells under hypoxic conditions is important in order to understand the progression of AMD.

Hypoxia can be induced in culture by the use of cobalt chloride ($CoCl_2$), a chemical hypoxia mimicking agent. Cobalt chloride induces hypoxia by stabilizing hypoxia-inducible factor, and is a quick and simple way to study hypoxia in tissues [52]. Cobalt chloride has also been used in RPE cells to induce hypoxia and study its effects on these cells [53].

1.4 Overview of 3,4 Dihydroxyphenyl Ethanol

3,4 Dihydroxyphenyl Ethanol (DPE), also known as hydroxytyrosol, is a polyphenol present in virgin olive oil. DPE originally generated interest among the scientific community due to its potential role in the reduction of mortality rates in Mediterranean countries of chronic disorders such as cardiovascular diseases and cancer, given that olive oil is a staple in the Mediterranean diet. In these countries, of the 25-50 mL of olive oil ingested daily, 9 mg are of olive oil polyphenols, and 1 mg is derived from free DPE. DPE is absorbed in the small intestine and colon 5-10 minutes after ingestion, and binds to circulating human lipoproteins before being excreted by the urine [54]. According to a study published in 2001, DPE has a half-life of 1-2 minutes in rat blood, and has been shown to be distributed to the kidneys, as well as the skeletal muscle, liver, lungs, heart, and brain (crossing the blood brain barrier) in smaller levels following injection. D'Angelo and colleagues proposed three metabolic pathways for DPE in this study: Oxidation via alcohol dehydrogenase and aldehyde dehydrogenase, methylation via catechol-O-methyltransferase, and methylation-oxidation. D'Angelo et al. also demonstrated the lack of toxic effects or macroscopic alterations in organs in rats after administration of DPE at a dose of 2g/kg of body weight, apart from brief piloerection [55].

DPE is a compound that has been shown to contain anti-atherogenic and cardioprotective properties, in addition to its anti-inflammatory properties. Carluccio and colleagues demonstrated a decrease in the expression of VCAM-1, a cell adhesion molecule, by endothelial cells, as well as a 25% reduction of VCAM-1 mRNA levels following treatment with DPE compared to the control. DPE was also able to inhibit the activation of NF-κB and AP-1, two transcription factors involved in the regulation of VCAM-1, by 50% and 40% respectively [56]. DPE functions by inhibiting the oxidation of low density lipoproteins (LDL). When LDLs are

oxidized, they become toxic to endothelial cells and lead to the production of atherosclerotic plaques. DPE has been shown to inhibit copper sulphate-induced LDL oxidation, thereby delaying the development of atherosclerotic plaques [57]. In addition to these cardioprotective functions, DPE displays anti-inflammatory properties involving the cardiovascular system. Zhang et al. demonstrated DPE's role in inhibiting the expression of two important pro-inflammatory cytokines, iNOS and COX-2, in human monocytic cells [58]. These anti-inflammatory actions are important in maintaining a normal and functional cardiovascular environment.

DPE has also demonstrated various anti-oxidant properties. In a study published in 2002, Casalino and colleagues demonstrated that the treatment of 9 mg/kg b.w. DPE in cadmium intoxicated rats resulted in a favourable effect given that there was no significant increase in TBARS in the liver following cadmium administration. DPE also induced the increase of glutathione, an endogenous anti-oxidant, without any significant formation of liver Thiobarbituric Acid Reactive Substances (TBARS) [59]. It is thought that DPE exerts its anti-oxidative effects by scavenging the hydrogen peroxide anion, but not the superoxide anion, during the respiratory burst in human neutrophils. O'Dowd et al. confirmed this during a study where human neutrophils were incubated with DPE for 15 minutes. The results showed that DPE was able to inhibit the luminol-amplified chemiluminescence but not the lucigenin-amplified chemiluminescence of human neutrophils. The inhibition of the hydrogen peroxide anion was confirmed when O'Dowd and colleagues showed that DCFH (an oxidation-sensitive dye that fluoresces when oxidized by H_2O_2) fluorescence was eliminated in DCFH-filled neutrophils [55].

DPE has been shown to inhibit proliferation and induce apoptosis in various tumor cell lines, as well as to prevent the formation of tumors due to its anti-oxidant capacities. Terzuoli et al. demonstrated these effects by investigating DPE in an HT-20 xenograft model, as well as in vitro in colon cancer cells which have been pretreated with IL-1β and prostaglandin E-2 (PGE-2). Terzuoli and colleagues found a down-regulation of the HIF-1α/mPGE-1/VEGF axis in vivo due to DPE treatment, a pathway involved in angiogenesis. Tumor growth was consequentially inhibited due to the reduction of blood vessel lumina and blood perfusion to the tumor [2]. These findings highlight DPE's anti-angiogenic properties. Manna et al. investigated the anti-oxidative effects of DPE on Caco-2, a line of human epithelial colorectal adenocarcinoma cells. The findings indicated that pre-treatment of Caco-2 cells with DPE can prevent the damage induced by H_2O_2 and xanthine oxidase by scavenging free-radicals produced by these oxidizing agents [60].

The effects of DPE following a hypoxic stimulus have been previously studied in vitro. González-Correa et al. subjected rat brain slices to hypoxia-reoxygenation before treatment with DPE in order to study DPE's neuroprotective properties. Tissue damage was measured by lactate dehydrogenase (LDH) efflux into the incubation medium. This study demonstrated DPE's ability to significantly reduce LDH efflux in a concentration dependant manner following hypoxic conditions [61]. As hypoxia has been shown to contribute to the progression of neovascular AMD, DPE treatment may be an effective approach to slow or reduce hypoxia-related damage in the RPE.

The effects of DPE on retinal pigment epithelial cells are currently under investigation. DPE's apparent anti-oxidative properties have initiated several studies looking at the possible therapeutic effects for ocular related diseases caused by reactive oxygen species, inflammation,

or choroidal neovascularization. In a study performed by Liu et al. in 2007, a retinal pigment epithelial cell line (ARPE-19) was exposed to acrolein (a major component of cigarette smoke responsible for oxidative damage and mitochondrial dysfunction) for 24 hours. Liu and colleagues demonstrated that DPE pre-treatment had a significant protective effect on the RPE cells, resulting in reduced oxidative damage and mitochondrial dysfunction [57]. In 2009, Zhu and colleagues further identified the mechanism of action of DPE in RPE cells. They showed that treatment of ARPE-19 cells with DPE resulted in the induction of phase II detoxifying enzymes, as well as the stimulation of mitochondrial biogenesis in response to acrolein-induced stress [60]. While a specific mechanism for DPE activity is not well understood, some basic studies, including those related to the retina, have been undertaken to determine pathways affected by DPE, and study its mechanism of action. Despite these studies, much remains to be done to understand DPE's activity in RPE cells, affecting RPE-related diseases. Nevertheless, according to these preliminary studies, DPE might be useful in the protection of retinal pigment epithelial cells from oxidative stress and delay the degeneration that is associated with diseases such as age-related macular degeneration.

1.5 Angiogenesis in AMD

Angiogenesis is defined as the growth of new blood vessels from pre-existing blood vessels. During this process, vessel sprouting occurs, and the vascular plexus is remodeled into an organized vascular network. The formation of new blood vessels is necessary for organ growth and repair, and it occurs during inflammation and wound healing. It is an action that is highly regulated, however when the balance between angiogenic stimulators and inhibitors is thrown off, it may lead to the development of various disorders and diseases. Currently, Angiogenesis is involved in over 70 disorders [62].

Angiogenesis is also the main process that occurs in neovascular AMD, in response to RPE and outer-retinal cell damage [63]. There are several pro-angiogenic cytokines, one of the most heavily studied ones being vascular endothelial growth factor (VEGF). The VEGF family contains several members, the most heavily investigated being VEGF-A. VEGF-A is a homodimer with 164 amino acids each, identified by Napoleone Ferrara in 1989. It is a cytokine that is critical for blood vessel formation, enlargement, branching, and proliferation [64]. VEGF's angiogenic potential was realized in the early 1990s, as it was identified in various highly vascularized areas, and endothelial cells contained VEGF binding sites both in vitro and in vivo. A better understanding of VEGF followed the development of neutralizing anti-VEGF monoclonal antibodies. These antibodies showed their potential to inhibit tumor vascularization, indicating a potential therapeutic role in various cancers. Bevacizumab was then developed in order to test this hypothesis [65]. Hypoxic conditions have been shown to stimulate the production of VEGF, which results in new blood vessel growth in the affected tissues [66]. Gain-of-function mice (where the bovine rhodopsin promoter is coupled to the VEGF gene) have

been used as an animal model to demonstrate increased VEGF expression in retinal tissues, resulting in subsequent intraretinal and subretinal neovascularization [67].

There are various pro-angiogenic cytokines other than VEGF that have been shown to promote angiogenesis in AMD [63]. Studies indicate that the inhibition of Platelet-derived Growth Factor (PlGF), a chemoattractant, dedifferentiator, and mitogen for RPE cells, showed an inhibition of retinal angiogenesis. Basic Fibroblast Growth Factor (bFGF), a pro-angiogenic cytokine produced by vascular endothelial cells of the choriocapillaries and RPE cells, has been shown to induce the secretion of VEGF by Muller glial cells [68, 69]. Conversely, Pigment Epithelium Derived Factor (PEDF), a cytokine found in retinal and choroidal cells, has been shown to display anti-angiogenic properties. Holekamp and colleagues investigated the presence of vitreous PEDF in neovascular eyes with AMD and found significantly decreased levels compared to controls [70]. Similarly, Funk and colleagues found increased aqueous VEGF expression and decreased aqueous PEDF expression levels in neovascular eyes with AMD [71]. Therefore one can infer that the neovascularization occurring in patients with wet AMD is due to the interplay of both angiogenic and anti-angiogenic cytokines present in the RPE and retina.

There are various therapies that are designed to target VEGF and its receptors, with the goal of inhibiting angiogenic signalling, a process that occurs during choroidal neovascularization. Anti-VEGF therapies such as Pegaptanib, Bevacizumab, and Ranibizumab are somewhat effective, however they do not represent a cure for neovascular AMD. Therefore combining anti-VEGF agents with other anti-angiogenic mediators is a field with great potential. Various anti-angiogenic agents may therefore be used to control the up-regulation of other angiogenic pathways that occur when one is down-regulated as compensation. Numerous potential combination therapies designed to inhibit ocular and tumor angiogenesis have been studied.

Dorrell and colleagues combined agents such as a VEGF aptamer identical to Macugen, a small-molecule integrin antagonist, and a VE-cadherin-mediated adhesion blocker (T2-TrpRS), which has been shown to provide synergistic effects with respect to inhibiting neovascular growth [5]. They strongly believe that the combination of anti-angiogenic treatments will prove to be more effective than monotherapy alone. Therefore various pro-angiogenic cytokines are currently being targeted along with VEGF for optimal anti-angiogenic agents for neovascular AMD.

Chapter 2

LITERATURE REVIEW

2.1 Initial Treatment Approaches

One of the first treatments for wet AMD was Photodynamic Therapy (PDT). During PDT, a photosensitizer dye (Verteporfin) is injected into the arm. Verteporfin associates with lipoproteins, and is therefore abundant in the neovasculature, which contains an increased expression of low-density lipoprotein (LDL) receptors [72]. Verteporfin therefore travels to the neovascularization in the choroid, and pools in the new blood vessels. It is then targeted with non-thermal laser light at 690 nm, which promotes release of ROS in the aberrant blood vessels, and consequential thrombotic cascade and neovessel closure, thereby discontinuing subretinal leaking. The dye does not accumulate in healthy retinal cells; therefore they are not subjected to the damage caused by the laser. This treatment, however, was only mildly successful in patients with neovascular AMD, and required repeated treatments. Therefore more effective therapies were required and sought after for the treatment of wet AMD.

2.2 Current Pharmacotherapies

Although the pathogenesis of AMD is yet to be determined, it is known that the wet form of AMD is characterized by choroidal neovascularization, and that VEGF plays an important role in regulating this process. Therefore, various therapies are tailored to blocking or inhibiting VEGF's pro-angiogenic effects. The three anti-VEGF compounds that are currently used for the treatment of patients with wet AMD are discussed below.

Pegaptanib

Pegaptanib (Macugen®) was the first anti-VEGF treatment to be approved by the US FDA in 2004 for use in patients with wet AMD [73]. It is a pegylated (polyethylene glycolated) anti-VEGF aptamer that binds selectively and specifically to the extracellular isoform of VEGF (called $VEGF_{165}$), thereby inhibiting binding to its receptors [73, 74]. According to various clinical trials, Macugen proved to be beneficial, safe, and well-tolerated in patients with choroidal neovascularization. A 2-year randomized controlled clinical trial of Macugen demonstrated favorable results for patients, however with serious but rare ocular complications [75, 76]. In addition, several patients who had been treated with Macugen for AMD reported the incidence of retinal pigment epithelial tears shortly after injection [75, 77]. Although the clinical efficacy of Macugen was proven to be beneficial for patients with wet AMD, it did not meet the expectations of the scientific community, and the search for the ideal therapy for wet AMD continued.

Ranibizumab

Ranibizumab (Lucentis®) is a recombinant, humanized monoclonal antibody fragment that binds and neutralizes VEGF-A with high affinity. It was approved for use in patients with neovascular AMD by the US FDA in 2006, and generated a great deal of interest in terms of its efficacy [78]. Ranibizumab is the current therapy of choice for neovascular AMD in Canada, according to the Canadian expert consensus [1]. Various clinical trials have tested ranibizumab's safety and effectiveness after being injected in patients with neovascular AMD, yielding promising results [79, 80]. Complications from the injections did arise, however serious adverse effects were minimal [79]. A drawback of Lucentis is that it is significantly more expensive than

Avastin – around 40 times the cost [81, 82]. A study published in 2010 comparing both Avastin and Lucentis over the period of 1 year was unable to demonstrate any differences in both visual and anatomical outcome post-treatment [83]. This conclusion was further confirmed by the CATT Research Group, who compared Avastin and Lucentis in a two year study. They reported that both drugs seem to have the same effect on CNV and visual acuity, with similar rates of adverse effects, however they concluded that further studies are required with increased amounts of patients to better understand the full effects of the drugs on the patients [82].

Bevacizumab

Bevacizumab (Avastin) is a monoclonal antibody that targets all isoforms of VEGF-A, giving it the potential to be more effective than Macugen. It was approved for the treatment of colorectal cancer by the FDA in 2004, which is the same year that a report came out describing the regression of CNV following systemic treatment with Avastin in patients with neovascular AMD [74]. However, systemic administration of Avastin was deemed unfavorable by physicians as various adverse effects were noted, such as increases in systolic blood pressure [84, 85]. Therefore, numerous clinical trials were performed to study the effectiveness of intravitreal injection of Avastin in neovascular AMD, which displayed promising results [86-90]. Thus, not only was Avastin shown to be clinically effective in patients with neovascular AMD, but it is also less costly per injection [74]. In addition, Avastin has demonstrated clinical efficacy in instances where Macugen has not, indicating that Avastin may be a superior therapeutic treatment option [91]. Therefore, Avastin is frequently the "off-label" choice for many physicians for use in patients with neovascular AMD.

2.3 Recent Advances in AMD Pharmacotherapies

Currently, researchers are investigating other therapeutic approaches for the treatment of neovascular AMD. They are concentrating on identifying other pathways involved in the pathogenesis of AMD that can be targeted by various therapies. In addition, an understanding of the role of Bruch's membrane and the role of various genes implicated in AMD is important in order to be able to manipulate the molecular mechanisms involved in AMD [92].

VEGF Trap-Eye

Aflibercept (Eyelea™) was accepted for treatment in patients with neovascular AMD by the US FDA in November 2011. It functions as a VEGF-Trap, binding to all VEGF-A isoforms, as well as placental growth factor (PlGF). Aflibercept is a recombinant chimeric molecule that utilizes VEGF-binding elements from the VEGFR1 and VEGFR2 extracellular domains, which are fused to the Fc portion of the human IgG1, and administered intravitreously [93]. It has shown to be more efficacious than either bevacizumab or ranibizumab in animal models, with a longer ocular half-life as well as a higher binding affinity to VEGF-A [94]. Various clinical trials have been performed in order to determine Aflibercept's effects on individuals with neovascular AMD. A phase I clinical trial studying the safety, tolerability, maximum tolerated dose, and bioactivity of intravitreal aflibercept confirmed its clinical efficacy and safety [95]. The phase II clinical trial was a multicenter, prospective, randomized, double-masked clinical trial testing various doses and frequencies of aflibercept administration. All groups displayed a decrease in central retinal thickness, with the groups dosed monthly showing the most improvement in visual acuity and retinal thickness (compared to the groups dosed quarterly) [1, 96]. Two phase III clinical trials were then undertaken in North America (VIEW 1) and the EU,

Asia Pacific, Japan, and Latin America combined (VIEW 2), testing the ideal frequency and dosage of aflibercept compared to ranibizumab. These two clinical trials showed that 95-96% of patients treated with aflibercept maintained their vision, compared to 94% of patients receiving ranibizumab alone. In addition, these studies confirmed the appropriate dose of 2 mg aflibercept in patients, with a loading dose of 3 monthly injections followed by administration every 8 weeks [94]. Therefore, aflibercept has thus far proven to be a novel choice of treatment for patients with neovascular AMD due to its higher half-life and consequential less frequent need for administration.

Combination Therapy

Although anti-VEGF therapies have shown to be effective in patients with neovascular AMD, researchers are currently trying to identify new therapeutic approaches that make use of the combination of anti-VEGF therapies with other agents, such as steroids. This approach has the potential to be more effective in patients with neovascular AMD due to the targeting of additional pro-angiogenic cytokines in combination with VEGF. Augustin and colleagues were looking into the use of a triple therapy, combining photodynamic therapy (PDT), bevacizumab, and triamcinolone as a possible treatment method for neovascular AMD [97]. Clinical trials are still ongoing to try and find the ideal combination of therapeutic agents to properly treat AMD. One ongoing study is comparing the safety and efficacy of iSONEP, an ocular formulation of an anti-sphingosine-1-phosphate monoclonal antibody that is known to inhibit angiogenesis, inflammation, cell survival, and proliferation. Four monthly injections of iSONEP are being tested both alone and in combination with Avastin or Lucentis for the treatment of AMD.

Chapter 3

MATERIALS AND METHODS

3.1 Cell Culture

One human retinal pigment epithelial cell line (ARPE-19) (ATCC, Rockville, MD) was used for all experiments performed for this thesis. The cell line was incubated at 37°C in a humidified 5% CO_2-enriched atmosphere. The RPE cells were cultured in a 1:1 mixture of Dulbecco's modified eagle medium (DMEM) and Ham's F12 (500 ml) (Invitrogen Life Technologies, Burlington, Ontario, Canada), supplemented with 10% heat inactivated fetal bovine serum (FBS; Invitrogen), 1% fungizone (Invitrogen) (1250 µg fungizone), and 1% penicillin-streptomycin (50,000 units). The cells were cultured as an adherent monolayer in 25cm² cellBIND™ surface technology flasks (Corning), and the media was changed twice weekly. The cells were passaged by treatment with 0.05% trypsin in EDTA (Corning) at 37°C, and washed in 5ml of their respective media before centrifugation at 120g for 5 minutes to form a pellet. A vital dye (Trypan Blue) as well as a hemocytometer was used to count the cells before seeding.

3.2 Treatment of RPE Cells

ARPE-19 cells were plated in 6 welled plates at a seeding density of $2.5x10^5$ cells/well and left overnight to adhere to the bottom of the well. After 48 hours, all media from all plates was removed and replaced with serum-free media. Plates intended to simulate hypoxic conditions were treated with 100 µM cobalt chloride ($CoCl_2$) (Sigma-Aldrich). Plates were then left for 24 hours, before treatment. All conditions were performed in triplicate. The treatment conditions included an alcohol-vehicle control, DPE alone (100µM) (Caymanchemical), bevacizumab alone (0.25mg/ml) (Roche), and the combination of DPE and bevacizumab. Media was harvested after 24 hours for sandwich ELISA-based angiogenesis arrays.

3.3 Quantibody Human Angiogenesis Array

Quantibody Human Angiogenesis Arrays (RayBiotech, QAH-ANG1), which is a sandwich ELISA-based angiogenesis array, were used in order to determine the secretion levels of the following ten pro-angiogenic cytokines: Angiogenin, ANG2, EGF, bFGF, HB-EGF, PDGF-BB, Leptin, PlGF, HGF, and VEGF-A. The arrays were set up and run as follows: 1) The array slides were allowed to equilibrate to room temperature for 30 minutes in the plastic bag, and for 2 hours outside the plastic bag. 2) 100µl Sample Diluent was added to each of the 16 wells for 30 minutes at room temperature to block the slide. Incubations were performed under gentle rotation. 3) Sample Diluent from each well was discarded and replaced with 100µl of pre-determined dilutions of the Cytokine Standard Mix for 2 hours at room temperature, as well as the experimental samples. 4) Both the dilutions of the Cytokine Standard Mix as well as the samples were discarded. The slide was washed five times in 150µl 1x Wash Buffer I at room temperature for 5 minutes each, followed by two washes of 150µl 1x Wash Buffer II at room temperature for 5 minutes each. Wash buffers were decanted after each wash step. 5) Wash Buffer II was discarded and replaced with 80µl of detection antibody cocktail in each well for 2 hours at room temperature. 6) Step 4 was repeated. 7) Wash Buffer II was discarded and replaced with 80µl Cy3 equivalent dye-conjugated streptavidin in each well for 1 hour. This step was performed in darkness and arrays were covered in aluminum foil to avoid light exposure. 8) Step 4 was repeated. 9) The slide device was disassembled, and was subjected to various wash steps. First it was washed in 30 ml Wash Buffer I for 15 minutes, followed by 30 ml Wash Buffer II for 5 minutes. Lastly, the slide was placed in the Washer/Dryer chamber provided, and centrifuged at 1,000 rpm for 3 minutes without a cap to dry the slide.

3.4 Statistical Analysis

A Student's t-test was used in order to determine statistical significance. The results for the angiogenesis array were directly compared to the control when the cells were treated with 100μM DPE. Results were considered statistically significant as follows: *: p-value <0.05, **: p-value < 0.001. The results for the angiogenesis array were directly compared to the bevacizumab control when the cells were treated with a combination of 100μM DPE + 0.25mg/ml bevacizumab. Again, results were considered statistically significant as follows: *: p-value <0.05, **: p-value < 0.001.

Chapter 4

RESULTS

4.1 Analysis of Secretion of Pro-Angiogenic Factors

Four of the ten pro-angiogenic cytokines measured were secreted by ARPE-19 cells. These pro-angiogenic cytokines included VEGF-A and Angiogenin, as well as ANG-2 and EGF, however these were secreted on a smaller scale (figure 1). Following treatment with bevacizumab, Angiogenin was significantly increased and VEGF-A was significantly decreased under normoxic conditions compared to the control (figure 2). In general, two cytokines (Angiogenin and VEGF-A) were significantly increased under $CoCl_2$-simulated hypoxia compared to normoxic conditions (figure 3). The secretion of HB-EGF and PlGF, although absent under normoxia, were slightly induced under hypoxia. The secretion of ANG-2 was increased (figure 4). Following treatment with DPE, secreted levels of Angiogenin and VEGF-A were significantly reduced under normoxic conditions (figure 5). The same pro-angiogenic cytokines were also significantly reduced following treatment with DPE in a hypoxic state (figure 6), in addition to a significant decrease of ANG-2 and PlGF, and a decrease in HB-EGF (figure 7). The secretion of Angiogenin was significantly reduced following treatment with the combination of bevacizumab with DPE under normoxic conditions (figure 8). Lastly, the secretion of Angiogenin was significantly reduced following combination treatment under hypoxia (figure 9).

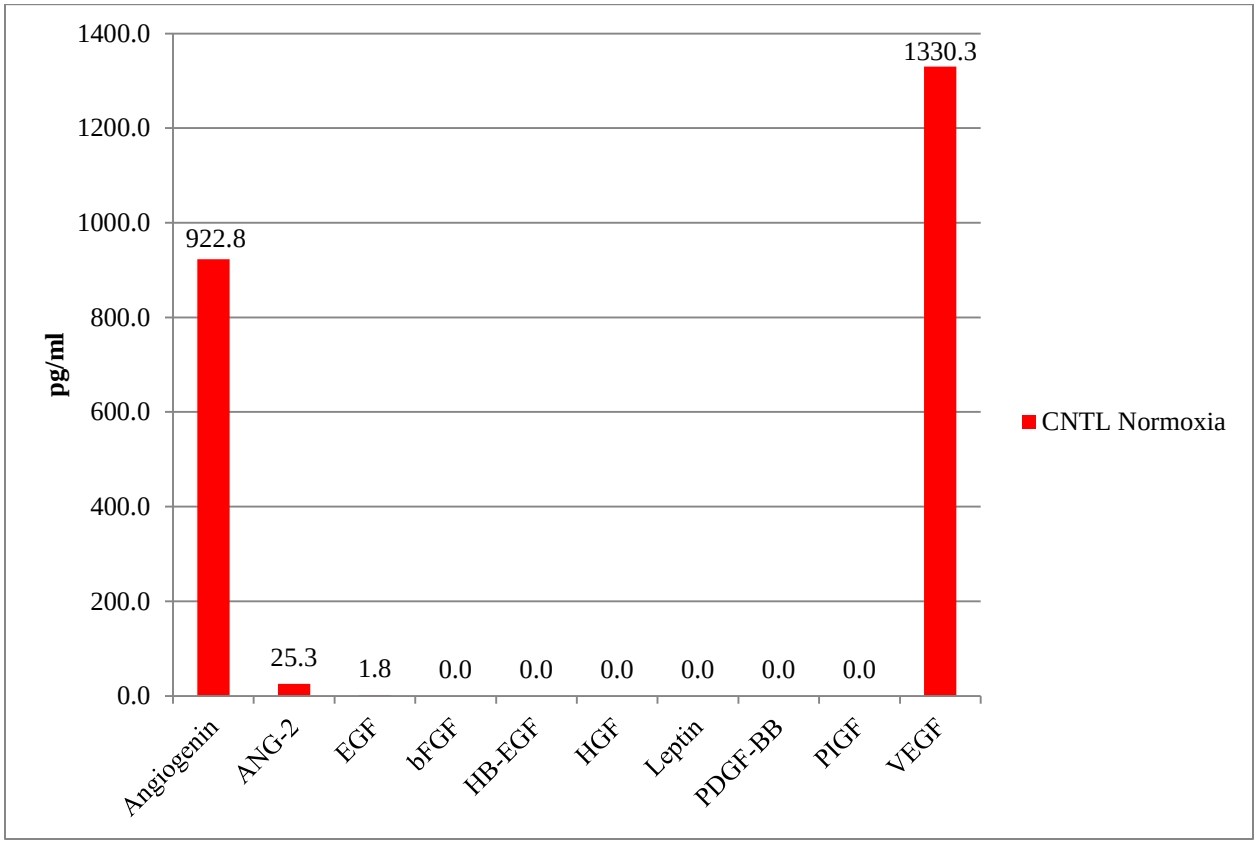

Figure 1. Secretion of pro-angiogenic cytokines from ARPE-19 cells under normoxia

ARPE-19 cells secreted four of the ten pro-angiogenic cytokines analyzed. The highest secreted pro-angiogenic cytokine was VEGF-A, which reached levels of 1330.3 pg/ml. Angiogenin was the next highest secreted pro-angiogenic cytokine, reaching levels of 922.8 pg/ml. ANG-2 (25.3 pg/ml) and EGF (1.8 pg/ml) were also secreted by ARPE-19 cells under normoxic conditions, but on a smaller scale.

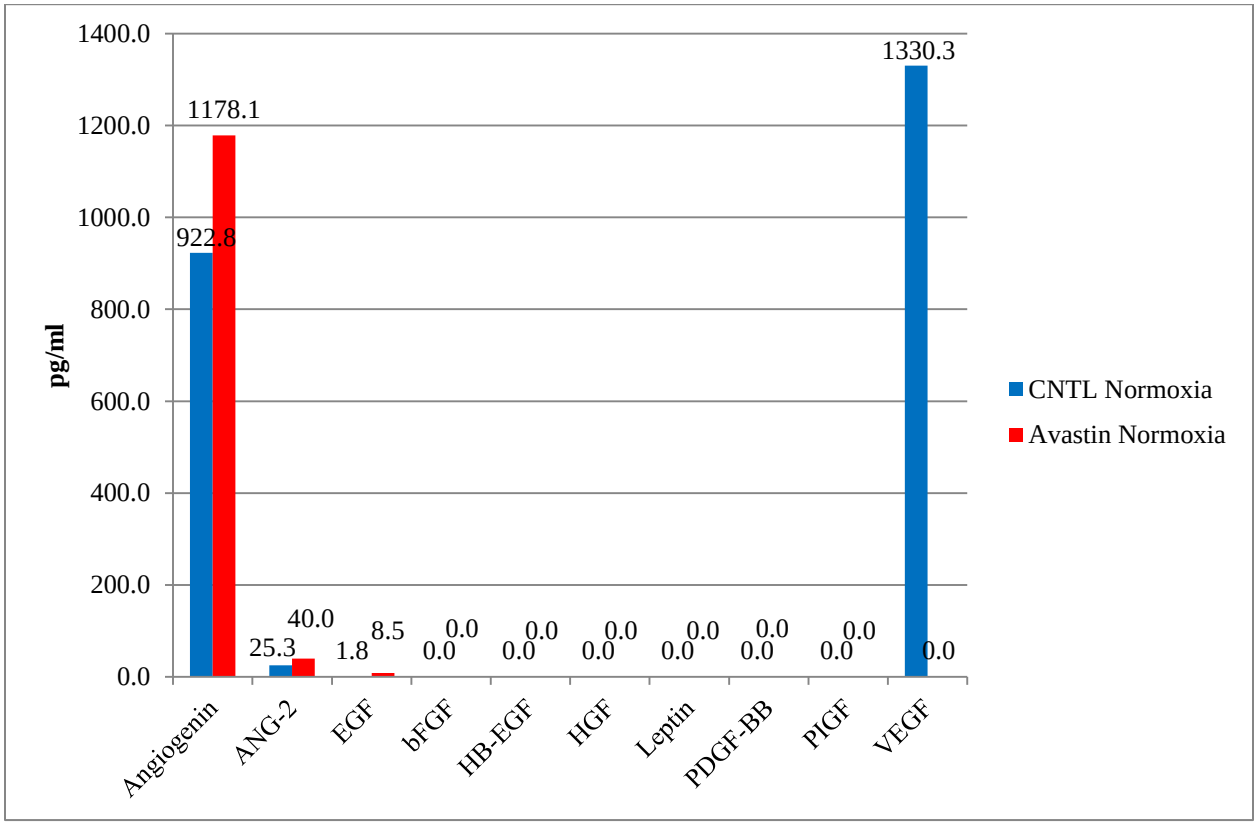

Figure 2. Secretion of pro-angiogenic cytokines following treatment with 0.25 mg/ml bevacizumab under normoxia

Secretion of three cytokines (Angiogenin, ANG-2, and EGF) was increased following treatment with 0.25 mg/ml bevacizumab under normoxia. Only the secretion of Angiogenin was significantly increased, from 922.8 pg/ml to 1178.1 pg/ml. ANG-2 secretion was increased from 25.3 pg/ml to 40.0 pg/ml. EGF secretion was increased from 1.8 pg/ml to 8.5 pg/ml. VEGF-A secretion is completely and significantly reduced from 1330.3 pg/ml to 0 pg/ml following treatment with bevacizumab.

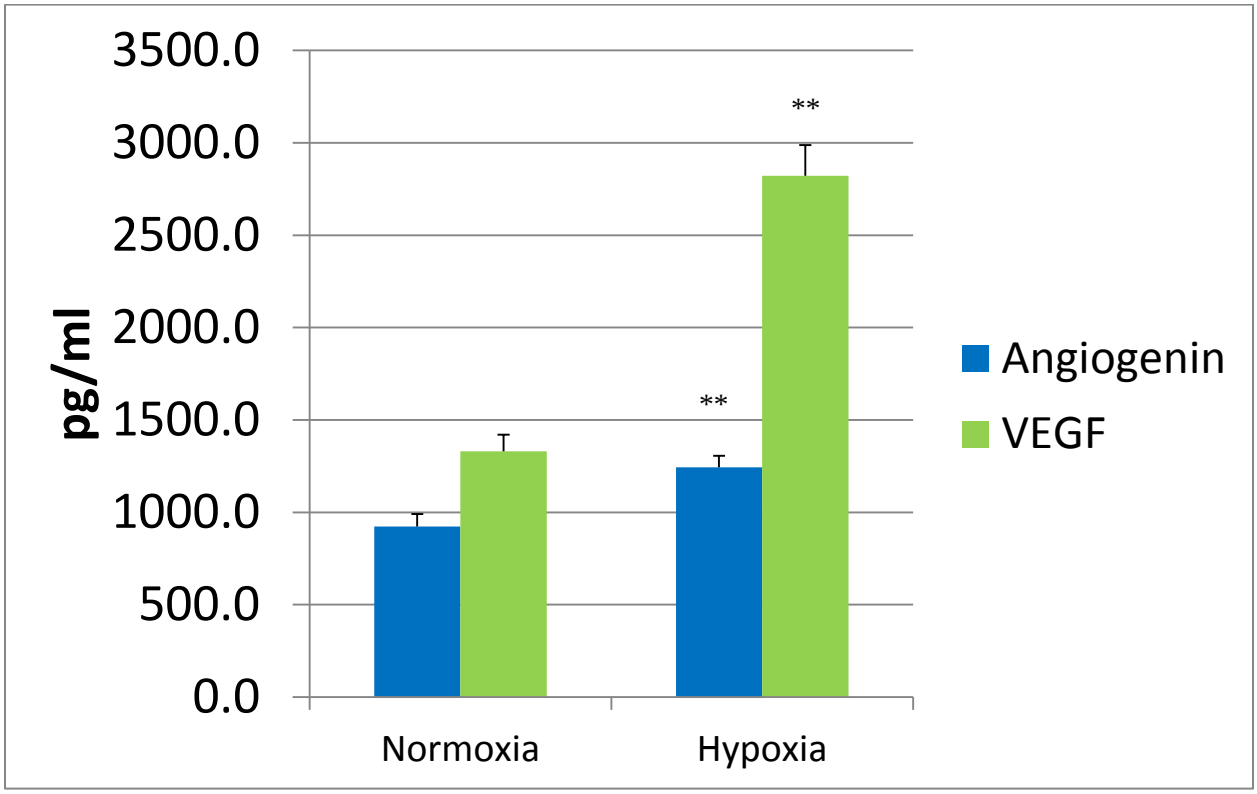

Figure 3. Secretion of various pro-angiogenic cytokines under normoxia compared to hypoxia

Various cytokines are known to increase under stimulated hypoxic conditions, such as VEGF-A [50]. Here we show that levels of both Angiogenin and VEGF-A were significantly increased under simulated hypoxic conditions in retinal pigment epithelial cells. Angiogenin secretion was increased from 922.8 pg/ml to 1243.6 pg/ml, whereas VEGF-A secretion was increased from 1330.3 pg/ml to 2821.9 pg/ml.

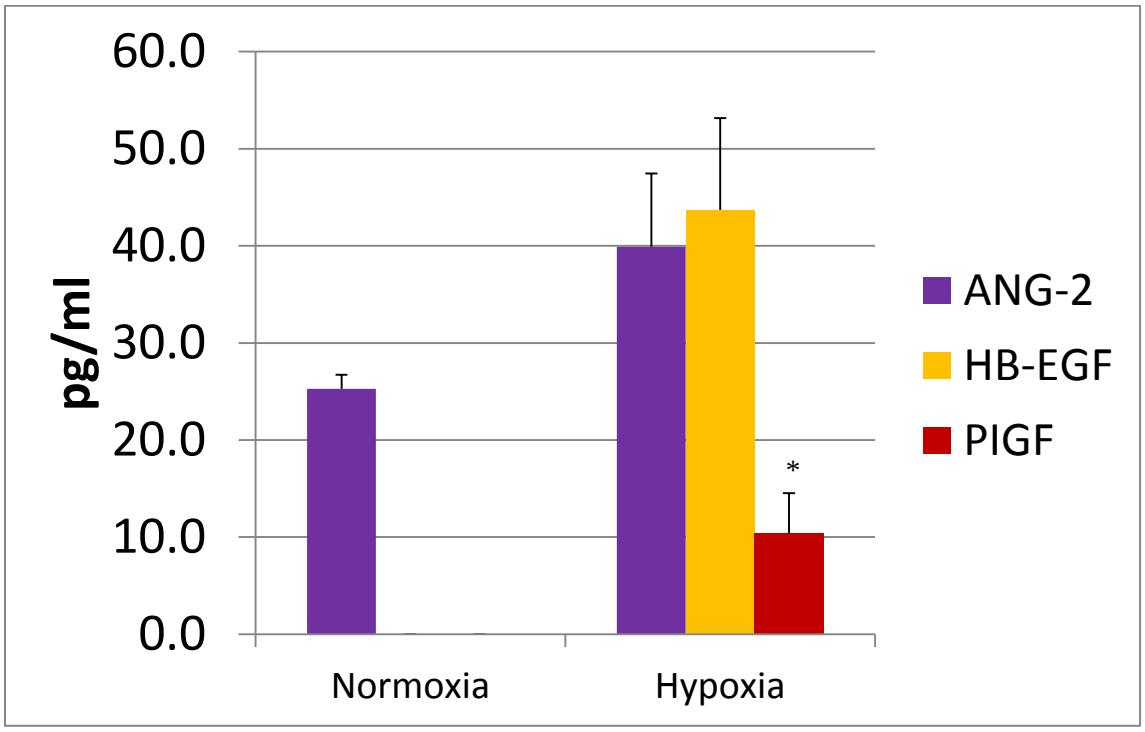

Figure 4. Induction of the secretion of pro-angiogenic cytokines under hypoxia

Levels of PlGF, originally not secreted at all, were significantly increased under hypoxia to 10.4 pg/ml. Levels of HB-EGF increased to 43.7 pg/ml, and ANG-2 increased from 25.3 pg/ml to 39.9 pg/ml, although these increases were not significant.

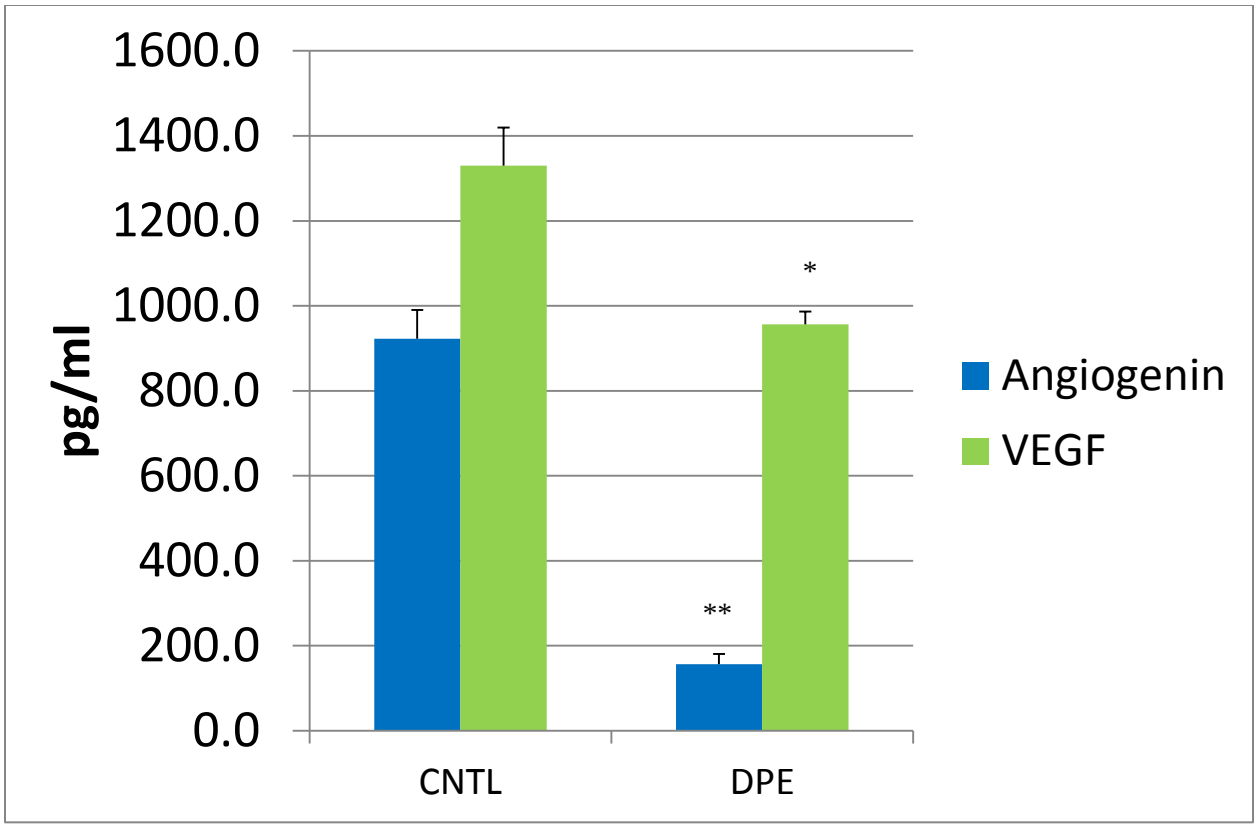

Figure 5. Secretion of pro-angiogenic cytokines following treatment with 100µM DPE under normoxia

The secretion of Angiogenin under normoxia reached levels of 922.8 pg/ml, however after the treatment with 100µM DPE, this value was significantly reduced to 156.9 pg/ml. This trend was also seen in the secretion of VEGF-A. Levels of VEGF-A originally reached 1330.3 pg/ml, however following treatment with 100µM DPE, secretion was significantly down regulated to values of 956.3 pg/ml.

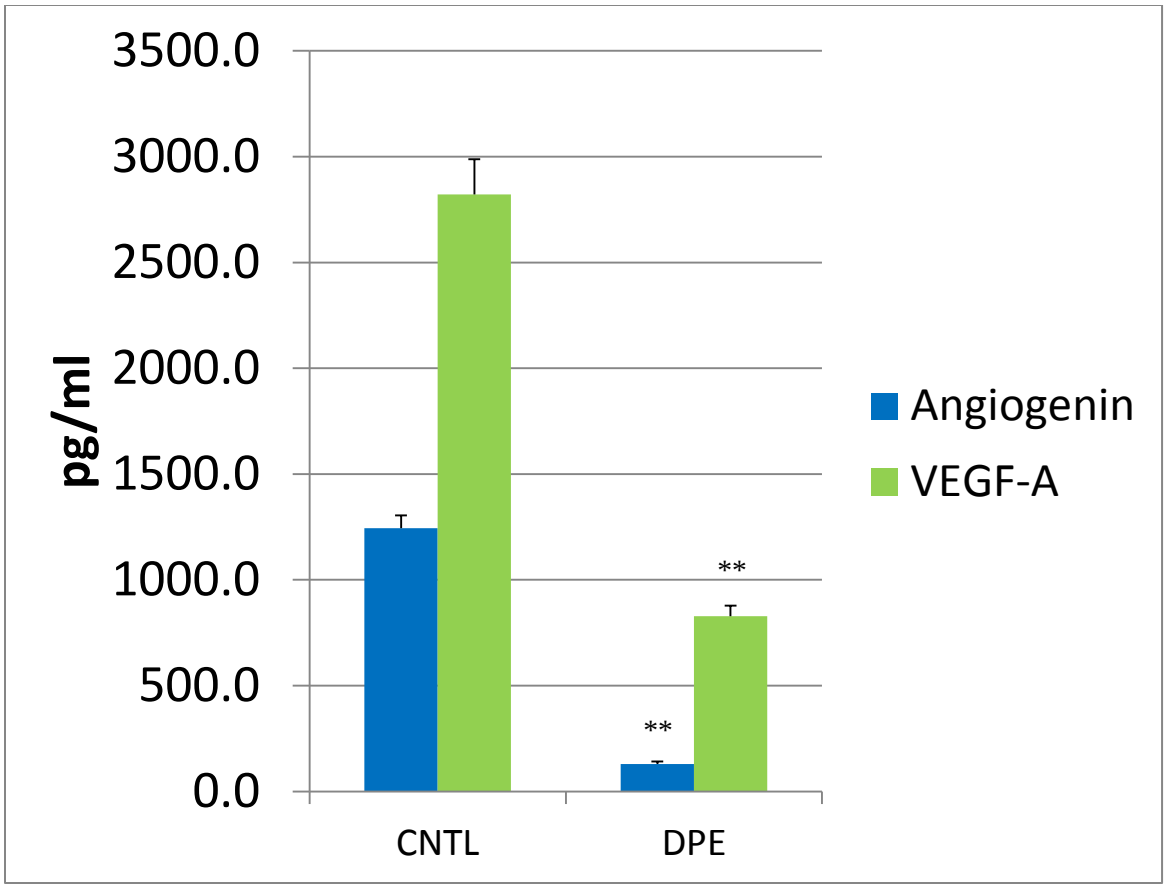

Figure 6. Secretion of pro-angiogenic cytokines following treatment with 100µM DPE under hypoxia

The secretion of Angiogenin under hypoxic conditions was elevated compared to normoxic conditions. However, after DPE treatment, levels of Angiogenin were significantly reduced from 1243.6 pg/ml to 128.9 pg/ml. In addition, levels of VEGF-A were significantly reduced from 2821.9 mg/ml to 828.7 pg/ml.

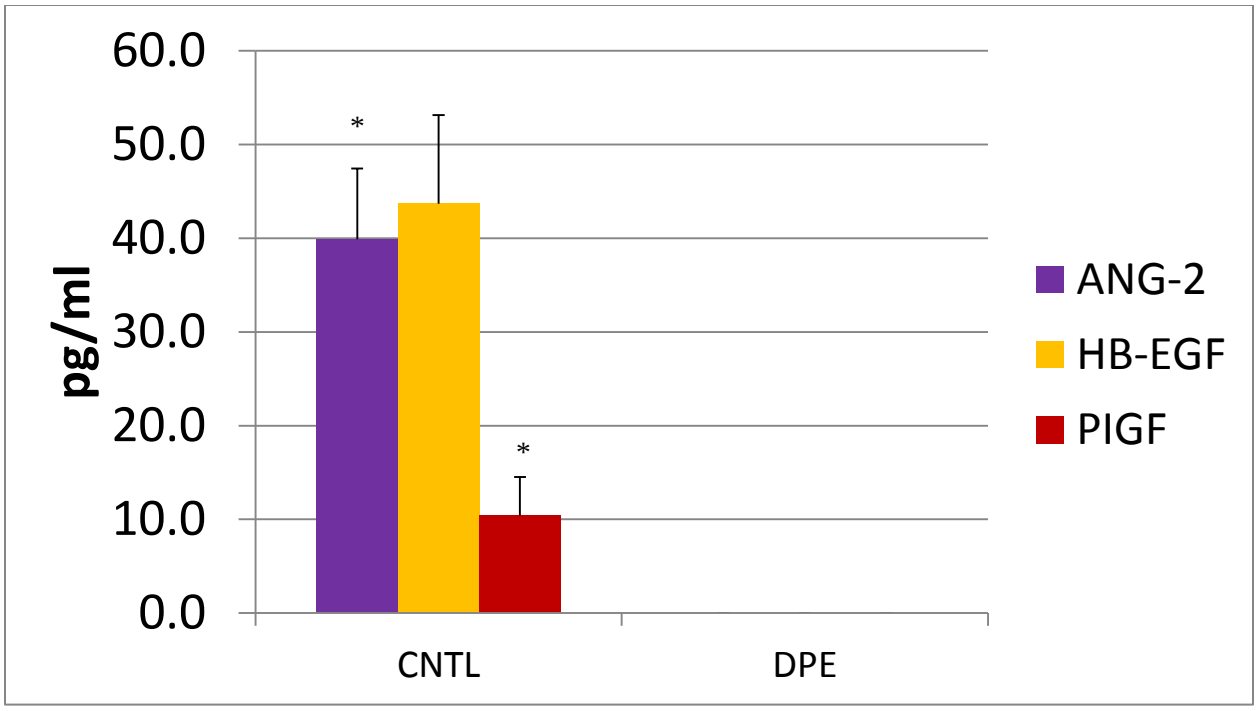

Figure 7. Secretion of pro-angiogenic cytokines following treatment with 100µM DPE under hypoxia

Levels of ANG-2, HB-EGF, and PlGF were reduced under simulated-hypoxia, however on a smaller scale compared to Angiogenin and VEGF-A. Levels of ANG-2 and PlGF were completely and significantly attenuated after the treatment with 100µM DPE, from 39.9 pg/ml and 10.4 pg/ml respectively. HB-EGF was reduced from 43.7 pg/ml, although not significantly.

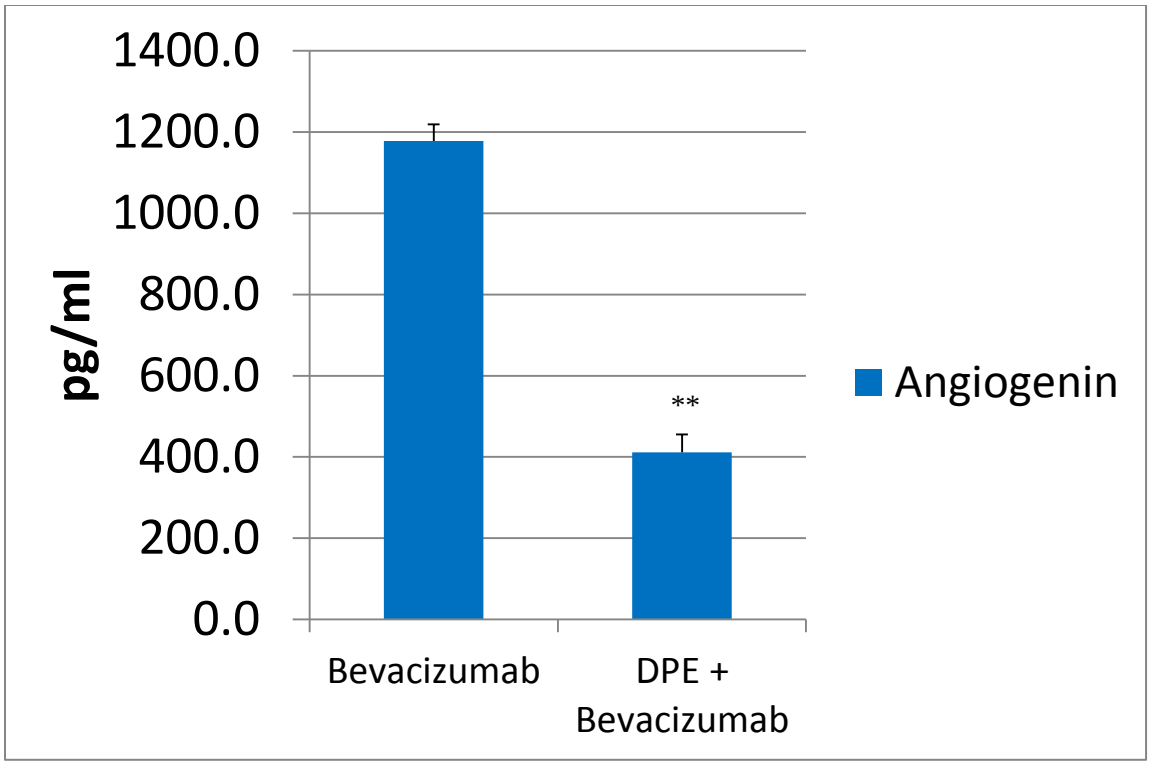

Figure 8. Secretion of pro-angiogenic cytokines following treatment with the combination of 100µM DPE and 0.25mg/ml bevacizumab compared to bevacizumab alone under normoxia

Cells were treated with both bevacizumab as well as the combination of bevacizumab and DPE under normoxia. Levels of Angiogenin after the combination treatment with bevacizumab and DPE were significantly reduced from 1178.1 pg/ml to 411.4 pg/ml.

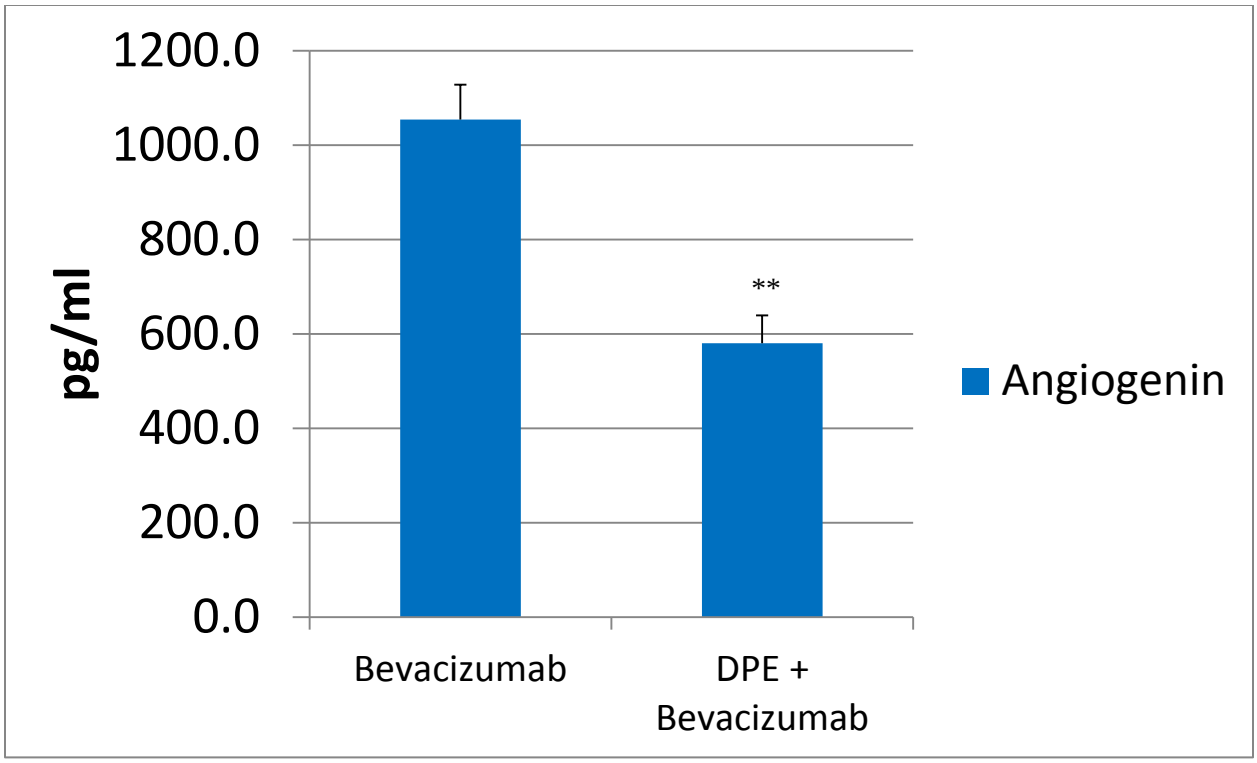

Figure 9. Secretion of Angiogenin following treatment with the combination of 100µM DPE and 0.25mg/ml bevacizumab compared to bevacizumab alone under hypoxia

The treatment with the combination of both DPE and bevacizumab resulted in significantly reduced secretion of Angiogenin under hypoxia. Before treatment with 100µM DPE and 0.25mg/ml bevacizumab, Angiogenin levels reached 1054.4 pg/ml. Following treatment, the secretion of Angiogenin was significantly reduced to 580.2 mg/ml.

Chapter 5

DISCUSSION

5.1 Hypoxia in AMD

Hypoxia has been shown to contribute to both the onset and the progression of AMD [49]. Although the pathogenesis of AMD is not fully known, researchers believe that hypoxia plays an important role in photoreceptor damage and cell death [84]. A hypoxic environment in the macula has been shown to trigger various cellular responses, such as protein misfolding in the ER, increased secretion of various pro-angiogenic proteins, and consequential choroidal neovascularization [84]. Further evidence that implicates hypoxia in the development of AMD is the presence of the transcription regulators HIF-1α and HIF-2α in CNV membranes [3]. VEGF, an important pro-angiogenic cytokine, has also been shown to be expressed in CNV membranes from human AMD eyes [4]. We have shown a significant increase in VEGF-A from RPE cells under a hypoxic stress (figure 3). This increase in VEGF-A under hypoxic conditions further implicate hypoxia in the development of neovascular blood vessels. These results also show that DPE decreased the secretion of pro-angiogenic cytokines under hypoxia. The combination of DPE and bevacizumab, a cheap and widely used alternative to ranibizumab, may be an effective approach because not only is VEGF targeted, but so is Angiogenin, which is another pro-angiogenic cytokine that is highly secreted under both normoxia and hypoxia by retinal pigment epithelial cells (figure 3). HB-EGF and PlGF were induced under hypoxic conditions, and the secretion of ANG-2 was increased (figure 4). The profile of the pro-angiogenic cytokines we observed under hypoxic conditions may reflect in vivo signalling pathways that are involved in the developmental progression of CNV.

Cobalt chloride was used to simulate hypoxia in RPE cells. It is an affordable alternative to incubators that allow adjustments to the oxygen concentration in the air mixture used in cell culture. However, this treatment represents an acute form of hypoxia, whereas the hypoxic stress in eyes with AMD is believed to be chronic. Under normoxic conditions, HIF-1α is degraded by the proteasome. Under hypoxic conditions, HIF-1α binds to HIF-1β, translocates to the nucleus, and stimulates hypoxia-induced gene expression. This will lead to hypoxia-induced angiogenesis. In vitro treatment with cobalt chloride up-regulates and stabilizes HIF-1α. This, in turn, up-regulates VEGF secretion and expression. Cobalt chloride is a viable alternative given that it stabilizes and activates HIF-1α, a master regulator of cellular hypoxic signalling.

5.2 Angiogenesis Assays

Bevacizumab, a widely used treatment for neovascular AMD, is a monoclonal antibody that targets all isoforms of VEGF-A. Therefore it is not surprising that VEGF-A secretion from RPE cells is completely attenuated following treatment with bevacizumab (figure 2). The opposite effect was seen with several other pro-angiogenic cytokines. Secretion of ANG-2 and EGF increased, although not significantly, as well as the secretion of Angiogenin, which was significant (figure 2). We believe that this increase in Angiogenin secretion may be due to compensatory mechanisms in response to bevacizumab treatment. Due to the inhibition of one important pro-angiogenic cytokine (VEGF-A), others such as Angiogenin may be increased consequentially. Compensatory changes in cytokines have been noted in cancer cases following treatment with bevacizumab. Systemic administration of bevacizumab in patients with colorectal cancer have shown significantly increased plasma levels of PlGF, further illustrating changes in pro-angiogenic cytokines following bevacizumab administration [98]. In addition, up-regulation of FGF [99], PlGF [100, 101], and erythropoietin [102] in the plasma have been documented as well following VEGF obstruction in various cancers. Ocular changes in cytokine profiles following bevacizumab administration have also been studied. Compensatory increases of intraocular inflammatory cytokine secretions (such as Il-8 and TGF-β_2) have previously been reported following intravitreal injection of bevacizumab in patients with proliferative diabetic retinopathy (PDR) [52]. This compensatory mechanism following VEGF down-regulation has also been reported in patients with retinopathy of prematurity (ROP), where the expression of Angiopoietin1 and bFGF1 has been shown to be increased in retinal vascular endothelial cells in low-expressing VEGF groups [96]. Therefore, it is possible that the increase in Angiogenin that

we have seen may be due to compensatory mechanisms that occur in the cell due to significant decreases in VEGF-A secretion.

Secreted levels of various pro-angiogenic cytokines in response to DPE were also determined. Both Angiogenin and VEGF secretion was significantly decreased following RPE treatment with 100µM DPE under normoxic conditions (figure 5), indicating DPE's potential to reduce the secretion of important angiogenic cytokines in RPE cells. While the standard of therapy for AMD targets VEGF alone, we demonstrate that DPE may be able to target VEGF and Angiogenin. DPE is also able to reduce pro-angiogenic secretion under hypoxic conditions. Secretion of Angiogenin, VEGF, ANG-2, and PlGF were significantly reduced under hypoxic conditions following treatment with DPE, further illustrating DPE's role in reducing pro-angiogenic signalling in RPE cells (figures 6&7). This concentration was chosen due to the fact that DPE has been previously studied in various in vitro systems at this concentration [2, 56, 58, 60, 103] in addition to in ARPE-19 cells [57, 60].

Due to DPE's effectiveness in reducing several pro-angiogenic cytokines secreted by the RPE cells in vitro under both normoxia and hypoxia, it was combined with bevacizumab to determine whether the combination offered potential benefits not seen with bevacizumab therapy. Bevacizumab (0.25 mg/ml) and DPE (100µM) were combined and used to treat RPE cells. Secretion of pro-angiogenic factors were quantitated to determine its effects in RPE cells compared to bevacizumab alone. Secretion of Angiogenin was significantly decreased in normoxic conditions compared to bevacizumab alone (figure 8). In addition, Angiogenin secretion was significantly reduced following combination treatment compared to bevacizumab alone in hypoxic conditions (figure 9), further validating DPE's role in reducing angiogenic signalling in RPE cells in combination with bevacizumab. The ability of DPE in combination

with bevacizumab to significantly reduce the secretion of Angiogenin, a pro-angiogenic cytokine that is up-regulated when RPE cells are treated with bevacizumab alone, illustrates their potential use as a combination therapy in AMD. The safety of 0.25 mg/ml of intravitreal bevacizumab in vitro in several ocular cells, such as ARPE-19, rat retinal ganglion cells, pig choroidal endothelial cells, and human microvascular endothelial cells was confirmed in several studies [85, 91].

5.3 Angiogenin secretion in AMD

According to our results, Angiogenin was the second highest secreted pro-angiogenic cytokine from RPE cells under both normoxia and hypoxia following VEGF-A. These findings warrant further investigation in the potential role of Angiogenin in the progression and pathogenesis of neovascular AMD. According to our results, Angiogenin would be a logical cytokine to target in combination with VEGF in order to reduce angiogenesis. Angiogenin functions as a potent inducer of angiogenesis, and does so by activating vessel endothelial and smooth muscle cells. Angiogenin is involved in vascular endothelial cell migration, invasion, proliferation, and tubular structure formation. It has been shown to have four main mechanisms of action in the cell: signal transduction, basement membrane degradation, and nuclear translocation, in addition to its ribonuclease activity [49]. Figure 4 and figure 5 show that the levels of secreted Angiogenin were reduced following treatment with DPE compared to the control during both normoxic and hypoxic conditions. In addition, the combination of DPE plus bevacizumab was more effective in reducing Angiogenin secretion compared to bevacizumab alone. These results highlight a potential therapeutic role for DPE in combination with bevacizumab in the treatment of choroidal neovascularization.

Angiogenin has been shown to bind to a 42-kDa cell surface actin, a 170 kDa cell surface protein, actinin, fibulin, and follistatin [20, 69-71, 104]. This interaction can potentially modify the cellular cytoskeleton, thereby contributing to Angiogenin's pro-angiogenic actions. Pyatibratov et al. demonstrated that human Angiogenin binds to both G-actin and F-actin, inhibiting G-actin polymerization, and causing F-actin stiffening [14]. Exogenous Angiogenin translocates into the nucleus of HUVECs, indicating its potential to regulate gene expression by binding to DNA. Angiogenin has been shown to be released by deoxyribonuclease I, which

further supports this hypothesis. In addition, HUVECs were shown to have an increased ability to take up Angiogenin at a low endothelial cell density [105]. Therefore anti-Angiogenin agents may represent a possible therapeutic approach in the dry form of AMD to prevent early stages of CNV formation.

Angiogenin also promotes angiogenesis through the phosphorylation of the protein kinase B/Akt. This PI3-kinase/Akt signal pathway, however, is independent of Angiogenin's ability to be translocated to the nucleus of endothelial cells [61]. As shown, Angiogenin has several pathways that are involved in promoting angiogenesis, and would therefore be a good target for diseases that occur due to blood vessel proliferation and migration, such as AMD.

Angiogenin expression has been shown to be up regulated in multiple types of cancers. In addition, in several types of cancer such as prostate cancer, the elevation of expression of Angiogenin is associated with disease progression, as prostatic epithelial cells advance from a benign to an invasive phenotype [104]. Yoshioka et al. demonstrate the ability of Angiogenin to stimulate prostate cancer cell proliferation both in vitro and in vivo. Upon blocking the translocation of Angiogenin to the nucleus in athymic mice, tumor growth was inhibited, in addition to a reduction in cancer cell proliferation and angiogenesis. These results further implicate Angiogenin in the promotion of angiogenesis [15].

Angiogenin's presence in the eye has also been studied. Vitreous levels of Angiogenin in patients with proliferative diabetic retinopathy (PDR) have been measured and compared to vitreous levels of Angiogenin in patients with proliferative vitreoretinopathy (PVR) and in patients with idiopathic macular epiretinal membrane (IERM). Patients with PDR and PVR have shown significantly increased vitreous levels of Angiogenin compared to patients with IERM, demonstrating Angiogenin's potential involvement in the breakdown of the blood-ocular barrier

in these eyes [106]. However, other studies have indicated a decreased concentration of Angiogenin in both the vitreous and serum in patients with diabetic retinopathy [107]. This conflicting evidence indicates that Angiogenin's role in the vitreous remains to be confirmed, however all studies support a potential role for Angiogenin in the aforementioned diseases, all of which include a pathologic vascular component.

Lastly, Angiogenin has been shown to play a role in neovascular AMD. Angiogenin has been shown to be expressed in both the retina and RPE cells. In addition, when incubated with chorioretinal endothelial cells, Angiogenin has been shown to be internalized by Rf/6a cells and translocate to the cytoplasm and nucleus [78]. Although Angiogenin has not been shown to play a role in chorioretinal endothelial cell migration, these findings do confirm Angiogenin's presence in the retina and RPE-choroid, and illustrate Angiogenin's potential role in other aspects of angiogenesis.

5.4 DPE and Angiogenic Signalling in RPE Cells

Not only does DPE decrease Angiogenin and VEGF-A secretion significantly under both normoxia and hypoxia compared to control conditions (figures 5 and 6), but we have also shown that it attenuates the secretion of several pro-angiogenic cytokines such as ANG-2, HB-EGF, and PlGF (figure 7). Although expressed at minimal amounts in RPE cells under hypoxia (figure 4), these cytokines are all heavily involved in angiogenesis. Among their various functions is a role in the proliferation of vascular endothelial cells.

Simon et al.'s results demonstrate the expression of ANG-2 at sites where vascular remodelling is taking place in order to promote angiogenesis [108]. These results, like ours, identify ANG-2 as a cytokine that is up regulated under hypoxic conditions, such as those seen in AMD. Given that this pro-angiogenic cytokine is expressed under hypoxic conditions, its inhibition or reduction in expression is important to reduce angiogenesis. DPE was able to completely and significantly reduce the secretion of ANG-2 under hypoxia, further solidifying DPE's potential anti-angiogenic role in AMD.

Fiedler et al. demonstrated the ability of ANG-2 to induce inflammation by sensitizing endothelial cells to TNFα, an inflammatory molecule [109]. Inflammation has also been shown to play a role in the death of the RPE and retina. Our results show a significant decrease in ANG-2 under hypoxic conditions following treatment with DPE (figure 7). These findings illustrate DPE's potential anti-inflammatory properties.

Ziche and colleagues have demonstrated PlGF's ability to promote neovascularization in rabbit corneas, and chick chorioallantoic membrane assays [110]. This is another pro-angiogenic cytokine that has been significantly up-regulated under hypoxic conditions (figure 4), and down-

regulated following treatment with DPE (figure 7). These results further illustrate DPE's potential ability to help retard neovascularization in the eye.

We have shown DPE's ability to inhibit the secretion of ANG-2, HB-EGF, and PlGF (figure 7), two pro-angiogenic cytokines that are secreted under hypoxic conditions, further demonstrating its potential anti-angiogenic and anti-inflammatory ability. Therefore, DPE may be useful in combination with an anti-VEGF agent such as bevacizumab due to its potential ability to target several anti-angiogenic pathways.

In 2010, Terzuoli and colleagues illustrated DPE's ability to inhibit the expression of HIF-1α, a major player in hypoxic signalling as well as neovascularization, in a HT-29 xenograft model [2]. The expression of these proteins has also been found in CNV membranes [3]. Therefore, in accordance with Terzuoli et al., we believe that DPE may function by down-regulating the HIF-1α/mPGEs-1/VEGF axis.

Zhu and colleagues have proposed a mechanism where DPE functions in two critical pathways. They first show that DPE induces phase II detoxifying enzymes in ARPE-19 cells [60]. Nuclear factor-E2-related factor 2 (Nrf2) is a transcription factor that is responsible for the induction of phase II detoxifying enzymes. It is kept inactive in the cytosol when bound to Keap1. However, upon phosphorylation, it will undergo a conformational change that separates it from Keap1, allowing it to translocate to the nucleus and activate the transcription of antioxidant response elements. This will lead to the induction of the expression of several anti-oxidant genes such as heme-oxygenase-1 (HO-1), NADPH (nicotinamide adenine dinucleotide phosphate)-quinone-oxidoreductase 1 (NQO-1), and γ-glutamyl-cysteinyl-ligase (γ-GCL). Zhu and colleagues demonstrated DPE's ability to activate Nrf2, consequently promoting the protein expression and activation of the previously mentioned anti-oxidant genes.

The second mechanism that Zhu et al. also proposed involves the stimulation of mitochondrial biogenesis and function. They have demonstrated DPE's ability to stimulate the expression of peroxisome proliferator-activated receptor coactivator 1 alpha (PPARGC1α), which is the key factor for mitochondrial biogenesis, as well as Tfam, a transcription factor that is also involved in mitochondrial biogenesis, and a downstream target for PPARGC1α. PPARGC1α signalling will promote mitochondrial replication and transcription. AMPK and e-NOS, two upstream regulators of PPARGC1α, have also been shown to be up-regulated by DPE.

Thus, DPE has been shown to reduce the secretion of several pro-angiogenic cytokines such as Angiogenin, VEGF-A, as well as ANG-2 and PlGF although their secretion is on a smaller scale. In the combination therapy that we tested, DPE may function by controlling compensatory mechanisms that occur in the cell in response to bevacizumab treatment. In addition, DPE has been shown to induce phase II detoxifying enzymes as well as stimulate mitochondrial biogenesis and function in ARPE-19 cells, and therefore may contribute to the reduction in pro-angiogenic cytokines through these mechanisms.

5.5 Antioxidants in AMD

Due to an increasing amount of evidence implicating oxidative damage in the pathogenesis of AMD, treatment with antioxidants may be beneficial to patients in order to reduce the progression of the disease. Studies investigating the anti-oxidative role of DPE as well as other antioxidants and their effects on AMD progression have been performed and are currently undergoing.

There have been several multiple prospective cohort observational studies (such as the Beaver Dam Eye Study, the Blue Mountains Eye Study, and the Carotenoids and Age-related Eye Disease Study (CAREDS)), that have shown that diets high in antioxidants such as zinc, vitamin C, vitamin E, and carotenoids are associated with a slower progression of AMD in patients with the disease [54, 111-113]. These studies are interesting because oxidative damage has been shown to be an important process that occurs in the progression of AMD [56]. Therefore, nutritional supplementation with antioxidants may be an interesting approach to either treat or delay the progression of this disease. There have been studies investigating the effects of vitamins A, C, E, zinc, lutein and zeaxanthin, α carotene, β carotene, β cryptoxanthin, and lycopene in both patients with and without AMD, with varying results [114, 115]. Although most antioxidants have not been shown to prevent early AMD, there are some that have shown some promise for the more advanced form.

The AREDS study (Age-Related Eye Disease Study) is the largest study to date which looks at the effects of antioxidants on the progression of AMD, with a sample size of 3,640, a study duration of 5 years, and follow-up duration of 7 years. This duration of the study as well as the follow-up is important when studying AMD, since its progression is slow and occurs over a number of years. In addition, a large sample size is also important since AMD progression into

an advanced form occurs at a low frequency in individuals. Other significant randomized control trials looking at nutritional supplements in AMD had smaller sample sizes of only 60-164 patients, with follow up times significantly smaller than the AREDS study, ranging from 6-24 months [57, 58, 74, 116, 117]. It is important to have larger follow-up times due to the fact that AMD progression is slow, and disease progression may be more apparent over a longer period of time. Consequently, the AREDS study is an important study that assesses the effects of nutritional supplementation in patients with AMD.

The AREDS Study

The AREDS study included two different trials – one for AMD and one for cataracts. The one for AMD included 3,640 participants between the ages of 55-80 that had at least the early form of AMD. Enrollment for the study was between the years of 1992 and 1998. Patients were divided into four categories. The patients in the first category (Category 1) had a few small or no drusen in one eye. The patients in the second category (Category 2) had early AMD, including multiple small drusen or a few medium sized drusen in one or both eyes. The patients in the third (Category 3) had intermediate AMD, consisting of several medium sized drusen or one or more large drusen in one or both eyes. The patients in the fourth category (Category 4) had advanced AMD, consisting of the advanced dry form or wet form in only one eye. The second eye had good vision and did not have advanced AMD. One eye of each patient had to be free of any vision-loss related disease besides AMD or cataracts, and not operated on other than for cataract surgery, as a constant for the study. Patients were randomly administered daily oral tablets consisting of Zinc alone (80 mg zinc oxide and 2 mg cupric oxide), Antioxidants alone

(500 mg vitamin C, 400 IU vitamin E, and 15 mg beta carotene), a combination of Antioxidants and Zinc, or a placebo [55].

The purpose of this study was to determine whether the nutritional supplementation of AMD patients with antioxidants and zinc could prevent or weaken the progression of AMD. Although no significant effects were observed in AMD patients in Category 1 and 2 (due to a slow rate of AMD progression), AMD patients in Category 3 and 4 demonstrated significant results, which were most effective in patients receiving the combination of Antioxidants and Zinc [111]. The study showed that patients with a high risk of developing advanced AMD had a 25% reduced risk of developing advanced AMD, as well as a 19% reduced risk of central vision loss in the Antioxidant + Zinc group. The study also showed that patients with a high risk of developing advanced AMD had a 21% reduced risk of developing advanced AMD, as well as a 11% reduced risk of central vision loss in the Zinc group. Lastly, patients with a high risk of developing advanced AMD had a 17% reduced risk of developing advanced AMD, as well as a 10% reduced risk of central vision loss in the Antioxidant group. The study concluded that there is a benefit in nutritional supplementation with Antioxidants and Zinc in patients with intermediate and advanced AMD [55]. However it is important to note the various potential harms, such as increases in mortality, congestive heart failure, as well as increased risk of prostate cancer, associated with the ingestion of vitamin E, which are associated with long term, high doses of vitamin E. Potential adverse effects for Zinc supplementation include increased urinary tract infections [111].

The AREDSII Study is a currently ongoing trial including 4,200 patients with intermediate and advanced AMD, and is scheduled to run until December 2012. This study is assessing the effects of carotenoids (lutein and zeaxanthin) and omega-3 fatty acids (DHA and

EPA), and whether their consumption can slow the progression of intermediate and advanced AMD [111]. In addition, this study will evaluate whether beta-carotene should be eliminated from the antioxidant mixture used in the AREDS study, as well as whether the concentration of zinc can be decreased.

Lutein and zeaxanthin are carotenoids of interest due to the fact that they are present in the macular pigment. In addition, studies have shown that there is a decrease in these substances in the macula in patients with AMD [76]. They are believed to function by protecting the photoreceptor cells in the macula from blue light [75]. There have been various studies investigating the effects of dietary lutein and zeaxanthin on the progression of AMD. A questionnaire filled out by 4,519 patients in the AREDS study indicated an inverse correlation between an increased dietary intake of lutein and zeaxanthin and advanced AMD [55]. The CAREDS study indicated that a diet rich in lutein and zeaxanthin may protect women less than 75 years old from intermediate AMD [54]. Lastly, the Blue Mountains Study demonstrated a reduced risk of developing early or neovascular AMD in individuals with a diet high in lutein and zeaxanthin [113]. Therefore, dietary lutein and zeaxanthin has shown some promise in terms of reducing AMD development and progression. The AREDSII study will be able to further confirm lutein and zeaxanthin's role in AMD.

Omega-3 fatty acids, such as DHA, are also currently of interest. DHA has been shown to be present in the outer segments of the photoreceptors [75]. The AREDS study showed that of 1,837 patients, those with the highest intake of omega-3 fatty acids (ALA, DHA, and EPA) demonstrated a 30% lower probability of developing advanced AMD over a 12-year period [55]. Other omega-3 fatty acids have been investigated as well. A study examining the use of supplemental omega-3 fatty acids such as L-carnitine and coenzyme Q10 has shown promise in

terms of slowing visual acuity loss in patients with AMD. However, it must be noted that most patients had early AMD, and there was a low sample size of only 107 patients [73]. More recently, a meta-analysis analyzed 9 different studies and demonstrated that individuals with a diet high in omega-3 fatty acids had a 38% reduction in their risk of developing AMD [77]. Further studies are necessary in order to determine whether omega-3 fatty acids should be recommended as nutritional supplementation to slow AMD progression.

DPE as an antioxidant to treat AMD

Considering our findings of DPE and the inhibition of angiogenic signalling in RPE cells, we believe that DPE should be further considered as an addition to current antioxidant treatment for advanced AMD. DPE has been shown to demonstrate chemopreventative effects which may be attributed to its antioxidant properties. Terzuoli et al. have shown DPE's ability to inhibit colorectal tumor growth due to the reduction of blood vessel lumina and blood perfusion to the tumor [2]. While few studies of DPE in vivo exist, there have been several in vitro studies of DPE in various cancer cell lines which have shown promising results [88-90]. Conversely, antioxidants such as vitamin A, vitamin E, and carotenes have not been shown to be effective in the chemoprevention of colorectal cancer [86, 87]. In fact, according to a review published in 2012, vitamin C and vitamin E's chemopreventative role still remains to be determined, is somewhat controversial, and requires further studies. It is in this fashion that DPE may be an interesting addition to current antioxidant nutritional supplementation for patients at risk for advanced AMD.

DPE has not been as extensively studied as the antioxidants used to treat AMD in the AREDS study, therefore more basic research is required to further understand its mechanisms of

action. Zhu and colleagues have previously proposed a mechanism by which DPE promotes the expression of several antioxidant genes in retinal pigment epithelial cells, primarily by inducing phase II detoxifying enzymes [60]. This previous evidence coupled with our findings provides strong support for DPE as a regulator of oxidative stress in RPE cells. The AREDS study indicated that the combination of antioxidants and zinc show the most benefits in terms of slowing disease progression. Therefore a possible therapeutic avenue for DPE would be to add it to existing formulas used to treat AMD, and compare the patients' response to the established formula to see if there might be any additional benefits of adding DPE to the antioxidant and zinc combination.

5.6 Conclusions

The data presented in this thesis has illustrated a possible therapeutic role for DPE alone or in combination with bevacizumab for the treatment of neovascular AMD. To the best of our knowledge, this is the first time that DPE has been studied in combination with bevacizumab with respect to its anti-angiogenic properties in RPE cells. Although bevacizumab is effective in targeting VEGF-A in AMD, there may be several compensatory mechanisms that allow for the up-regulation of various other pro-angiogenic cytokines, inducing CNV. DPE may help target these pro-angiogenic cytokines, such as Angiogenin. DPE's mechanism of action remains to be determined in terms of its anti-oxidative and anti-angiogenic capacities, however our findings coupled with previous findings of DPE in RPE cells suggest that DPE may be a potential candidate for the treatment of AMD in combination with bevacizumab. Further studies should investigate the effects of DPE in vascular endothelial cells to determine its potential to reduce the proliferation of this cell type. In addition, the use of an animal model may further the understanding of DPE's effects on the progression of AMD in vivo.

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