Effects of Grape Seed Extract, Lutein, and Omega-3 Fatty Acids on Lens Epithelial Cell Behavior *In Vitro* and *Ex Vivo*

THESIS

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Abstract

The purpose of this study was to determine if grape seed extract (GSE), lutein, and omega-3 fatty acids (O3FA), alter oxidative stress, migration, and proliferation in lens epithelial cells (LECs). An antioxidant reductive capacity assay determined the reducing capability of each antioxidant. Then following antioxidant treatment, the following was performed: 1) dichlorofluorescein (DCF) assay to evaluate reduction in LEC reactive oxygen species (ROS) production; 2) a protein array to determine changes in cytokine expression; 3) an MTT assay to evaluate the antioxidants effects on LEC viability; 4) a scratch wound assay to evaluate LEC migration and proliferation; 5) an ex vivo model of posterior capsular opacification (PCO) to evaluate LEC migration and proliferation. GSE’s antioxidant reductive effects surpassed the positive control, while lutein and O3FA showed little reductive ability. The DCF assay corroborated this data; GSE reduced ROS production in LECs compared to positive controls. Lutein was pro-oxidative and O3FA had negligible effects on ROS production. GSE decreased IL-6, IL-8, CCL3, and CCL5 expression compared to controls. Lutein and O3FA showed increased or similar cytokine expression compared to controls. All tested antioxidants decreased in vitro LEC viability compared to controls. All tested antioxidants also decreased LEC migration in the scratch wound assay with presumed toxic effects on the cells. Ex vivo PCO was increased following treatment with O3FA, while GSE and lutein treatments were similar to controls. In conclusion, only GSE showed substantial
antioxidant capabilities and reduced ROS generation. Lutein and O3FA demonstrated no antioxidant abilities and lutein proved pro-oxidative in vitro. Following antioxidant treatment, LECs showed altered expression of cytokines influencing redox signaling, migration, and proliferation. O3FA increased cell proliferation and migration in an ex vivo PCO model while GSE and lutein demonstrated little effect. Careful conclusions should be made regarding the effects of the studied antioxidants on LECs due to findings of variable and limited reducing power.
Dedicated to my late father Dr. Billy Miller
for his inspiration to follow this career
and to my wife Jamie for always
standing by my side as it continues.
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Chapter 1: Introduction

The crystalline lens is a refractive tissue of the eye that works in conjunction with the cornea to focus light onto the retina (Samuelson, 2013). The lens also functions as a filter to prevent damaging ultraviolet light from reaching the retina (Mainster, 1978). The canine lens is biconvex and has an average central thickness of 7mm with an approximately 10mm diameter at the equator. Its function is dependent upon transparency, which is created by the regular arrangement of the anterior epithelium and lens fibers, and the lack of pigmented or vascular structures (Samuelson, 2013). The lens is surrounded by the basement membrane of the lenticular epithelium termed the lens capsule. The lens capsule is a specialized basement membrane important to both structure and function of the lens and is comprised mostly of type IV collagen, laminin, and proteoglycans (Danysh & Duncan, 2009). Anterior lens capsule thickness increases with age and differs dramatically from that of the posterior capsule with the anterior capsule being approximately 50-70μm and the posterior capsule measuring 2-4μm in adult dogs (Samuelson, 2013). The posterior capsule of the canine lens is attached to the anterior vitreous by the hyaloideocapsular ligament and is situated in the patellar fossa (Samuelson, 2013). Zonular ligaments, arising from the ciliary body, insert into the capsule surrounding the equator. These zonules attach to the anterior and posterior capsule at the equator and become homogenous with the capsule matrix (Hansson,
The zonules maintain the proper position of the lens and are necessary for lens accommodation in species with this function. The lenticular epithelium is a monolayer of cells located anteriorly beneath the lens capsule. Active mitosis of lens epithelial cells (LEC) occurs in a germinative region near the equator throughout life. Equatorial lens epithelial cells then are displaced or migrate toward the lens bow where they undergo terminal differentiation into lens fibers. These lens fibers elongate anteriorly and posteriorly meeting fibers from the opposite equatorial region and joining at the lens sutures (Samuelson, 2013). This process begins in utero with newly formed lens fibers surrounding the embryonic nucleus that subsequently form the adult nucleus and cortex of the lens. Lens fiber development is sustained throughout life; as the surrounding new fiber layers form, older, fully differentiated fibers are internalized and compressed resulting in a natural aging change known as lenticular sclerosis (Samuelson, 2013).

The aqueous humor provides the necessary nutrients for lens metabolism and energy generation and is responsible for waste removal. Glucose is the main energy source utilized by the lens and is acquired by diffusion through the lens capsule. Energy within the lens is necessary for ion transport and to fuel its abundant protein production (Gum & MacKay, 2013). Lenticular metabolism consists of both aerobic and anaerobic pathways with glycolysis being the predominant pathway for energy generation due to low oxygen tension within the lens (Beebe, 2011). While deeper fiber cells with very low oxygen availability rely mostly on glycolysis, some oxidative metabolism is utilized by the superficial differentiating fibers and the epithelial cells. Oxidative metabolism, along
with mitochondrial activity, and exposure to light and UV radiation results in the generation of oxygen radicals and subsequent oxidative stress (Beebe, 2011).

Although oxygen tension around the lens is relatively low, oxidative stress does occur necessitating the maintenance of an oxidative balance to prevent cellular damage and maintain transparency. Lenticular oxidative balance is primarily maintained by glutathione (GSH). Glutathione is a tripeptide antioxidant made of the amino acids cysteine, glutamine, and glycine (Beebe, 2011). High concentrations of GSH, mainly derived from synthesis by LEC and differentiating fiber cells, can be found within the lens. The protective effects of GSH are exerted through maintaining lens protein thiols in a reduced state and reducing reactive oxygen species (ROS) (Reddy, 1990). Ascorbic acid is also found in the lens and can reduce oxygen free radicals via oxidation to dehydroascorbate (Lou, 2003). It has been noted that the presence of ascorbic acid may be lower in canine lenses (Gum & MacKay, 2013). Vitamin E and carotenoids along with enzymatic systems are also utilized to maintain oxidative balance in the lens (Lou, 2003).

Several enzymatic systems contribute to preserving a reduced state and repairing lens protein damage created by oxidation. Superoxide dismutase forms peroxides in the mitochondria decreasing oxygen radicals (Beebe, 2011). Catalase and glutathione peroxidase work to reduce peroxides. Together NADPH and glutathione reductase influence the regeneration of GSH from oxidized glutathione (Lou, 2003). Glutathione dependent thiotransferase and NADPH dependent thioredoxin help to maintain protein solubility and restore damaged proteins therefore sustaining transparency. Alterations in
this delicate oxidative balance result in damage to LEC and fibers due to decreased protein solubility, lipid peroxidation, and DNA fragmentation (Lou, 2003). Decreased antioxidant capabilities within the lens can naturally occur with age and have been associated with cataract formation (Giblin, Chakrapani, & Reddy, 1976).

Cataracts are defined as any opacity altering the transparency of the lens. Cataracts can occur for a number of reasons including UV exposure, oxidative stress, toxins, imbibition of fluid, and altered gene expression. Cataracts are the leading cause of vision loss in humans accounting for 51% of cases (about 20 million) worldwide (Pascolini & Mariotti, 2012). Cataracts are also a leading cause of vision loss in dogs with one large study showing that 50% of dogs develop cataracts by age 9.4 and by age 13.5 all dogs had some lens opacity (Williams, Heath, & Wallis, 2004). Restoration of vision lost to cataracts currently requires surgical intervention.

Surgical removal of lens fibers and epithelial cells via extracapsular phacoemulsification is currently the treatment of choice for cataracts resulting in vision disturbances in humans and animals. Phacoemulsification involves a small corneal or scleral incision to approach the anterior chamber. An opening or capsulorrhexis is created in the anterior capsule allowing access to the lens material, which is emulsified using ultrasonic energy and aspirated until the capsule is largely devoid of cellular material. After phacoemulsification a clear visual axis is restored and an artificial intraocular lens (IOL) is placed within the capsule to restore the refractive power and result in emmetropia. Following extracapsular cataract surgery, posterior capsule opacification (PCO) can occur in all species resulting in a reoccurrence of visual disturbance.
Posterior capsule opacification is the most common complication following cataract surgery (Wormstone, Wang, & Liu, 2009). This complication reportedly occurs following phacoemulsification in up to 100% of canine patients (Bras, Colitz, Saville, Gemensky-Metzler, & Wilkie, 2006; Gift, English, Nadelstein, Weigt, & Gilger, 2009) and up to 50%(Wilhelmus & Emery, 1980) to 100% (Pandey et al., 2001) of adult and pediatric human patients, respectively. Through migration, proliferation, and differentiation of residual LEC, posterior capsule opacification can obstruct the visual axis after cataract surgery. The wound healing response of surgery likely stimulates this LEC behavior (Wormstone et al., 2009). Two major morphologic changes can occur during the formation of PCO. Lens epithelial cells can undergo epithelial-mesenchymal transformation (EMT) and result in fibrosis and wrinkling of the capsule or equatorial cells may proliferate, undergo cortical regrowth and form swollen bladder cells called Elschnig’s Pearls (Garg, Pandey, Chang, Papadopoulos, & Maloof, 2005). When PCO-related vision disturbances occur the current standard of care in human patients is neodymium:yttrium-aluminum-garnet (Nd:YAG) laser posterior capsulotomy (Murrill, Stanfield, & Van Brocklin, 1994). The Nd:YAG laser procedure carries rare but serious complications (Apple et al., 1992) and is the second largest surgical expense of the United States Medicare system (Apple et al., 2000). No consistent effective treatment exists for canine patients with PCO. For this complication to be eliminated, thorough knowledge of the pathogenesis is crucial. The full pathogenesis of PCO is not completely understood but oxidative stress, cytokines, and growth factors have been shown to play a
role in migration and proliferation of residual LEC (Lewis, 2013; Wormstone et al., 2009).

Cytokine growth factors such as transforming growth factor β (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) may function to regulate PCO formation. These growth factors are normally present in the eye and increased expression of these growth factors occurs following cataract surgery (Wormstone et al., 2009). Aqueous humor collected following cataract surgery stimulated cultured LEC proliferation in a process that was thought to be growth factor mediated (Wallentin, Wickström, & Lundberg, 1998). Transforming growth factor-β has garnered the most attention and evidence suggests a major role in PCO formation (Wormstone et al., 2009). Transforming growth factor-β and FGF are capable of inducing lens fiber differentiation in experimental models (McAvoy & Chamberlain, 1990; Wormstone, Tamiya, Anderson, & Duncan, 2002). This coupled with the fact that these factors are up regulated following cataract surgery could be the reason for LEC transdifferentiation and Elschnig’s Pearl formation. Transforming growth factor-β is also likely responsible for induction of α-smooth muscle actin (α-SMA), a muscle related protein that can be utilized as a marker for lens epithelial to myofibroblast differentiation (Nagamoto, Eguchi, & Beebe, 2000; Wormstone et al., 2002). Myofibroblastic cells are contractile and are capable of causing capsule wrinkling and production of extracellular matrix, both of which are major components of PCO (Nagamoto et al., 2000). Additionally, TGF-β and α-SMA have been directly demonstrated in PCO fibrous tissue (Wormstone et al., 2002).
Other cytokines such as interleukin (IL)-6, IL-8, and chemokine C-C motif ligand 2 (CCL2) may also play a role in the pathogenesis of PCO. These cytokines are reported to be increased in aqueous humor following cataract surgery and may, in fact, be produced directly by the residual LEC (Kawai et al., 2012; Malecaze et al., 1991; O. Nishi, Nishi, & Ohmoto, 1996). Levels of IL-6 were detected at one week and seven weeks in human LEC cultures taken from lens capsules during cataract surgery (Malecaze et al., 1991). Chemokines play a critical role in many inflammatory processes, (Yadav, Saini, & Arora, 2010) lending to their potential involvement in PCO. Cytokines may work upstream, signaling a series of events and other mediators such as TGF-β and protein kinases that may lead to PCO or work directly to stimulate migration, proliferation, and differentiation of LEC’s. Interleukin-6 likely plays an integral role in uveitis following cataract surgery and can stimulate cellular proliferation and differentiation in a variety of other inflammatory processes throughout the body (Lewis, 2013; Malecaze et al., 1991). Interleukin-6 has been demonstrated in PCO tissue of human lens capsules (Shigemitsu et al., 1999). Evaluation of PCO tissue from rabbits revealed elevated levels of CCL2 and in work performed by Kawai, et al (Kawai et al., 2012), levels of IL-8 were significantly associated with CCL2. In another study CCL2 stimulated collagen synthesis by lung fibroblasts from rats, which was thought to be co-mediated by TGF-β (Denholm, 1996). Interleukin-8 is involved in many different ocular inflammatory processes and has been shown to stimulate mitogen activated protein kinases (MAPK) (Ghasemi, Ghazanfari, Yaraee, Faghihzadeh, & Hassan, 2011). Protein kinases are key regulators of cell proliferation and differentiation
(Pearson et al., 2001) and their inhibition may help to reduce PCO formation (Chandler et al., 2010). Although mechanisms have not been completely elucidated, cytokines are likely important factors in the pathogenesis of PCO.

The pathogenesis of PCO may also involve the presence of ROS. Reactive oxygen species have a variety of harmful cellular effects including DNA damage, apoptosis, and inflammation. These detrimental events are associated with progression of many diseases including Alzheimer’s, macular degeneration, and cataract (Chan, 2001; Gough & Cotter, 2011; Spector, 1995). While a large body of research has focused on elucidating the damage that ROS production has on cells and developing methods to reduce these effects, recent data suggests that ROS production at sub-lethal levels can be beneficial or even necessary for cell survival and function (Finkel, 2011; Gough & Cotter, 2011; Sundaresan, Yu, Ferrans, Irani, & Finkel, 1995). Of particular importance, growth factor stimulation of low level ROS production is known to promote migration and proliferation in a variety of cells (Bae et al., 1997; Hsieh, Wang, Wu, Chu, & Yang, 2010; Huo et al., 2009; Junn et al., 2000; Lo & Cruz, 1995; Nam, Park, Yoon, Cho, & Lee, 2010). It has been recognized that ROS can act as signal intermediates for several growth factors, including the earlier described TGF-β and EGF (Bae et al., 1997; Krieger-Brauer & Kather, 1995; Lo & Cruz, 1995). Within the lens, platelet-derived growth factor (PDGF) expression results in ROS generation; in turn, ROS regulates diverse downstream signaling pathways within the cell (Chao-Wei Chen, Zhou, Xing, Krysan, & Lou, 2004; K. C.-W. Chen, Zhou, Zhang, & Lou, 2007). Previous research using human LEC has demonstrated that proliferation and mitogenic signaling can be
ROS-dependent (Chao-Wei Chen et al., 2004; K. C.-W. Chen et al., 2007). While others have suggested that ROS-mediated signaling pathways are important to lenticular development and physiologic function, to date, the role of low level ROS production in relation to PCO formation is unknown.

The ROS responsible for this novel concept of cellular signaling may be generated from several enzymatic systems including membrane-bound enzymes, mitochondria, and a variety of intracellular enzymes. NADPH oxidase is a membrane-bound enzyme for which the only known function is the generation of ROS (Finkel, 2011). NADPH oxidases can be up-regulated in response to stimuli such as growth factors and cytokines and the enzyme has previously been localized to LEC (Li, 2003; Rao, Maddala, John, & Zigler, 2004). Reduction reactions resulting in ROS production also occur via Complex 1 in the mitochondria and through intracellular cytokines such as lipoxygenase metabolism of arachidonic acid (Finkel, 2011). While both lipoxygenase and Complex 1 can mediate growth factor signaling, in several cell types, including human LEC, they are not thought to be the main contributor to ROS production (Cui & Douglas, 1997; Huo et al., 2009; J. Zhang, Jin, Liu, & Rhoades, 1998). Depending on the mechanism of action used to generate ROS, downstream signaling can change. Migration, proliferation, and adhesion have been demonstrated as different cellular responses depending on the specific enzymatic system accounting for ROS production (Finkel, 2011; Huo et al., 2009; Rao et al., 2004; J. R. Stone & Yang, 2006). This concept may explain some of the complexity of PCO and represent one of the many factors associated with understanding
this complication. The pathogenesis of PCO formation is multifactorial and multimodal lending to the difficulty in elucidating the exact mechanisms by which it occurs.

Despite the incomplete understanding of mechanisms behind PCO formation many advances have reduced the frequency of this complication, but fail to prevent it entirely. Perhaps the most significant advances contributing to current reduction in PCO severity are surgical and mechanical factors. Surgical methods attempting to remove residual LEC’s such as hydrodissection, pulsed fluid, and capsule polishing/vacuuming have been described and may reduce or delay the onset of PCO but do not appear to have significantly reduced the need for Nd:YAG procedures in humans (Khalifa, 1992; O. Nishi & Nishi, 1991; Peng et al., 2000; Vasavada, Dholakia, Raj, & Singh, 2006; Yoo & Bhatt, 2007). Intraocular lens design and biomaterial seem to have a greater impact on reducing PCO and the need for Nd:YAG capsulotomy. One study comparing PMMA, silicone, and acrylic lens biomaterials showed a 2-year incidence of PCO of 43, 33, and 11% respectively (Ursell et al., 1998). Another study showed Nd:YAG capsulotomy rates of 28.9% for PMMA, 14.4% for silicone, and 4.2% for acrylic lenses within 2 years of surgery (K. Hayashi, Hayashi, Nakao, & Hayashi, 2001). Several studies have suggested that an IOL with sharp edge optic design versus traditional rounded optic design impedes LEC migration and significantly reduces PCO (Auffarth, Golescu, Becker, & Völcker, 2003; Buehl et al., 2005; K. Hayashi & Hayashi, 2005; O. Nishi & Nishi, 1999). However, another study evaluating optic edge design, biomaterial, and degree of anterior capsulorrhesis/optic overlap suggested that 360° capsulorrhesis/optic overlap was more important for PCO reduction than lens design (S. R. Smith, Daynes,
Hinckley, Wallin, & Olson, 2004). Recently, placement of endocapsular tension rings for the prevention of PCO in human and canine cataract surgery patients has been evaluated. In several human trials statically significant reductions in PCO rates and need for Nd:YAG capsulotomy were demonstrated following the placement of capsule tension rings (Halili, Mutlu, Erdurman, G Ndogan, & Kilic, 2013; J.-H. Kim, Kim, & Joo, 2005; Menapace et al., 2008; O. Nishi, Nishi, Menapace, & Akura, 2001). In a canine study by Stone et al. a reduction in PCO was noted in ex vivo lens capsules implanted with both an IOL and capsule tension ring compared to controls however, no statistically significant reduction in PCO between eyes implanted with a tension ring and control eyes was detected in vivo (S. G. Stone, 2008).

Regardless of the above surgical and mechanical advances clinically significant PCO still exists and further discoveries will be necessary to eradicate this postoperative complication. Much current focus is being directed at pharmacological methods to remove residual LEC’s. Pharmacologic agents can be injected directly during surgery or delivered via implanted devices or the IOL itself (Wilkie & Colitz, 2013). The goal of this therapy is to specifically damage or inhibit growth of residual LEC’s without consequence to other ocular tissues, most specifically the corneal endothelium and anterior uvea (Garg et al., 2005). Some of the pharmacological substances evaluated include chemotherapeutic agents such as 5-fluorouracil (5-FU) or mitomycin (Fernandez et al., 2004), anti-inflammatories such as diclofenac or dexamethasone (Inan UU et al., 2001), and more specific immunological agents such as thapsigargin or RGD peptide (G. Duncan, Wormstone, Liu, Marcantonia, & Davies, 1997; O. Nishi et al., 2013). While
some of these methods have proven effective at mitigating PCO, none have been consistently utilized due to toxicity concerns or difficulties with drug delivery. Recently, a sealed capsule irrigation device allowing delivery of pharmacologic agents to the capsular bag without contact of other tissues has been developed (Maloof, Neilson, Milverton, & Pandey, 2003). One in vivo safety study performed in rabbits demonstrated no detrimental effects to any ocular tissues 48 hours after instillation of 5-FU but did not evaluate the effects on PCO (Abdelwahab, Kugelberg, Seregard, & Zetterström, 2007). A canine study evaluating instillation of 5-FU in six clinical patients showed subjectively less PCO six months post surgery but leakage occurred in one patient and a 24.9 percent increase in corneal thickness and increased aqueous flare were reported (Histed, Nadelstein, & English, 2009). A single human case report noted a clear central and peripheral posterior capsule and no clinically detectable abnormalities one year after use of the device to instill 5-FU (Milverton, 2008). Another study in 17 human patients evaluated the effects of distilled water administered utilizing the device and detected no detrimental effects and no significant difference in PCO scores compared to controls 24 months post-operatively (Rabsilber, Limberger, Reuland, Holzer, & Auffarth, 2007). Utilization of this device will inevitably add time and cost to cataract surgery procedures but could prove to be of value if effective reductions of PCO are achievable. Continued work in this area will likely provide beneficial therapeutic avenues for eradication of PCO in the future.

A novel approach with the potential to reduce PCO formation is the administration of antioxidants in the perioperative period. The use of antioxidants to prevent oxidative
damage to cells is not uncommon. A large industry exists promoting the use of antioxidants to slow aging and prevent disease. The positive effects of antioxidants are thought to be due to a reduction of high level ROS mediated cell damage. Ocular examples of this include the ability for grape seed extract (GSE), lutein, and omega-3 fatty acids to exert beneficial effects related to cataract formation, macular degeneration, and oxidative damage to retinal pigmented epithelial cells, respectively (Barden, Chandler, Lu, Bomser, & Colitz, 2008; Jia, Song, Zhao, Wang, & Liu, 2011; L. Lu, Hackett, Mincey, Lai, & Campochiaro, 2005; Richer et al., 2004). Additionally, antioxidants may exhibit a positive effect by reducing sub-lethal levels of ROS that support cell migration and proliferation. As discussed above, ROS likely play a crucial role in cell signaling and growth factor stimulation (Bae et al., 1997; Krieger-Brauer & Kather, 1995; Lo & Cruz, 1995). Reactive oxygen species have also been shown to be important mediators of corneal epithelial cell migration and wound healing (Huo et al., 2009). The use of antioxidants to prevent these essential ROS may help reduce the migration, proliferation, and differentiation of LEC resulting in PCO formation.

Grape seed extracts are proanthocyanidins obtained from *Vitis vinifera* (common grape seeds). These proanthocyanidins exhibit potent scavenging of ROS among other properties. The polyphenolic nature of these compounds made up mostly of flavanoids is likely responsible for their ability to accept electrons and therefore act as antioxidants (Clouatre, Kandaswami, & Connolly, 2005). In one *in vitro* study without cellular influences, grape seed extracts out performed Vitamins C and E in oxygen radical scavenging ability (Bagchi et al., 1997). Some of the protective effects of GSE involving
the lens are represented in the following studies. In two separate studies, oral supplementation with GSE prevented hereditary and selenite-induced cataract formation in rats (Durukan et al., 2006; Yamakoshi, Saito, Kataoka, & Tokutake, 2002). Grape seed extracts also protected human and canine LEC from oxidative stress in two other in vitro studies. In canine LEC, the protective effects were thought to be due to reduced activation of MAPK and phosphoinositide-3 kinase (PI3K) signaling pathways (Barden et al., 2008). As discussed previously MAP kinases are known to be key regulators of cellular migration, differentiation, and proliferation (Pearson et al., 2001). It has been shown that PI3K signaling is necessary for TGF-β mediated EMT in mammary epithelial cells (Bakin, 2000). In human LECs, GSE’s protective effects against oxidative stress were thought to also involve reduced activation of MAPK but unlike the canine study, signaling of another pathway nuclear factor kappa-B (NF-κB) instead of PI3K was altered (Jia et al., 2011). Nuclear factor kappa-B is a transcription factor known to control genes responsible for cell apoptosis and proliferation among other functions (S. Ghosh & Karin, 2002). Therefore, the potent ROS scavenging capability and likely ability of GSE to decrease activation of MAPK, PI3K, and NF-κB pathways suggests the possibility that these extracts could reduce PCO formation.

Another family of antioxidants, the carotenoids, may also affect PCO formation. Carotenoids are fat-soluble plant pigments that are found in many plants and vegetables. These pigments account for the bright colors of foods like tomatoes, spinach, and carrots (Johnson & Russell, 2005). Lycopene, β-carotene, and lutein are a few of the compounds in this family that have potential benefits in disease prevention and
treatment (Johnson & Russell, 2005). Due to their fat solubility they are absorbed similarly to dietary fat and are transported in circulation via lipoproteins (Johnson & Russell, 2005). To obtain their potential benefits, dietary intake of carotenoids is necessary as mammals have no ability to biosynthesize these compounds (SanGiovanni, Chew, & Johnson, 2005). Lutein is a polar dihydroxycarotenoid that mainly comes from green leafy vegetables (SanGiovanni et al., 2005). Lutein is a large component of retinal macular pigment that absorbs light at wavelengths capable of inciting phototoxicity and oxidative damage (SanGiovanni et al., 2005). In a study of age-related macular degeneration, supplementation with lutein improved visual acuity compared to placebo controls (Richer et al., 2004). Lutein has also been shown to inhibit proliferation of a human monocyte cell line suggesting a possible role in cell cycle regulation (McDevitt, Tchao, Harrison, & Morel, 2005). Further supporting lutein’s potential role in cell proliferation, the antioxidant decreased bovine LEC proliferation and migration rate in a scratch wound model (Hu & Xu, 2008). In vitro lutein protected human LEC from oxidative damage induced by H$_2$O$_2$ but was unable to provide protection when GSH levels were depleted suggesting that cellular GSH levels must to be maintained for lutein to provide protection (Gao et al., 2011). Cumulatively these findings suggest lutein could possibly affect the cellular proliferation and migration responsible for PCO formation.

Omega-3 fatty acids are a group of highly unsaturated fatty acids present in many tissues throughout the body. The main compounds that make up this family of fatty acids are α-linolenic acid, eicosapentaenoic acid, docosapentaenoic acid, and
docosahexaenoic acid (Harris, 2005). Like the carotenoids, mammals are unable to biosynthesize fatty acids making dietary intake necessary to maintain natural omega-3 functions in the body. These fatty acids are produced by microalgae and are subsequently consumed by marine and freshwater fish that then serve as the main source for mammalian intake (Harris, 2005). Omega-3 fatty acids are mainly found in membrane phospholipids, circulating cells, and lipoproteins of plasma. In stressed tissues omega-3 fatty acids are cleaved by phospholipase-A2 to become arachidonic acid, which eventually forms prostaglandins and leukotrienes. These metabolites can exhibit both anti-inflammatory and pro-inflammatory effects (Harris, 2005). In a large study of hypercholesterolemic patients supplemented with eicosapentaenoic acid, frequency of heart attack and stroke were reduced compared to controls (Yokoyama et al., 2007). These protective effects are thought to be due to stabilization of membrane ion channels (Kang, Xiao, & Leaf, 1995). Inconclusive evidence suggests a protective effect of omega-3 fatty acids against macular degeneration and cataract formation, although the mechanism by which this may occur is unclear (Seddon, 2001; 2007; W. Smith, 2000). Although omega-3 fatty acids are known antioxidants little information exists to support a major functional role in the prevention of PCO.

In the year 2000 almost two million cataract surgeries were performed in human patients in the United States alone. During that same year over six hundred thousand Nd:YAG laser capsulotomies were performed representing a still very significant impact of PCO on post-operative cataract surgery patients (Maloof et al.,
2003). While surgical and mechanical factors have reduced the frequency of PCO formation it appears likely that further advancements will be in the form of pharmacological methods to eliminate this vision threatening complication in human and canine patients. Surgical advances such as accommodative IOL’s for human patients are becoming more standard and their function depends on preserved capsule integrity (McLeod, Vargas, Portney, & Ting, 2007; MS et al., 2013; Werner et al., 2004). With advances like this coupled with the fact that there is no proven therapy for affected canine patients; a great need exists to reduce or eradicate PCO. Precedent exists to suggest that antioxidants can alter LEC behavior and are therefore a reasonable potential therapeutic approach for investigation in PCO prevention. This study will evaluate the effects of GSE, lutein, and omega-3 fatty acids, on oxidative stress, migration, and proliferation in LEC. The purpose of the study is to determine if these commonly available antioxidants can prevent or modify PCO in an ex vivo canine model.
Chapter 2: Materials and Methods

Antioxidant preparation

The grape seed extract, lutein, and omega-3 fatty acids were provided free of charge by Animal Necessity. Concentrations used were based on the commercially available veterinary product Ocu-GLO Rx Vision Supplement™. Omega-3 fatty acid containing fish oil (FO) was dissolved in dimethyl sulfoxide (DMSO) and diluted with Dulbecco’s minimum essential medium (DMEM; Gibco, Carlsbad, CA) to achieve a 50mg/L solution. Grape seed extract (GSE) was dissolved in DMSO and diluted with DMEM to achieve a final solution of 25mg/L. Lutein was dissolved in 100% ethanol (EtOH) and diluted with DMEM to achieve a 15mg/L solution. The lutein product was supplied as a paste and would bind to containers used for measuring. Due to this phenomenon and estimating an ~50% loss of product the starting concentration was doubled to 30mg/L. When experimentally appropriate, N-acetyl cystine (NAC) was used as a positive antioxidant control and was prepared in deionized water and diluting with DMEM to achieve a 1mM solution. Each antioxidant was derived from naturally occurring sources (i.e. lutein was derived from marigolds) making the purity of all tested antioxidants unknown.
Antioxidant Reductive Capacity Assay

A commercially available antioxidant reductive capacity assay (Antioxidant Reductive Capacity Assay, Northwest Life Science Specialties LLC, Vancouver, WA) was used to determine the relative antioxidant power of the tested antioxidants. The assay determines the ability of aqueous samples to reduce Cu^{2+} to Cu^{+}. Cu^{+} reacts with bathocuproine (BC) to form a complex with a colorimetric absorbance between 480-490nm. Therefore, measurement of samples at 450nm before and after the addition of BC will create a net difference that is directly proportional to the reductive capacity. The net absorbance values are compared to a standard curve generated using Trolox, a water-soluble analog of Vitamin E. The provided 2uM Trolox standard was serially diluted to create six standards with concentrations ranging from 2uM to 0.063uM. After preparation, each antioxidant sample and standard was diluted to 1:40 concentration using the provided dilution buffer (containing BC) and 200uL of each was added to a 96 well microplate. The absorbance of each sample or standard was read at 450nm using an automated microplate reader (Tecan, Infinite® 200, Mannedorf, Switzerland). The Cu^{2+} solution was added to each well and allowed to incubate for 3 minutes at room temperature. The provided stop solution was then added to each well and the absorbance was again read at 450nm. The difference in absorbance for each well was calculated and compared to the difference of each standard to create a standard curve. Results were reported in copper reducing equivalents.
Canine Tissue

All protocols were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by The Ohio State University Institutional Animal Care and Use Committee. Canine LEC cultures were generated from enucleated, grossly normal eyes from dogs euthanized for reasons unrelated to this study. The globes were collected within 1-2 hours of euthanasia and rinsed with iodine solution. Globes were then stored for up to 24 hours in 1x phosphate buffered saline (PBS) at 4°C until time of cell harvest.

Canine Primary Lens Epithelial Cell Cultures

To harvest canine LEC, globes were stabilized and the cornea was removed by creating a circumferential grooved incision in the sclera approximately 2mm caudal to the limbus using a scalpel blade. The incision was completed using Steven’s tenotomy scissors and the cornea removed. Vannas iris scissors were then used to remove the iris, first by transecting radially from the pupil to the base, then transecting circumferentially at its base. Once full exposure of the anterior lens surface was obtained, a continuous curvilinear capsulorrhexis (CCC) was performed by creating a small capsular opening using Vannas iris scissors and completing a continuous circular tear using Utrata capsulorrhexis forceps. The removed axial anterior capsules and associated LEC were then placed in a microcentrifuge tube containing 0.5mL of 0.25% trypsin (Gibco) and incubated at 37°C for 7-10 minutes. Following incubation, trypsin’s enzymatic activity was halted by adding 0.5mL of DMEM containing 10% fetal bovine serum (FBS; Gibco).
The cells were then centrifuged for 5 minutes at 1800 rpm. The supernatant was removed and the cells/capsules were re-suspended in 1mL DMEM before being transferred to a 25cm\(^2\) laminin-coated culture flask (Beckton-Dickinson, Franklin Lakes, NJ). Four-milliliters DMEM was added to each culture flask for total volume of 5mL and flasks were incubated at 37°C and 5% CO\(_2\) until cells became confluent.

Ex Vivo Capsule PCO Model

Mock cataract surgery was performed on canine globes. Enucleated globes were stabilized and the cornea and iris were removed as described above. Once full exposure of the anterior lens surface was obtained, a CCC was performed in the same manner as described above. The lens fibers were removed using a lens loop and residual LEC were removed via gentle irrigation with a 22-gauge cannula. The lens was freed from its zonular attachments using Vannas iris scissors and placed in a cell culture dish with DMEM or DMEM containing GSE 25mg/L, FO 10mg/L, or lutein 15mg/L (n= 6 capsules per treatment group). The medium was changed every two days throughout the treatment period. Inverted phase contrast microscopy was used to score capsules and obtain photomicrographs on day zero and every two days until day fifteen when capsules were placed in formalin for histologic evaluation. The following scoring system was used to follow capsules and determine percent confluence:

<table>
<thead>
<tr>
<th>Score</th>
<th>Microscopic Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No identifiable cells present in capsule</td>
</tr>
</tbody>
</table>
1 Residual or inactive cells present in equator, peripheral posterior capsule, or anterior capsule but central posterior capsule clear

2 ≥ 1 spindloid like cell in equator, peripheral posterior capsule, or anterior capsule but central posterior capsule clear

3 ≤ 50 percent central posterior capsule coverage

4 51-95 percent central posterior capsule coverage

5 100 percent central posterior capsule coverage

Following the fifteen-day evaluation period the capsules were sectioned and stained with hematoxalin and eosin (H&E) for histologic evaluation and cell counts. The results for capsule scoring and histology were reported as average capsule score per evaluation or average cell number per histologic section respectively.

Human Lens Epithelial Cell Cultures

Human LEC cultures were obtained using the immortalized human lens cell line SRA 01-04. Frozen cells were rapidly thawed by agitating the cryovial in a 37°C water bath. The tube was then dipped in 70% EtOH to prevent contamination and the contents were transferred to a 25cm² uncoated tissue culture treated flask (Beckton-Dickinson) along with 5mL DMEM warmed to 37°C. The culture flask was incubated overnight at 37°C and 5% CO₂. The DMEM was replaced the following day. Cells were allowed to grow to desired confluence before use.
Scratch Test Model of LEC Migration and Proliferation

Cultured canine and human LEC were plated in a 24 well plate with DMEM and incubated overnight at 37°C until confluent. A central 1-mm scratch was made vertically on the cellular surface. Cells were then treated with fresh DMEM or DMEM containing GSE 25mg/L, GSE 12.5mg/L, FO 50mg/L, FO 10mg/L, lutein 15mg/L, or lutein 7.5mg/L. Each treatment was performed in triplicate. Digital images were taken immediately after the initial scratch and 24 hours later. Image J (U. S. National Institutes of Health, Bethesda, MD) was used to quantify the area of the scratch at times 0 and 24 hours. The change in area for each group was averaged and percent change relative to control was graphed.

Dichlorofluorescein (DCF) Assay for Detection of ROS Generation

Cultured canine LEC were plated in a 96 well laminin-coated microplate at approximately 10,000 cells/well and incubated at 37°C with DMEM overnight. The DCF reagent (Molecular Probes Inc, Eugene, OR) was diluted to 5uM with PBS according to the manufacturers instructions, prior to adding 100ul to each well. Cells were incubated with the DCF reagent at 37°C for 20 minutes. Following incubation the DCF reagent was removed and cells were washed gently with PBS. Cells were then treated with fresh DMEM or DMEM containing GSE 25mg/L, FO 50mg/L, Lutein 15mg/L, or NAC 1uM and incubated at 37°C for 6 hours (n= 10 replicates per treatment group). To create a positive control with induction of ROS, selected wells were exposed to ultraviolet radiation (UV; 600 J/m²) for 45 seconds. Fluorescence was then read at 490nm excitation
and 520nm emission at 37°C using the Infinite® 200 (Tecan). The values from wells containing only DMEM were subtracted from treatment wells to correct for background fluorescence. This experiment was repeated in the same fashion using cultured human LEC.

MTT Assay of Cell Proliferation and Viability

A commercially available MTT 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay kit was used to carry out the experiments (Vybrant® MTT Cell Proliferation Assay, Molecular Probes Inc). Cultured canine LEC were plated in a 96 well laminin-coated microplate with DMEM and incubated at 37°C with 5% CO₂ overnight. The following day the medium was changed to fresh DMEM or DMEM with GSE 25mg/L, GSE 12.5mg/L, FO 50mg/L, FO 10mg/L, lutein 15mg/L, lutein 7.5mg/L, or mitomycin C (MMC) 0.002mg/L and cells were incubated for 24 hours as above (n=8 replicates per treatment group). The medium was then replaced with fresh DMEM and 10uL of 12μM MTT was added to each well followed by incubation for 4 hours at 37°C. The addition of MTT labeled living cells via absorption and subsequent reduction to formazan. After labeling, the medium was mostly removed leaving 25uL in each well; 50uL of DMSO was then added. Following a 10 minute incubation period at 37°C, the absorbance was read at 540nm using the Infinite® 200 (Tecan). The experiment was repeated in the same fashion using cultured human LEC. The results were reported as percent absorbance change relative to control.
Cytokine Inflammation Protein Array

Cultured human LEC were plated in a 24 well microplate with DMEM and incubated overnight at 37°C. The following day the cells were treated with fresh DMEM or DMEM containing GSE 25mg/L, GSE 12.5mg/L, FO 50mg/L, FO 10mg/L, lutein 15mg/L, or lutein 7.5mg/L (n=3 replicates per treatment group) and incubated for 24 hours at 37°C. Following incubation the medium was removed and centrifuged at 1800 rpm for 5 minutes to remove any cells or debris; 1mL of supernatant was obtained for array testing. The array was performed according to manufacturer’s instructions (Quantibody® Human Inflammation Array 3, RayBiotech Inc, Norcross, GA). Briefly, the lyophilized cytokine standards were prepared. The provided glass chips were blocked to reduce non-specific binding by incubating with sample diluent at room temperature for 30 minutes. The standards or samples were added to each well and incubated overnight at 4°C. The glass chips were washed using wash buffer and were then incubated with the detection antibody cocktail for two hours at room temperature. Washing was repeated and the glass chips were incubated for one hour at room temperature with Cy3 equivalent dye-conjugated streptavidin for labeling. After labeling with Cy3, fluorescence was detected using the Typhoon 9410 Variable Mode Imager (Amersham Biosciences, Amersham, UK). ImageQuant TL (GE Healthcare Biosciences, Pittsburg, PA) was then used quantify absorbance and cytokine concentrations (pg/mL) were determined using the standard curve.
Chapter 3: Results

Antioxidant Reductive Capacity Assay

A commercially available antioxidant reductive capacity assay determined the reducing power of the test antioxidants. NAC acted as the positive control. As determined by the Trolox standard curve the FO group produced 23.1 Cu++ reducing equivalents, while lutein, GSE, and NAC control produced 9.4, 2116.3, and 488.1 Cu++ reducing equivalents respectively (Figure 1).

![Figure 1: Antioxidant Reductive Capacity Assay](image)

GSE surpassed the positive control NAC in reductive capacity while lutein and FO exhibited little ability to reduce oxidized Cu++. 

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Ex Vivo Capsule PCO Model

Capsules were evaluated using inverted microscopy. Data reported are the average of six capsules for each treatment group recorded as percent confluence +/- SD. Lens capsules treated with FO (10 mg/L) reached 72 +/- 19.4% confluence by day 8 and 100% confluence within 15 days. After 8 days of lutein (15 mg/L) treatment, LEC migration and proliferation were slowed and the lens capsules only achieved approximately 5 +/- 5% confluence and reached just 11 +/- 10.7% by day 15. Grape seed extract (25 mg/L) treated capsules reached 38 +/- 14.7% confluence by day 8 and showed large variability in migration and proliferation rates throughout the entire culture period with a final confluence of approximately 50 +/- 22.8% by day 15. Control capsules reached 10 +/- 5.8% confluence on day 8 and 50 +/- 15.3% by day 15 (Figure 2). Capsules were formalin fixed at day 15 and histologic cell counts were performed. Data reported are the average total cell count of six capsules for each group. Capsules from the FO group contained 406.8 cells while lutein, GSE, and control capsules contained 54.0, 211.2, and 229.9 cells respectively. These counts compared favorably with percent confluence as determined by inverted microscopy (Figure 3).
Figure 2: Ex Vivo PCO Model

FO increased rate of PCO formation compared to controls with 100% capsule coverage by day 12, GSE exhibited PCO similar to controls with 50% capsule coverage by day 15, and lutein slowed PCO formation with 11% capsule coverage at the day 15 end point.

Figure 3: Histologic Cell Counts

Results corroborated PCO model with LEC counts similar to capsule scores.
Scratch Test Model of LEC Migration and Proliferation

A scratch test determined the migration and proliferation of canine and human LEC. Inverted microscopy was used to obtain photomicrographs immediately following scratch formation and 24 hours later. Image J software (U. S. National Institutes of Health) was used to determine change in scratch area. Results are reported as percent change relative to control. All treatment groups showed less migration and proliferation when compared to controls. In the canine LEC the 50mg/L FO treatment resulted in 85.4% less change in scratch area and the 10mg/L FO treatment resulted in 37.5% less change compared to controls. The lutein treatment at 15 and 7.5mg/L resulted in 61.7% and 41% less change, respectively, while GSE treatment at 25 and 12.5mg/L, resulted in 107.4% and 79.2% less change in area, respectively (Figure 4). In the human LEC the 50mg/L FO treatment resulted in 84.9% less change in scratch area while the 10mg/L FO treatment resulted in showed 8.1% less change compared to controls. The 15 and 7.5mg/L lutein treatment groups resulted in 21.1% and 36.1% less change, respectively, while GSE treatment at 25 and 12.5mg/L resulted in 97.7% and 103% less change in area respectively (Figure 5).
Figure 4: Scratch Wound Assay Canine LEC

All groups increased scratch area in a concentration dependent manner relative to control.

Figure 5: Scratch Wound Assay Human LEC

GSE scratch area was similar to controls while lutein and FO increased scratch area relative to control.
Dichlorofluorescein (DCF) Assay for Detection of ROS Generation

A DCF assay determined ROS production in LEC treated with the test antioxidants in stressed or unstressed conditions. UV stress induced ROS production in all groups; only GSE and the positive control NAC were able to reduce the generation of ROS following UV exposure by 8.7% and 63% respectively. Lutein increased ROS production in unstressed LEC and was unable to reduce UV induced ROS. FO slightly increased ROS in unstressed LEC and was unable to reduce ROS production following UV exposure (Figure 6).

Figure 6: DCF Assay for Detection of ROS generation

GSE decreased ROS generation in UV stressed and unstressed LEC, lutein and FO increased ROS generation in stressed and unstressed LEC.
MTT Assay of Cell Proliferation and Viability

An MTT assay was performed to evaluate LEC viability and proliferative activity. Averaged absorbance values of treated wells were compared to averaged values of control wells. After 24 hours of incubation, GSE, lutein, and FO all reduced cell viability and prevented proliferation at all concentrations relative to controls. The reduction in cell viability decreased in a concentration dependent manner within all treatment groups (Figure 7).

![MTT Assay of Cell Proliferation and Viability](image)

**Figure 7: MTT Assay of Cell Proliferation and Viability**

All groups decreased LEC viability in a concentration dependent manner except for GSE where the lower concentration decreased viability greater than the higher concentration.
Cytokine Inflammation Protein Array

A cytokine inflammatory protein array was performed to determine if the expression of selected cytokines was altered following exposure to the test antioxidants. Forty different cytokines were evaluated but only cytokines with a greater than 5-fold change in at least one test group were included in the results. Results are reported as percent change relative to controls. The following results are represented in graphs (Figures 8-11). Following exposure to GSE (25mg/L), cultured LEC decreased expression of IL-6 by 82%, IL-8 by 69.7%, CCL2 by 93.3%, and CCL5 by 99.9% compared to controls. GSE (12.5mg/L) decreased expression by 37.3% for IL-6, 23.9% for IL-8, 17.6% for CCL2, and 68.8% for CCL5. Following exposure to lutein (15mg/L), cultured LEC increased expression of IL-6 by 72.5%, CCL2 by 49%, and CCL5 by 132.3% compared to controls. Lutein (7.5mg/L) increased expression by 17.5% for IL-6, 25.5% for CCL2, and 33.7% for CCL5. Following exposure to FO (50mg/L), cultured LEC increased expression of IL-6 by 39.6%, CCL2 by 37.2%, and CCL5 by 167% compared to controls. Fish oil (10mg/L) increased expression by 89.1% for IL-6, 74.6% for CCL2, and 197.6% for CCL5. Exposure to lutein and FO exhibited very little effect on expression of IL-8 compared to controls.
Figure 8: Cytokine Protein Array IL-6
GSE decreased expression of IL-6 while lutein and FO increased IL-6 expression relative to controls.

Figure 9: Cytokine Protein Array IL-8
GSE decreased expression of IL-8 while lutein and FO showed IL-8 expression similar to controls.
Figure 10: Cytokine Protein Array CCL-2

GSE decreased expression of CCL-2 while lutein and FO increased CCL-2 expression relative to controls.

Figure 11: Cytokine Protein Array CCL-5

GSE decreased expression of CCL-5 while lutein and FO increased CCL-5 expression relative to controls.
Chapter 4: Discussion

In the year 2010 an estimated thirty-nine million people suffered from blindness worldwide with cataracts accounting for fifty-one percent of the cases (Pascolini & Mariotti, 2012). Cataract surgery is the most common surgery performed among Medicare beneficiaries (Thilen et al., 2013) and with an aging population this frequency is likely to grow. Cataracts are also the leading cause of vision loss in dogs affecting nearly every dog that reaches 13.5 years of age according to one survey (Williams et al., 2004). Surgical removal is currently the only effective means to restore vision lost to cataracts. Despite advances in reduction and prevention, PCO remains the most common complication affecting cataract surgery outcomes in canine (Sigle & Nasisse, 2006) and human patients (Wormstone et al., 2009). This complication reportedly occurs following phacoemulsification in up to 100% of canine patients (Bras et al., 2006; Gift et al., 2009) and up to 50% (Wilhelmus & Emery, 1980) to 100% (Pandey et al., 2001) of adult and pediatric human patients, respectively. Residual LEC migrate, proliferate, and differentiate following surgery due to a multitude of factors (Wormstone et al., 2009). This LEC behavior often results in vision disturbing opacities of the lens capsule (Wormstone, 2002). We attempted to alter this LEC behavior with a novel approach utilizing antioxidants.
Reactive oxygen species are molecules with unpaired electrons that are highly reactive with other molecules. Damage to proteins, lipids, and DNA by ROS is termed oxidative stress (Lü, Lin, Yao, & Chen, 2009). Antioxidants may reduce ROS and, in turn, oxidative stress through a variety of mechanisms. The basis of antioxidant function is direct reduction of ROS or indirect reduction through inhibition of intracellular free radical generating enzymatic systems or enhancement of intracellular antioxidant enzymatic systems (Lü et al., 2009). Specific antioxidant properties may vary in acellular systems, cell cultures, and cells within biological systems due to the presence or absence of these enzymatic systems and other unknown factors (Lü et al., 2009). Therefore, antioxidant properties may vary with different experimental modalities and this should be taken into account when evaluating antioxidant function.

Antioxidants appear to have many benefits related to ocular health (Bagchi et al., 1997; Coates, 2005; Durukan et al., 2006; Richer et al., 2004; Seddon, 2007; Yamakoshi et al., 2002). In previous studies, GSE prevented experimentally induced cataract formation in rats and also protected human and canine LEC from oxidative stress (Barden et al., 2008; Durukan et al., 2006; Jia et al., 2011; Yamakoshi et al., 2002). Lutein has also been shown to protect human LEC from oxidative stress (Gao et al., 2011). This carotenoid can protect the retina from phototoxic damage (SanGiovanni et al., 2005) and lutein administration improved visual acuity in a study of age related macular degeneration (Richer et al., 2004). Omega-3 fatty acids may help prevent cataract formation, protect retinal pigmented epithelial cells from oxidative damage, and slow progression of macular degeneration (L. Lu et al., 2005; Seddon, 2001; 2007). A
large body of evidence also suggests that antioxidant reduction of ROS may alter cellular migration and proliferation through redox signaling (CAI et al., 2011; Hsieh et al., 2010; Hu & Xu, 2008; McDevitt et al., 2005; Nam et al., 2010; Pan, Qiu, Huo, Yao, & Lou, 2011; Y. Wang & Lou, 2009).

We hypothesized that three common commercially available antioxidants, GSE, lutein, and omega-3 fatty acids, may decrease PCO formation by altering the behavior of LEC in vitro. First we determined the reductive capability of the antioxidants. Assessment of the reductive capacity and ability to reduce ROS was important, as a reduction of ROS would likely correlate with our proposed mechanism for altering LEC behavior. Two assays evaluated the effectiveness of our test antioxidants. One assay determined overall reducing capacity without other cellular influences and the second more specifically determined the ability to reduce ROS generated by LECs. For the overall reductive capacity we determined that, in the absence of LEC, only GSE was actually functioning as a true antioxidant. Additionally, using the DCF assay, we determined that GSE was the only antioxidant with the ability to reduce ROS generation in stressed and unstressed LECs. Grape seed extract has been shown to possess significant reducing power and one study demonstrated that GSE could scavenge oxygen radicals better than vitamins C & E (Bagchi et al., 1997). Grape seed extract has also been previously shown to reduce oxidative stress in human and canine LEC cultures (Barden et al., 2008; Jia et al., 2011). The reason for lutein and FO’s insignificant reducing power in the reductive capacity assay was unknown. This may be explained by inadequate quantities of lutein and omega-3 fatty acids in the extracts tested. The reason
for lutein’s pro-oxidative effect in the DCF assay was also unknown. Other studies have shown that carotenoids can have a pro-oxidative effect with varying concentrations, oxygen tension, and biological system (Palozza, 1998). The reason for this shift from antioxidant to pro-oxidant is not fully understood but this could explain our findings. The antioxidants’ effects on cell viability may have played a role and will be discussed later. Another possible explanation is that the reductive capacity assay evaluated the ability of aqueous solutions to reduce oxidized copper and we experienced difficulty achieving homogeneous aqueous solutions from the FO and lutein due to their physical properties. Therefore, without knowing the purity of the substances and with difficulty creating aqueous solutions, determination of accurate antioxidant concentrations was not possible and could account for these results.

Sub-lethal or low level ROS can function to stimulate growth factors, cytokines, and cell migration and proliferation (Finkel, 2011; Gough & Cotter, 2011; Huo et al., 2009; Lo & Cruz, 1995). Rao et al. described the presence of an NADPH oxidase system in human LEC and found that ROS production by this system could be stimulated by the addition of several growth factors including PDGF, EGF, and TGF-β. The same study also determined that ROS production could be inhibited by the addition of antioxidants (Rao et al., 2004). In a study by Chen et al., it was determined that human LEC stimulated by PDGF resulted in low level ROS production and subsequent proliferation; when inhibitors of protein kinase and PI3K pathways were applied, ROS production and cell proliferation were halted. The inhibition of these pathways also decreased activation of MAPK pathways, a change potentially mediated by the down
regulation of ROS (K. C.-W. Chen et al., 2007). As discussed previously, MAPK pathways are known to be key regulators of cellular migration, proliferation, and differentiation (Pearson et al., 2001). These findings suggest that ROS are an integral component of LEC proliferation in vitro and may be an important factor in PCO cell proliferation. The addition of antioxidants may alter LEC behavior by reducing the low level ROS production that is involved in LEC migration and proliferation and by decreased expression of cytokines important for LEC signaling. We utilized GSE, omega-3 containing FO, and lutein in an in vitro scratch test model of LEC migration and proliferation and an ex vivo capsule model of PCO to determine the effects of these antioxidants on PCO formation.

The scratch test model evaluated the antioxidants’ effects on migration and proliferation of cultured LEC. We demonstrated that in both human and canine cells, the tested antioxidants did not stimulate migration and proliferation; in most treatments, the scratch area did not change while in some treatments, the scratch area increased compared to controls. While this was the hypothesized result, changes in cell morphology were noted on microscopy and the lack of cellular ingrowth was presumed to be due to a toxic effect from the compounds. Increased cell death should not be significantly influenced by a natural decrease in cell viability as all cultures were performed utilizing first or second passage cells and control treatments maintained viability. Without the ability to reduce ROS generation, as shown in previous assays, the observable lack of migration and proliferation seen in the present study, is likely not due to the antioxidants’ ability to influence redox signaling but rather related to a toxic effect (Figure 12).
Figure 12: Phase Contrast Microscopy (100x)

Changes in cell morphology in the GSE, lutein, and FO groups relative to vehicle control likely represent a toxic effect to LEC.

The morphologic changes of the LECs in the scratch test prompted the evaluation of cell viability using the MTT assay. The MTT assay confirmed that, compared to controls, cell viability was decreased by all the tested antioxidants. This decrease in viability was concentration dependent except for GSE where the lower concentration resulted in a greater loss of viability compared to the higher concentration. Minotti and
Aust have shown that antioxidants can result in lipid peroxidation and subsequent cellular
damage by affecting the redox cycling of iron (Minotti & Aust, 1992). This phenomenon
could explain our results of decreased cell viability caused by the addition of
antioxidants.

An *ex vivo* canine lens capsule model was utilized to determine the possible
effects of antioxidants on PCO formation. This established method was first described in
dogs by Davidson et al. but has been described in other species as well (Davidson et al.,
2000). Capsule bag models provide an ideal mechanism for the study of PCO because
they create a natural capsule and LEC environment similar to that of *in vivo* cataract
surgery yet allow for controlled monitoring and application of treatments (Wormstone,
2002). At fifteen days, the GSE and control capsules had reached fifty percent
confluence, the FO capsules had reached one hundred percent confluence, and the lutein
supplemented capsules had reached eleven percent confluence. These results were
corroborated by histologic cell counts. The decreased PCO noted in the lutein capsules
compared to the other treatment groups does not appear to be related to the hypothesized
decreased redox signaling as the lutein was unable to reduce ROS in the DCF assay and
actually increased ROS production compared to controls. This result could be explained
by ROS induced apoptosis or autophagy due to lutein’s pro-oxidative effect. Much
evidence suggests that oxidative stress and ROS play a role in apoptotic and autophagic
mediated cell death (Dröge, 2002; Dumont et al., 1999; Kannan & Jain, 2000;
Orrenius, Nicotera, & Zhivotovsky, 2010; Slater, Stefan, Nobel, van den Dobbelsteen,
& Orrenius, 1995). However, in contrast to this possibility, the lutein group that was
pro-oxidative in other experiments did not promote a substantially larger decrease of LEC viability compared to the anti-oxidative groups in the MTT assay. It is possible that lutein slowed PCO formation by another mechanism not evaluated in this study or the effect may have been induced by other components in the impure lutein compound.

Interestingly, the FO group increased the rate of PCO compared to controls reaching one hundred percent confluence by day twelve. This result was unexpected and not completely understood. It could be that the slight increase in ROS production compared to controls shown in the DCF assay also occurred in the capsule model and provided the appropriate ROS production to promote redox signaling. Another possibility is an unknown positive interaction between the LECs and capsule generated by the presence of FO. Basement membranes are known to be important regulators of cellular adhesion and function (Berrier & Yamada, 2007; Kalluri, 2003). Several in vitro studies show that the presence of the lens capsule or its matrix components promotes adhesion, migration, and proliferation of LEC (Oharazawa, Ibaraki, Lin, & Reddy, 1999; Olivero & Furcht, 1993). Therefore the presence of the lens capsule in the ex vivo model may have played a role in the increased PCO recognized in the FO group if the presence of FO promoted supportive changes in the capsule matrix components. Overall, it does not appear that the tested antioxidants have the ability to reduce PCO in a clinically relevant manner. When compared to controls, even with a slowed rate of ex vivo PCO formation in the lutein group, a continuous increasing trend of LEC proliferation and migration was noted; this cellular behavior would likely continue with time. However, with differences in the ability to remove residual LEC during in vivo phacoemulsification surgery using
standard irrigation/aspiration techniques and the lack of an IOL barrier in the *ex vivo* model utilized in this study our results may not accurately represent clinical PCO outcomes. Further studies would be necessary to confirm or deny this hypothesis.

No precedent exists to determine useful concentrations of antioxidants to prevent oxidative stress or simply lower ROS production within the lens. The concentrations utilized in this study were based on a commercially available antioxidant supplement for canine ocular health (Ocu-GLO Rx Vision Supplement™). Evidence suggests that over supplementation with antioxidants can be toxic. This effect may be related to the possibility that some antioxidant metabolites become pro-oxidative (Bast & Haenen, 2002). Minotti and Aust reported that a reduction of transition metals by antioxidants can lead to generation of free radicals (Minotti & Aust, 1992). As discussed previously a pro-oxidative state could theoretically induce cellular apoptosis and or autophagy (Dröge, 2002; Dumont et al., 1999; Kannan & Jain, 2000; Orrenius et al., 2010; Slater et al., 1995). Omega-3 fatty acid induction of ROS-mediated apoptosis and autophagy has been shown in some cancer cell lines (Shin et al., 2013). Therefore if the concentrations utilized in this study represented over supplementation, it is possible that a pro-oxidative state could have resulted in cell death. Future assays to determine the type of cell death occurring would be needed to confirm this possibility.

An inflammatory cytokine protein array was used to determine the effects of the antioxidants on LEC cytokine expression. The array determined the expression of 40 different cytokines but only cytokines with a greater than five fold change in at least one antioxidant test group were reported. This large change in expression was noted for
interleukins IL-6 and IL-8 and chemokines CCL2 and CCL5. Previous work suggests that LEC are capable of producing IL-6, IL-8, and CCL2 (Kawai et al., 2012; O. Nishi et al., 1996; O. Nishi, Nishi, Wada, & Ohmoto, 1999). To the authors’ knowledge no previous reports exist regarding expression of CCL5 by LEC or a role of CCL5 in PCO formation.

It has been recognized that ROS production may be an important regulator of cytokine expression (DeForge et al., 1993; Remick & Villarete, 1996; Satriano et al., 1993; Schreck & Baeuerle, 1991), and that antioxidants may alter cytokine expression in various cell types (Ma et al., 2003; Roy, Sannigrahi, Majumdar, Ghosh, & Sarkar, 2011). Our data suggests this may also be true for LEC. This hypothesis is supported by the fact that GSE reduced the expression of all the reported cytokines and was the only antioxidant tested with the ability to reduce ROS production in our experiments.

Furthermore, the pro-oxidative lutein and FO groups increased the expression of these same cytokines with the exception of IL-8 where no difference or only a slight increase was detected relative to controls. It does not appear that altering expression of these cytokines generates a major inhibitory effect on ex vivo PCO formation. This is assumed because the lutein group reduced ex vivo PCO formation and the FO group increased this behavior in the capsule model but the lutein and FO groups produced very similar results in their effects on LEC cytokine expression. Additionally, GSE decreased the expression of all the reported cytokines in our study but resulted in PCO formation equal to that of the controls in the capsule model. Further studies are needed to investigate the role of these cytokines in the formation of PCO before more definitive conclusions can be drawn.
There were several limitations to this study starting with the antioxidants. As previously discussed we experienced difficulty in determining exact concentrations. This problem was due to several factors. First, the compounds are not completely pure, likely containing other unknown substances; therefore, precise antioxidant concentrations could not be produced and the cellular effects specific to the antioxidants could not be conclusive. Second, the compounds exhibited physical properties that created difficulty with making solutions for the addition to the culture medium. The lutein substance would bind significantly to any glass or plastic container and the FO was very difficult get into aqueous solution. Another limitation was the lack of precedent for a safe or effective range of antioxidant concentrations to utilize in LEC or other cell types. A further limitation included the inability of our lutein and FO groups to act as antioxidants and reduce ROS production. Our working hypothesis was that the antioxidant effects on LEC behavior would be through a reduction of ROS important for cellular functions (i.e redox signaling). Without the ability to reduce ROS with some of the tested antioxidants, the hypothesis could not be fully tested or proven. A limitation of most in vitro studies is the absence of naturally occurring processes, such as the release of inflammatory mediators, which may influence cellular behavior. The ex vivo capsule model is a technique that has been widely used to evaluate methods of PCO prevention but lacks the influence from other ocular tissues that may ultimately play a role in LEC behavior following cataract surgery. Finally, our study lacked the placement of an IOL barrier. As discussed previously the capsule bend created by the IOL plays a significant role in altering the migration of LECs.
Future directions of this work should include establishing a technique to detect the levels of these antioxidants in aqueous humor following oral or topical administration. Without this information, further work with these antioxidants may not be of value. This knowledge could lay the groundwork to determine useful concentrations for further experimentation. Our finding of decreased cytokine expression by the GSE treated LEC could be an area of investigation if ocular penetration is proven. While this change did not seem to alter PCO formation it could be investigated for benefits in other ocular disease processes. Also, further work would be needed to determine if lutein has a potential benefit in PCO reduction. As previously discussed, lutein slowed PCO formation in the capsule model but the significance of this finding is unknown as there was a continued increase in PCO formation at the fifteen-day end point.

In conclusion, we sought to determine if GSE, lutein, and FO could alter the behavior of LEC in vitro and ex vivo. Of our test antioxidants only GSE was able to act as an antioxidant at the concentrations tested. We determined that all the test antioxidants had a negative effect on the viability of cultured lens epithelial cells. We also determined that the test antioxidants altered the expression of cytokines known to affect cellular migration and proliferation. Finally, in the ex vivo PCO model lutein slowed PCO formation, FO increased PCO formation, and GSE produced similar results compared to vehicle controls. However we realize careful conclusions should be drawn regarding the ability of antioxidants to alter PCO formation, as the overall antioxidant power in this study was weak.
References


