Induction of Autophagy-Mediated
Lens Epithelial Cell Death Using Cyclosporine A to
Prevent Posterior Capsule Opacification

Thesis

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Abstract

Cataracts are the leading cause of blindness throughout the world, and cataract surgery is the most common ophthalmic surgery performed in America. Posterior capsule opacification (PCO) is the leading complication following cataract surgery. During routine cataract surgery, lens fibers and lens epithelial cells (LEC) found on the anterior capsule are removed. However, due to surgical constraints it is impossible to remove all LEC. The remaining LEC eventually proliferate and migrate around and behind the intraocular lens implant causing a progressive reduction in vision. This opacification of the posterior lens capsule following cataract surgery is known as PCO. The only current treatment for PCO is Nd-YAG capsulotomy, in which a laser is utilized to remove the LEC from the posterior capsule of the lens. However, this procedure has complications including damage to the intraocular lens, cystoid macular edema, intra-ocular pressure spikes, and retinal detachments. This procedure is also difficult to perform in children, is costly, and is not widely available in rural areas and underdeveloped countries. Several surgical and pharmacologic procedures have been implemented to reduce the incidence of PCO; however, PCO can still occur in up to 50% of adults and 100% of children and canines following cataract surgery. Recent research has used Cyclosporine-A (CsA) to prevent PCO formation \textit{ex vivo}. In the present study, it was determined that 10 $\mu$g/mL CsA for 7 days was the necessary dose needed to prevent PCO formation in an \textit{ex vivo} extra-capsular cataract surgery model using cadaveric canine lenses. The mechanism
whereby LEC death occurs was subsequently evaluated using transmission electron microscopy, western blots for microtubule associated light chain 3 (LC3) protein, and immunofluorescence (specifically targeting LC3, F-actin, and acridine orange). Specific results included the presence of double-membrane autophagosomes, increased expression of LC3-II, accumulation of acridine orange, and inhibition of cell death in the presence of an autophagy inhibitor. Cumulatively, the evidence supports autophagy-mediated LEC death following exposure to CsA.
This work is dedicated to my loving parents,

Jeffery and Rosemary Hydeman. Your support throughout
my educational journey has brought me farther than I imagined possible.

Thank you for your love and for always believing in me,

and for teaching me that faith in God

is the most important aspect of life.
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The Crystalline Lens

The crystalline lens is a very important structure within the eye to help maintain a clear and focused image on the retina. It is a biconvex structure located directly posterior to the iris and centered behind the pupil. Light that is refracted by the tear film on the cornea enters the pupil of the eye and is again refracted by the lens. The lens is roughly composed of 66% water and 33% protein (Remington, 1998). This is an extremely high protein content (double that of any other tissue in the body), giving the lens its unique ability to refract light onto the retina. The lens is also avascular and lacks nerves, promoting transparency to allow for light transmission and refraction. The lens must maintain its transparency through carefully controlled metabolism (Remington, 1998).

The lens has several unique anatomical features that relate to its functions. Early in the embryological development of the lens (four to six weeks in utero), a thickening of surface ectoderm cells, called the lens placode, invaginates to form a hollow sphere. This is known as the lens pit, which eventually forms a hollow sphere to become the lens vesicle. During the formation of this sphere, the apical surface of the epithelial cells becomes oriented internally. The basal aspect of the cells are oriented externally and secretes a basement membrane. These cells are now known as the lens epithelial cells (LEC). The epithelium on the posterior aspect of the lens terminally differentiates into
primary lens fiber cells, which continue their growth anteriorly to completely diminish the lens vesicle lumen. These primary lens fibers ultimately form the embryonic nucleus of the lens at two months of fetal development (Remington, 1998).

The lens is unique in the fact that new fibers continue to grow throughout one’s lifetime without the destruction of older fibers. Because of the apical to apical interaction established during embryological development of the anterior LEC, newer lens fibers are found on the external aspect of the lens, and older fibers are condensed towards the internal aspect of the lens. The post-natal lens epithelium is a single layer of cells located directly between the anterior capsule of the lens and the lens fiber cells (Remington, 1998). These LEC are divided into four zones. The central zone, as the name implies, is located on the visual axis in the center of the anterior lens epithelium. The cells here have small nuclei, few organelles, and are cuboidal and very regular in shape. This is the most metabolically active portion of the lens. The pre-germinative and germinative zones are found more equatorially in the lens epithelium. These zones represent areas of increased mitosis and decreased metabolism. Cells in the germinative zone are transiently amplifying cells; daughter cells produced in the germinative zone go on to create the equatorial zone. In the equatorial zone, LEC adopt a columnar morphology, decrease mitotic and metabolic activity, and begin the process of terminal differentiation. These cells will eventually elongate from the central portion of the cells to become mature lens fibers cells (Kuszak, 2008).

Generation of lens fibers through terminal differentiation continues throughout life. Because it occurs within a closed system, growth shells of new fibers are formed near the external aspect of the lens, while older fibers are pushed towards the center of
the lens. This layering process gives the lens an onion-like appearance. As the lens ages, the fibers become more and more compressed, decreasing the space between the once highly organized fibers. This causes them to lose their highly organized pattern and makes it more difficult for nutrients to diffuse through the lens. Over time, the lens hardens, and disorganized opacities can form within. These opacities are most common within the nucleus, cortex, and below the posterior capsule of the lens (Remington, 1998). Opacifications in the lens formed over time are known as age-related cataracts (Kanski, 2011).

*Cataracts and Cataract Surgery*

Cataracts are the leading cause of blindness throughout the world. In the United States, cataract surgery is the most common type of surgery covered by Medicare. In 2010, cataract surgery and related medical visits cost $3.2 billion dollars to Medicare (McElroy, 2014). Cataracts are defined as any opacification of the normally clear crystalline lens of the eye (Ehlers, 2008). Cataracts are most commonly due to age-related changes and can be exacerbated by exposure to ultraviolet (UV) light, inadequate nutrition, cigarette smoke, high alcohol intake, diabetes, and long-term use of antipsychotic medications or corticosteroids (Francis, 1999). Specifically, longer wavelength UV A (300-400 nm) and UV B (290-320 nm) may be more likely to cause cataracts (Raj, 2013). Eating a diet high in leafy green vegetables may be protective against developing cataracts (Klein, 1999). Children can also be born with congenital cataracts, and it is imperative to remove these to prevent the eye from becoming amblyopic (Ehlers, 2008). Even in adults, it is indicated to remove clinically significant cataracts that are causing a reduction in a patient’s vision. Cataracts are also the most
common ocular pathology found in dogs, leading to reduced vision and secondary uveitis (Bras, 2006).

Cataracts can occur in any location of the crystalline lens. Nuclear cataracts are the most common age-related cataract and are more common in women (Klein, 1999). According to the Beaver Dam Study, cigarette smoking and alcohol consumption modestly increase the risk of developing nuclear cataracts over a five year period (Klein, 1999). Cortical cataracts are the second most common type of age-related cataract. These are more common in African American patients as well as in diabetics (Klein, 1999). UV-B exposure is most commonly associated with cortical cataractogenesis (McElroy, 2014). Posterior subcapsular cataracts (PSC) are the third most common age-related cataract and are a known risk factor with corticosteroid use (Klein, 1999). Ionizing radiation and blunt trauma have also been found to cause PSC (Wong, 2002).

Cataract surgery is the most common surgical procedure in the United States today. The previous method of cataract removal was known as intracapsular cataract extraction. During this procedure, the entire lens and lens capsule was extracted from the eye to fully remove the cataract (Raj, 2013). Today, extracapsular cataract extraction is employed as the surgical technique of choice for removing cataracts. This surgery involves the removal of all of the lens fibers within the lens capsule, leaving most of the lens capsule intact. The lens capsule is then used as anchorage for the implantation of an intraocular lens (Raj, 2013).

Phacoemulsification is the preferred surgical technique for extracapsular cataract extraction. To perform this surgery, a small limbal incision is made to access the
intraocular structures. This small incision is beneficial in decreasing post-operative astigmatism and in stabilizing the patient’s refractive error following surgery (Kanski, 2011). Intraocularly, a small tear, known as the continuous curvilinear capsulorhexis, is made in the anterior capsule of the lens. The cortex is separated from the nucleus and capsule using a technique known as hydrodissection. The cataract is then rapidly removed with a phacoemulsification tip that vibrates at ultrasonic frequencies. The emulsified lens material is then aspirated from the eye through the incision. The original equatorial and posterior lens capsule is left intact, and an artificial IOL is placed within the eye to restore visual acuity (Kanski, 2011).

**Posterior Capsule Opacification**

While cataract surgery is a common procedure, it is not without complications. The most common complication following cataract surgery is visually significant posterior capsule opacification (PCO). PCO is also commonly referred to as ‘secondary cataract’ or ‘after cataract’, and frequently occurs a few months to years following routine cataract surgery (Raj, 2013). PCO can cause reduced visual acuity, problems with glare, impair contrast sensitivity, and result in monocular diplopia (Kanski, 2011). During cataract surgery, most of the LEC are removed from the anterior lens capsule. However, due to surgical constraints, it is not possible to remove all of the cells, and some residual LEC are left behind on the capsule (Raj, 2013). These surgical constraints include decreased intraoperative times (to reduce the risk of post-operative uveitis) and anatomical constraints due to working in the confined space of the eye. These residual LEC migrate onto the posterior capsule of the lens, causing it to become cloudy. PCO is therefore a physiological consequence of routine extracapsular cataract surgery.
During PCO formation, the remaining LEC undergo epithelial-to-mesenchymal transition (EMT), proliferation, and migration from the anterior capsule of the lens to the posterior capsule of the lens behind the IOL. Collagen is deposited on the posterior capsule along with formation of regenerated lens fibers. Once enough of these changes occur, the posterior capsule becomes hazy, and the patient can experience a reduction in vision that may be worse than the initial cataract itself (Awasthi, 2009). In more advanced forms of PCO, the capsule can become wrinkled due to the fibrous depositions on the lens and contraction of the LEC (Raj, 2013). It has been shown in both rabbit and human models that LEC remaining following cataract surgery undergo hyperplasia soon after the procedure; by post-operative day four, they can begin to change morphologically into spindle-shaped myofibroblast cells (McDonnel, 1983). This begins at the location of the capsulorrhexis, and by one week, a fibrotic ring can be seen encroaching upon the posterior lens capsule (Spalton, 2003). Soon, the cells become surrounded by proteoglycans (dermatan sulfate, chondroitin sulfate, and heparin sulfate), as well as collagen fibrils (types I, IV, V, and VI) (Spalton, 2003).

There are two main clinical signs of PCO. The first is known as Elschnig pearls, which are residual equatorial epithelial cells from the lens bow that migrate to the posterior capsule and form modified fibers with vacuoles. These vacuoles contain the protein crystalline and often cause a large reduction in vision. The second clinical sign is called capsular fibrosis. This is typically seen earlier than Elschnig pearls, if it occurs. Capsular fibrosis is due to the LEC that undergo EMT, leading to cellular contraction and wrinkling of the posterior capsule (Kanski, 2011). Histologically, capsular fibrosis contains an accumulation of extracellular matrix and elongated fibroblastic cells (Raj,
cataract surgery itself causes the LEC to undergo a wound-healing response, which induces the changes seen with PCO. While the molecular mechanisms of PCO formation are not entirely clear, the process seems to be mediated by several inflammatory cytokines and growth factors (Awasthi, 2009).

On the molecular level, cytokines play a role in the pathogenesis of PCO. Cytokines are defined as peptides that are secreted from cells that act on target cells in either a paracrine or autocrine fashion (Spalton, 2000). Several different types of cytokines will exist in the anterior chamber of the eye following cataract surgery. The main cytokines found to play a role in PCO formation are Transforming Growth Factor β (TGF-β), basic Fibroblast Growth Factor (b-FGF), and interleukin 1 and 6 (Nishi, 1996). TGF-β is a serine-threonine kinase that plays a central role in the growth and differentiation of many cell types. It is secreted by macrophages, and has been found to play a central role in wound healing, specifically by regulating cell growth and cell-to-cell interactions, by inducing mitosis and playing a role in inflammatory cascades. TGF-β has also been found to play a role in the increased deposition of extracellular matrix and in the inhibition of the turnover of extracellular matrix by matrix metalloproteinases during PCO pathogenesis (Spalton, 2000).

b-FGF is a polypeptide involved in the proliferation and differentiation of various cell types. It plays a role in lens development and has been found to stimulate lens fiber differentiation in a dose dependent manner (Spalton, 2000). b-FGF is also found in LEC, and its levels have been shown to be increased thirty days following cataract surgery (Spalton, 2000). In culture, b-FGF increases mitosis and collagen production in LEC (Spalton, 2000).
The interleukins play central roles in the inflammatory cascade and wound healing. IL-1 can induce LEC mitosis and collagen formation, which may play a role in PCO formation. Both IL-1 and IL-6 levels are increased following cataract surgery (Cortina, 1997). Despite the association between cytokines and PCO, it is clear that the direct role inflammation plays in PCO formation is obscure. As one example, the use of diclofenac, an anti-inflammatory medication that inhibits cyclooxygenase, does not prevent the formation of PCO (Spalton, 2000). Collectively, current research confirms that the development of PCO is complex and involves various cellular pathways and processes.

PCO occurs in human adults and children, as well as many other species including canines. There is a 30-50% incidence of PCO in adults and an 80-100% incidence in children and canines following cataract surgery (Pandey, 2004). It has recently been shown that 25% of patients undergoing modern cataract surgery develop PCO within five years (Totan, 2008). In adults, visually significant PCO formation can take up to five years to form, while it commonly occurs within one to two years in children (Pandey, 2004). Clearly, PCO is very common and is especially concerning in pediatric patients. Because the incidence of PCO is similar in the canine population, it makes a suitable naturally-occurring model for human PCO. In a study of 265 canine eyes, 100% of the eyes developed PCO twelve months following cataract surgery (Bras, 2006). No difference in the incidence of canine PCO was found relative to etiology of the cataract (breed related vs. diabetes induced), age, sex, or presence of previous inflammation inside of the eye (Bras, 2006).
The current treatment for PCO is a procedure known as Neodymium:Yttrium-Aluminum-Garnet (Nd:YAG) laser posterior capsulotomy. The procedure includes an Nd:YAG laser that is focused at the posterior capsule of the lens to remove the central posterior capsule and the subsequent PCO. While this is a very common procedure, serious, though rare, complications can result. These include damage to the existing IOL, intraocular pressure spikes, rhegmatogenous retinal detachments, cystoid macular edema, posterior IOL subluxation, and chronic endophthalmitis (Kanski, 2011). The procedure is difficult to do in children, often resulting in a need to put the child under general anesthesia. This alone can pose a high risk, but may be necessary in order to perform an Nd:YAG posterior capsulotomy successfully on a child.

Nd:YAG posterior capsulotomy is a costly procedure. While cataracts are common in all parts of the world, they are extremely prevalent in underdeveloped nations. Vision care is particularly lacking in many of these countries (Vasvada, 1997). Even if these people do receive cataract surgery, many ultimately lose their vision due to PCO (Apple, 1992). It is often not possible to perform Nd:YAG capsulotomy in these areas of the world due to the high cost. The high cost also puts an immense burden on healthcare systems throughout the world emphasizing the need to decrease the incidence and prevalence of PCO (Vasvada, 1997).

Much research has been focused on decreasing PCO following cataract surgery. There is some promise in recent advances in IOL design, allowing it to act as a mechanical barrier between the IOL itself and the posterior capsule of the lens to prevent PCO formation (Clark, 2013). Apple and Werner identify several factors to reduce the incidence of PCO. One technique is hydrodissection-enhanced cortical clean-up to
remove as many LEC as possible during the surgical process. Another is to form a small diameter continuous curvilinear capsulorhexis that is smaller than the IOL optic. This creates a tighter interaction between the posterior capsule and the IOL optic to mechanically prevent the migration of LEC onto the posterior capsule. There are also several important IOL design factors to reduce PCO formation. A biocompatible IOL material, such as silicone or acrylic, should be used to reduce the stimulation of cell growth and inflammation. Also, an IOL with a square, truncated edge decreases LEC migration (Apple, 2001). However, even with these advances, PCO is still an obvious complication following cataract surgery, and occurrences are not declining enough despite these improvements in surgical techniques (Clark, 2013). Clearly, another method is needed to treat, and ideally prevent, PCO.

Cyclosporine A

In recent years, efforts have been made to find a pharmacologic means to prevent and/or treat PCO. Various pharmacologic agents to decrease the proliferation of the LEC have been tested, including mitomycin-C, ethylenediaminetetraacetic acid, 5-fluorouracil, retinoic acid, and caffeic acid phenethyl ester (Fernandez, 2004). Unfortunately, even those that proved effective in reducing LEC growth have been shown to be toxic to other cells within the eye, specifically the corneal endothelium (Totan, 2008). Recently, interest has been placed on the use of Cyclosporine-A (CsA) to treat PCO. Promising studies have shown that CsA is an effective medication to decrease PCO development, and it is not toxic to other tissues within the eye. Pei et al. (2013) have shown that CsA is not toxic to the corneal endothelium in in vivo rabbit eye models. Our lab has also shown that CsA is not toxic to the corneal endothelium in ex vivo models (unpublished).
Cortina et al. (2013) speculated that CsA inhibits the proliferation of human LEC in culture in a dose dependent manner. Other *in vivo* models have shown promise in the ability of CsA to decrease LEC migration and proliferation in a dose dependent manner in rabbit lenses following cataract surgery (Totan, 2008). The rabbit lens is similar in morphology to the human lens, so it is a sufficient model for lens studies (Gwon, 1990). For the treatment group in this study, 0.1mL (0.5mg/mL) of CsA was injected into the capsular bag following cataract surgery in rabbit eyes. PCO development was assessed weekly through biomicroscopy, as well as through haematoxylin and eosin staining following enucleation. Of ten rabbits in the CsA treatment group, none of the eyes developed severe PCO, one developed moderate PCO, four developed mild PCO, and five had clear capsules. This was found to be significantly less than the PCO developed in the control group (Totan, 2008).

CsA is a commonly used systemic immunosuppressant drug following organ transplants. It is also used as a topical ocular drug, most commonly in the form of Restasis, to decrease inflammation for treatment of chronic keratoconjunctivitis sicca. CsA is a metabolite of the fungus *Tolypocladium inflatum*. It is neutral and hydrophobic, and is composed of a cyclic chain of eleven amino acid residues. CsA inhibits T-cell activation and reduces the formation of certain cytokines, such as IL-2, IL-3, IL-4, and interferon gamma (Leonardi, 2001). IL-1 is produced by human LEC, so it is thought that CsA may work to inhibit LEC migration by decreasing this and several other inflammatory factors (Nishi, 1996). Intraocular inflammation increases following all cataract surgery, and the risk for PCO increases with a higher amount of intraocular inflammation (Elgohary, 2006). Therefore, while the exact mechanism by which CsA
reduces the formation of PCO is not fully elucidated, an anti-inflammatory component may be involved (Totan, 2008).

While CsA shows promise in the reduction and possible prevention of PCO, the mechanism responsible for LEC death is currently unknown. Previously, it was hypothesized that LEC exposed to CsA undergo an apoptotic process of cell death. Leonardi et al. (2001) demonstrated that CsA induces apoptosis in conjunctival fibroblasts. However, recent work in our laboratory has shown that the LEC exposed to CsA are not undergoing apoptosis nor necrosis (unpublished). Due to these findings, we hypothesize that the LEC are undergoing autophagy when exposed to CsA.

**Autophagy**

Autophagy, or “self-eating,” is a homeostatic process found in eukaryotic organisms. During times of stress, a cell will undergo autophagy to recycle intracellular components and preserve nutrients (Heymann, 2006). Autophagy is a catabolic pathway in which intracellular components of the cytoplasm are embedded into double membrane-bound sacs, called autophagosomes. These autophagosomes then fuse with lysosomes within the cell cytoplasm to form autophagolysosomes (Heymann, 2006). Once these fuse, the contents are degraded by the hydrolytic enzymes within the lysosomes, known as acid hydrolases, and the contents are disposed from the cell for metabolic recycling. Autophagy has been found to be a common response of the cell to various diseases, such as cancer, cardiovascular disease, neurodegenerative processes, and diseases of metabolism (Choi, 2013). Also, it is a common cellular response during times of either
senescence or exercise, two examples of when a cell is trying to maintain nutrient supply (Choi, 2013).

During times of stress, autophagy allows the cell to produce metabolic precursors, as well as rid itself of waste (Heymann, 2006). During the early stages of autophagy, it has been found that the cytoskeletal protein, actin, plays an important role in the initial formation of the autophagosome (Aguilera, 2012). Soon, mitochondria and other organelles are degraded in the autophagosome (Barth, 2010). Also, proteins and lipids, which accumulate during times of stress, are removed through autophagy (Barth, 2010). The main function of autophagy is to protect the cell from dying; however, there is new experimental evidence that suggests that unregulated autophagy can promote cell death (Choi, 2013). Additionally, the autophagy pathway is tightly linked with the apoptotic pathway, and the two can facilitate programmed cell death (Pallet, 2009).

Autophagy also plays a role in immunity (Heymann, 2006). Bacteria and viruses are degraded through a specialized autophagy process known as xenophagy. Interestingly, autophagy is also involved in the inhibition of inflammatory processes, playing a role in interferon and cytokine down-regulation, as well as in adaptive immunity through interactions with major-histocompatibility-complex II (Choi, 2013). It is known that PCO development is increased with high levels of post-operative inflammation, and CsA is known to decrease inflammation, possibly through stimulation of the autophagy process.

The exact role that autophagy plays in pathogenesis is unclear as autophagy is up-regulated in some disease processes and down-regulated in others (Heymann, 2006).
Recent research has focused on targeting the autophagy pathway to fight human disease. Sirolimus, an immunosuppressive and anti-cancer agent, has been shown useful in increasing autophagy (Heymann, 2006). Chloroquine and hydroxychloroquine are two immunosuppressive agents that have shown promise in the modulation of autophagy by decreasing the formation of lysosomal acid hydrolases (Choi, 2013). Much research is needed to determine how pharmaceutical interventions can be used to protect against disease and how autophagy may play a role in that mechanism. It is our supposition that CsA decreases the formation of PCO post-cataract surgery by causing the LEC to undergo autophagy.

Several molecular markers for autophagy have been identified. A family of autophagy proteins, known as autophagy-related (Atg) proteins, has been identified in mammals. To date, more than thirty Atg proteins have been identified. Several major regulators of autophagy are of interest for our research purposes. The first is the Beclin-1 interacting complex, which is necessary for the initiation of autophagy (Chifenti, 2013). The complex is formed through the interaction of phosphatidylinositol-3-kinase and Atg14L (Chifenti, 2013). When activated, this complex stimulates the formation of the autophagosome (Cherra, 2010). The ULK complex also contributes to autophagy induction by integrating signaling from the mammalian target of rapamycin (mTOR) (Ciechomska, 2013). mTOR is a negative regulator of autophagy and it’s inhibition leads to autophagy initiation (Ciechomska, 2013).

Another major regulator is microtubule-associated protein light chain 3 (LC3). During initiation of the autophagic pathway, LC3 is conjugated with lipids to become the soluble form, known as LC3-II. The presence of LC3-II is indicative of autophagy. In the
cytoplasm of cells, a shortened form of LC3 (LC3-I) exists; this is converted to the soluble LC3-II form during autophagosome formation (Choi, 2013). In immunofluorescence staining, LC3-II appears as distinct, punctate areas of bright fluorescence when autophagosomes are present. Brennan et al. (2012) showed an increase in LC3-II-positive puncta in serum starved LEC in culture, indicating a role of autophagy during times of stress to the crystalline lens. They concluded that autophagy is an important cellular process to maintain clarity of the crystalline lens and prevent cataract formation (Brennan, 2012). Importantly, LC3-II is mainly seen in autophagosomes, for the acid hydrolases found within autophagolysosomes degrade LC3-I (Eskelinen, 2005).

Another well-established method to identify a cell undergoing autophagy is through morphological analysis utilizing transmission electron microscopy (TEM). There are several distinct morphological characteristics of autophagic cells that can be identified using TEM. Along with using the protein marker LC3, TEM analysis of cells is the most sensitive method to identify cells undergoing autophagy. Eskelinen outlines the key identifiers needed to accurately classify an autophagosome using TEM. As stated above, autophagy occurs when an environmental signal causes a flat membrane cistern, derived either from the endoplasmic reticulum or mitochondria, to engulf organelles and/or cytoplasm. This forms a double-membrane bound compartment, known as an autophagosome, which contains organelles or other cellular components. These autophagosomes eventually mature when they fuse with lysosomes to form autophagolysosomes.
These double-membrane autophagosomes can be identified through TEM. It is important to note that the cytoplasm within the compartments should have the same morphology and electron density as the cytoplasm outside of the autophagosome (Eskelinen, 2008). Autophagosomes often contain organelles, especially ribosomes, within their double-membrane walls (Eskelinen, 2008). Even though double-membrane bound compartments morphologically characterize autophagy, both membranes are not always fully visible using TEM due to differences in the preservation of membrane lipids during the technical process of sample preparation (Eskelinen, 2008). Therefore, these double-membrane compartments cannot solely be used to indicate that a cell is indeed undergoing autophagy (Eskelinen, 2008).

Once an autophagosome fuses with a lysosome (to form an autophagolysosome), the degradation of cellular organelles begins. While new autophagosomes usually contain intact organelles and many ribosomes, older autophagosomes and autophagolysosomes contain partially degraded contents, which mostly consist of ribosomes (Eskelinen, 2005). These appear as dark, granular, amorphous, electron dense clusters. Therefore, the presence of dense clusters within an autophagosome can be indicative of the age of the compartment (Eskelinen, 2005). It is useful to be aware of this morphological variant of an autophagosome seen with TEM to avoid confusion.

Further confusion can also arise while differentiating the endoplasmic reticulum (ER), which often wraps around mitochondria, from double-membrane bound autophagosomes. A key differentiating factor is the presence of ribosomes on the ER (Eskelinen, 2008). Autophagosomes do not have ribosomes directly incorporated into their membranes (Eskelinen, 2008). Mitochondria themselves are also double membrane
bound organelles. It is important to look for the presence of a folded inner membrane (known as cristae) found in all mitochondria in order to distinguish this organelle from an autophagosome (Eskelinen, 2008). Vacuoles within a cell could also be confused with autophagosomes; however, vacuoles are empty compartments, and autophagosomes contain cytoplasmic components (Eskelinen, 2008).

The size of autophagosomes can also vary. Diameters range from 300 nm to several micrometers with an average diameter of 600 nm. They can be seen as single compartments, or they may fuse with one another to form large autophagic vacuoles. Also, several autophagosomes can fuse with one lysosome, and autophagosomes can also fuse with older autophagolysosomes (which, as stated above, are characterized by clusters of electron rich, partially degraded ribosomes). While TEM is the most sensitive technique available to identify autophagic vacuoles, it is important to keep all of these variants in mind while identifying cells that are undergoing autophagy (Eskelinen, 2008).

Various links have been discovered between CsA and autophagy. Several studies have been conducted on the effects of CsA on renal cells due to the use of CsA following organ transplants. CsA nephrotoxicity is a known complication of long term immunosuppressive therapy following organ transplantation. Pallet et al. (2009) has shown that CsA induces endoplasmic reticulum stress in renal tubular cells, which ultimately leads to an activation of autophagy. The ER has a complex “unfolded protein response” pathway that is activated during times of stress. In this instance, autophagy is acting as a feedback mechanism to reduce cell death caused by CsA. Interestingly, it has been found that acute exposure to CsA has led to the activation of both the ER stress
response (and thus, autophagy) and apoptosis. Conversely, chronic exposure to CsA has led to a more apoptotic response and less autophagy (Yoon, 2009).

Ceichomska et al. (2013) have shown that CsA induces both apoptosis and autophagy in malignant glioma cells through the ER unfolded protein response and through the inhibition of the mTOR pathway. The autophagy induction in this instance also appears to be cyto-protective. It seems that cellular stress caused by CsA leads to misfolded proteins within the cell. This ER stress induces autophagy to rid the cell of the misfolded proteins. Lim et al. (2012) also showed that CsA induces autophagy in mouse kidney cells. Clearly, a link exists between CsA and the induction of autophagy.

Purpose and Hypothesis

The first aim of this research is to determine the amount of time of CsA exposure needed to prevent PCO formation in cadaveric canine lenses following ex vivo cataract surgery. It is hypothesized that LEC death will increase in a time and does dependent manner. The second aim of this research is to determine the cellular and molecular mechanism whereby CsA causes cell death in LEC following ex vivo cataract surgery. It is hypothesized that these cells are dying through an autophagy-related pathway.
Chapter 2: Materials and Methods

Cells and Tissues

Normal eyes were obtained by enucleation from dogs between the ages of 3 – 6 years, deemed to be in good general health, that were humanely euthanized for reasons unrelated to this study. All protocols are in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and have been approved by The Ohio State University Institutional Animal Care and Use Committee.

Ex Vivo Cataract Surgery and LEC treatment with CsA

An extracapsular cataract extraction with lens capsule dissection was performed as previously described (Cleary, 1998). Residual LEC and cortical material were removed via gentle manual irrigation and aspiration with PBS using a coaxial cannula, but no effort was made to polish the anterior and posterior lens capsule. The capsules were then excised from their zonular and vitreal attachments and placed in cell culture dishes with unsupplemented DMEM containing 1% antibiotic/antimycotic (Gibco, Carlsbad, CA). Each lens capsule was treated with 10 µg/mL CsA for 4, 5, 6, or 7 days. 27 total lenses were treated with CsA. During CsA treatment, drug and culture media was replaced daily. Following CsA treatment, lens capsules were incubated in unsupplemented DMEM containing 1% antibiotic/antimycotic for a total of 28 days; this experiment evaluated the effect of CsA on long-term ex vivo PCO formation to determine the amount of treatment time needed to prevent PCO formation with CsA. The vehicle
(DMSO) group contained 24 lenses. The control (DMEM) group contained 24 lenses. LEC growth was observed and recorded on several days throughout the 28 days, and a final observation of cell growth on the posterior capsule of the lens was made on day 28. LEC growth was graded on a scale of 0-6:

- 0: No cells
- 1: Central posterior capsule (CPC) clear, inactive cells on peripheral posterior capsule (PC), anterior capsule (AC), or equator (Eq)
- 2: One or more (1-20%) activated epithelial-mesenchymal transition cells (EMT) present anywhere on AC or Eq
- 3: Any degree (21-40%) of EMT encroachment on CPC (axial to capsulorhexis)
- 4: 41-60% of EMT encroachment or confluent LECs on PC
- 5: 61-95% of EMT encroachment or confluent LECs on PC
- 6: Entire PC confluent (95-100%). May have small clear areas on AC or at Eq
- 6+: Entire capsule 100% confluent, often with capsule wrinkling

Representative photomicrographs were taken of LEC using inverted light microscopy.

*Primary Canine Lens Epithelial Cell Cultures*

Anterior lens capsules with adherent LEC were incubated in trypsin (0.25% trypsin and 1X EDTA, Gibco) for five minutes at 37°C. After incubation, the solution and lens capsule were centrifuged for two minutes at 300 x g. Fluid was decanted and supplemented DMEM (10% fetal bovine serum and 1% antibiotic/antimycotic [Gibco]) was then added. The solution, including the lens capsule, was transferred to a laminin-coated culture flask (Beckton-Dickinson, Franklin Lakes, NJ) and incubated in a
humidified incubator at 37°C and 5% CO₂. LEC were grown until 90% confluence prior to re-plating.

Transmission Electron Microscopy

Canine LEC were treated with 0, 5, or 10 µg/mL CsA for 24 hours prior to fixation with 3% gluteraldehyde in a 0.1M cacodylate buffer for 1 hour. Following fixation, samples were post-fixed in 1% OsO₄ in the same buffer for 30 minutes. Ultra-thin sections were then observed under TEM (FEI Tecnai G2 Spirit, Houston, TX).

Western Blot Analysis of LC3

Sample protein content was determined using the Pierce 660 Protein Assay microplate procedure (Thermo Scientific, Rockford, IL). A standard curve was prepared by plotting the average blank-corrected 660nm measurement for each bovine serum albumin (BSA) standard (50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, 800 µg/mL, and 1600 µg/mL) versus its concentration in µg/mL. The standard curve was used to determine the protein concentration of our sample. Equal amounts of protein were added to each well, along with the NuPAGE Sample Buffer (4X) and the Reducing Agent. The gel was run at 200V for 50 minutes in 1X NuPAGE SDS Running Buffer (20X MOPS diluted in deionized water). Next, the gel was removed from the apparatus and allowed to equilibrate in 50mL of 2X NuPAGE Transfer Buffer (20X NuPAGE Transfer Buffer with 10% methanol, 0.1% NuPAGE Antioxidant, and deionized water) for 10 minutes with shaking. Proteins were transferred from the gel onto a nitrocellulose membrane using the semi-dry transfer apparatus at 200 mAmps for 1.5 hours. Next, the membrane was swirled in Ponceau S stain for 30 seconds and rinsed with distilled water. The membrane
was then blocked in blocking buffer (rinse buffer: 5M NaCl, 1M Tris HCl ph 7.6, and Tween 20 with 5% nonfat dry milk) overnight at 4°C. Next, the primary antibody was diluted in blocking buffer and added to the blot overnight at 4°C. The secondary antibody was prepared in blocking buffer, and the blot was incubated for 1 hour at room temperature. The blot was rinsed 5 times in rinse buffer (10 minutes each). The blot was then developed using the SuperSignal West Femto Chemiluminescent detection assay (according to manufacturer’s instructions) and visualized on the Kodak imager.

**LC3 Immunofluorescence**

Canine anterior lens capsules with adherent LEC were removed from normal lenses and immediately placed cell-side up in culture dishes. Cell culture medium containing vehicle control, 5 or 10 µg/mL CsA (n=6) was added to each culture dish and all LEC and capsules were incubated at 37°C for 24 hours. Culture medium was then aspirated and LEC were fixed using 10% formalin. Following two phosphate buffered saline (PBS) washes, cells were permeabilized with PBS containing 0.5% Tween-20. Cells were washed and nonspecific binding was blocked using 10% goat serum in PBS for one hour at room temperature. Following removal of the blocking buffer, LC3 antibody (1:200, rabbit, Novus Biologicals) was applied overnight at 4°C. Cells were subsequently washed with PBS and placed in secondary antibody (1:250, goat anti-rabbit; AlexaFluor 488, Invitrogen) for one hour at room temperature. Finally, LEC were washed three times and VectaShield (Vector Laboratories, Burlingame, CA) containing DAPI was used to mount the LEC.

**Acridine Orange Immunofluorescence**
Canine anterior lens capsules with adherent LEC were removed from normal lenses and immediately placed cell-side up in culture dishes. Cell culture medium containing vehicle control, 5 or 10 µg/mL CsA (n=6) was added to each culture dish and all LEC and capsules were incubated at 37°C for 24 hours. Culture medium was then aspirated, cells were washed twice with pre-warmed PBS (37°C, pH 7.4), and LEC were fixed using 3.7% formaldehyde solution in PBS for 10 minutes at room temperature. Following two PBS washes, cells were permeabilized with PBS containing 0.1% Triton X-100 for 5 minutes. LEC were stained with acridine orange (0.5 µg/mL in PBS) for 15 minutes at room temperature. LEC were subsequently washed twice with PBS, allowed to air dry, and VectaShield (Vector Laboratories, Burlingame, CA) containing DAPI was used to mount the LEC.

F-actin Immunofluorescence

Canine anterior lens capsules with adherent LEC were removed from normal lenses and immediately placed cell-side up in culture dishes. Cell culture medium containing vehicle control, 5 or 10 µg/mL CsA (n=6) was added to each culture dish and all LEC and capsules were incubated at 37°C for 24 hours. Culture medium was then aspirated, cells were washed twice with pre-warmed PBS (37°C, pH 7.4), and LEC were fixed using 3.7% formaldehyde solution in PBS for 10 minutes at room temperature. Following two PBS washes, cells were permeabilized with PBS containing 0.1% Triton X-100 for 5 minutes. LEC were washed and nonspecific binding was blocked using Image-iT FX signal enhancer for 30 minutes at room temperature. Following removal of the blocking solution, cells were stained on the coverslips with fluorescent phalloidin (AlexaFluor 488 phalloidin, Invitrogen) with 5µL methanolic stock solution diluted in
200µL PBS and 1% bovine serum albumin (BSA) for 20 minutes at room temperature in a covered container. LEC were subsequently washed with PBS, allowed to air dry, and VectaShield (Vector Laboratories, Burlingame, CA) containing DAPI was used to mount the LEC.

Statistical Analysis

SPSS computer software was utilized for statistical analysis. Two way ANOVA, one way ANOVA, and Tukey post-hoc analyses were performed to evaluate the growth of LEC on the posterior capsule of the lens in one of three treatment groups [10 µg/mL CsA, DMSO (vehicle), or DMEM (control)] over a time period of twenty-eight days.
Chapter 3: Results

Effect of CsA on Long-term ex vivo PCO Formation and Statistical Analysis

The purpose of this experiment was to determine the amount of time of CsA exposure needed to prevent PCO formation in cadaveric canine lenses following ex vivo cataract surgery. It was previously determined in our lab that a concentration of 10 µg/mL CsA is needed to prevent PCO formation following ex vivo cataract surgery (Lutz, 2013). It is hypothesized that the growth of LEC onto the posterior capsule of the lens will be increasingly inhibited by longer exposure times to CsA. Ex vivo cataract surgery was performed and capsules with remaining LEC were assigned to one of the following treatment groups [CsA (10 µg/mL), vehicle (DMSO), or control (DMEM) for 4, 5, 6, or 7 days]. Following treatment, capsules were placed in unsupplemented culture medium, and LEC growth was observed and recorded for a total of 28 days. LEC growth was graded on a scale of 0-6, as described in the methods section.

Figure 1 depicts three representative photomicrographs of inverted light microscopy of LEC growth following CsA treatment. As observed in Figure 1A, when control LEC were not exposed to CsA, the cells had a very normal, healthy morphology. They form a uniform, cobblestone-like monolayer of cells in culture. By comparison, Figure 1B depicts LEC that were treated with 10 µg/mL CsA for 7 days. Any remaining observable cells appear to be in the process of dying; they have a rounded morphology and can only be seen on the peripheral lens capsule. Finally, Figure 1C shows LEC
treated with 10 µg/mL CsA for 7 days, then subsequently cultured for a total of 28 days in unsupplemented culture medium. There are no viable LEC remaining on the lens capsule in this photomicrograph.

Figure 1. Inverted light microscopy of LEC growth on the lens capsule. The red, dotted line in the photomicrographs represents the capsulorhexis edge. (A) growth of LEC in the control treatment group (culture media only) after 28 days of culture. Note that only the central posterior capsule is represented. (B) LEC treated with 10 µg/mL CsA for 7 days. Note the change in morphology and limited viability of LEC. (C) LEC treated with 10 µg/mL CsA for 7 days, and subsequently in unsupplemented medium for a total of 28 days. Note the lack of LEC on the lens capsule. Scale bar: 100 µm.
Growth plots of LEC over time following treatment with CsA can be observed in Figure 2. CsA treatment groups included 10 µg/mL CsA for 4, 5, 6, or 7 days (A-D, respectively) before being switched into unsupplemented DMEM for a total of 28 days. Figure 2 clearly demonstrates that LEC growth is increasingly inhibited with increased exposure time to CsA. LEC growth was most inhibited in the 7 day treatment group; by day 28, there were no viable cells detectable on the posterior capsule of the lens in the 7 day treatment group (Figure 2D). Of note, the 7 day treatment group was the only group in which PCO formation was completely inhibited. Cell growth was also inhibited in the LEC exposed to CsA for a total of 6 days (Figure 2C). Most of the capsules in the 6 day treatment group were given a score of ‘2’, which is indicative of one or more activated LEC undergoing morphologic changes consistent with epithelial-mesenchymal transition (EMT) present anywhere on the capsule. Cell growth was inhibited in the 5 day treatment group (Figure 2B), but to a lesser extent when compared to the 6 and 7 day treatment groups. Cell growth was minimally inhibited in the LEC exposed to CsA for 4 days (Figure 2A).
Figure 2. Lens epithelial cell growth over time. Plots depict LEC treated with 10 µg/mL CsA for 4, 5, 6, or 7 days (A – D, respectively). All cells were switched to unsupplemented DMEM for a total of 28 days after their specific CsA-treatment times, and cell growth was graded on a scale from 0-6 throughout the experiment.

Statistical analyses were completed to analyze the growth of LEC following the various exposure times to CsA. The outcome measure was the grade (0-6) of the cell growth on day 28 of cell culture. The two independent comparison groups were the treatment groups (control, vehicle, or 10 µg/mL CsA) and exposure times to the specific treatment (4, 5, 6, or 7 days). Data was normalized using the Shapiro-Wilk and Kolmogorov-Smirnov tests of normality (see Appendix A, figure 2A). A two-way ANOVA was run using SPSS software. Values were assumed to be continuous (comparing means) and independent of each other (different lenses were used for each group). The distribution of the dependent variable (cell growth) was assumed to be
normal, and the variances were assumed to be equal for the two comparison groups. The following three null hypotheses were tested:

- **H₀** for interaction: there is no interaction between treatment group and exposure time.
- **H₀** for treatment group: the means of cell growth for treatment group are equal.
- **H₀** for exposure time: the means of cell growth for exposure time are equal.

A significant interaction between treatment group and exposure time was found (p<0.0001) (see Appendix A, figure 4A).

Three one-way ANOVAs were run for each treatment group (control, vehicle, or 10µg/mL CsA) to analyze the differences in cell growth in each treatment group for the four different exposure times. The following null hypothesis was tested:

- **H₀** = the means for each exposure time within a treatment group are equal.

For the control group, we fail to reject the null hypothesis (p=0.059) (see Appendix A, figure 5A). There is no difference in cell growth based on exposure time for the control group. For the vehicle group, we fail to reject the null hypothesis (p=0.213) (see Appendix A, figure 6A). There is no difference in cell growth based on exposure time for the vehicle group. For the CsA treatment group, a test of homogeneity of variances was run, and equal variances were assumed (p=0.116). For the CsA treatment group, we reject the null hypothesis (p<0.0001) (see Appendix A, figure 7A). There is a difference in cell growth based on exposure time. At least one group mean for the exposure times is different from the others.
To analyze the differences between the exposure times in the CsA treatment group, a post-hoc Tukey HSD test was run.

- Group 1 was the 7 day exposure time.
- Group 2 was the 6 day exposure time.
- Group 3 was the 5 day exposure time.
- Group 4 was the 4 day exposure time.

A significant difference in cell growth was found between group 1 and 2, 1 and 3, 1 and 4. (p<0.0001) (see Appendix A, figure 8A). A significant difference in cell growth was also found between group 2 and 4 (p=0.001) (see Appendix A, group 8A). A significant difference in cell growth scores on the posterior capsule of the lens was found in the CsA treatment group only. Specifically, capsules exposed to CsA for seven days had significantly lower cell growth scores than capsules exposed to CsA for any other exposure times. Capsules exposed to CsA for six days also had significantly lower cell growth scores than capsules exposed to CsA for four days.

*Transmission Electron Microscopy*

TEM analysis of cells is the most sensitive method to identify cells undergoing autophagy (Eskelinen, 2005). Autophagy occurs when an environmental signal causes a flat membrane cistern, derived either from the endoplasmic reticulum or mitochondria, to engulf organelles and/or cytoplasm, forming a double-membrane bound compartment, known as an autophagosome (Eskelinen, 2008). Figure 3 depicts two different cells viewed through TEM. Figure 3A represents a LEC that was cultured for 24 hours in vehicle control. Figure 3B represents a cell undergoing apoptosis. Clearly, the
morbidity of these two cells is very different. The cell in 3B has many small blebs of cytoplasmic material breaking off of the main body of the cell. The nucleus (black dot) is very condensed and small. The LEC in 3A has a defined cell membrane with a large nucleus that contains four well defined nucleoli within its nuclear membrane. The cytoplasm and organelles within are also intact.

**Figure 3.** TEM comparison of morphologic change in LEC death. (A) represents a LEC in the vehicle control treatment following 24 hours of exposure. (B) represents a LEC undergoing apoptosis; note the apoptotic bodies and pyknotic nuclei.

Figure 4 represents TEM of LEC exposed to 5 µg/mL CsA for 24 hours. Figure 4A demonstrates the gross morphology of the treated LEC. Similar to the vehicle control (Figure 3A) the cell membrane is well defined; however, small vacuoles can be detected within the cytoplasm of the CsA-treated cell. The nucleus of the CsA-treated LEC has a defined membrane, and three dark, distinct nucleoli can be seen within the nucleus. Higher magnification of the CsA-treated LEC (Figure 4B) reveals that the many vacuoles are filled with cellular components and organelles, such as mitochondria. With increasing
magnification (Figure 4C), a more detailed view of a vacuole can be observed in a CsA-treated LEC. The double black arrows identify a double membrane for the vacuole, a characteristic structural component of an autophagosome. The double membrane extends around the entire circumference of the vacuole.

Figure 4. TEM of LEC exposed to 5 μg/mL CsA for 24 hours. As the magnification progressively increases (A – C) morphologic changes consistent with autophagy can be observed. (A) The cellular membrane remains intact and normal in appearance; no overt changes in the nucleus can be noted. The cytoplasm is filled with vacuoles. Closer examination of the vacuoles reveals that the vacuoles have sequestered cytoplasmic contents (B) and consist of a double membrane structure (black arrows; C).
Figure 5 represents TEM of LEC exposed to 10 µg/mL CsA for 24 hours. Figure 5A depicts the gross morphology of the LEC cytoplasm where large vacuoles can be seen (light gray circles). These vacuoles are more numerous and are larger than those apparent in LEC treated with 5 µg/mL CsA (Figure 4). Higher magnification in Figure 5B reveals a double membrane structure (double black arrows). Again, this autophagosome is filled with cytoplasmic components and organelles.

LC3 Expression in CsA-treated LEC

Microtubule–associated protein light chain 3 (LC3) is a known major regulator of autophagy (Mehta, 2014). Two forms of LC3 exist within cells (Jiang, 2010). The first is LC3-I, which exists in an insoluble form in the cytoplasm of cells (Jiang, 2010). All cells have basal expression of LC3-I in their cytoplasm (Avagliano, 2013). During the initiation of autophagy, LC3-I is lipiddated to become the soluble form of LC3-II (Cherra,
LC3 is a well-known molecular marker for autophagosomes, and can be found in mature autophagosome membranes as LC3-II (Avagliano, 2013). In Figure 6, expression of LC3-I and LC3-II in CsA-treated LEC was first evaluated using western blot analysis; the upper band is LC3-I and the lower band is LC3-II. Lane E contains LC3 proteins extracted from LEC exposed to the vehicle only. It is clear that only a small amount of normal background levels of LC3-I and trace amounts of LC3-II are expressed in these cells.

Lane B (Figure 6) represents LEC treated with 10 µg/ml CsA while Lane E represents LEC treated with 5 µg/mL CsA. Robust expression of LC3-I and LC3-II can be seen in cells treated with CsA, indicating an upregulation of autophagy. Additionally, the response appears to be dose-dependent; more robust expression of LC3-II can be seen by the thicker band in the LEC exposed to 10 µg/mL CsA when compared to LEC exposed to 5 µg/mL CsA.

3-Methyladenine (3-MA) is a known inhibitor of autophagy. 3-MA inhibits autophagy by blocking autophagosome formation via the inhibition of type III phosphatidylinositol 3-kinases (PI-3K). In Figure 6, LEC were treated with 10 µg/mL CsA plus 3-MA (Lane A) or with 5 µg/mL CsA plus 3-MA (Lane C). The expression of LC3 was reduced in both treatments compared to the cells treated with CsA alone. Additionally, LC3-II expression was decreased the most (as compared to LC3-I expression) when 3-MA was applied to the cells.
LC3 is frequently used to identify autophagosomes with immunofluorescence staining, where LC3-II appears as distinct, punctate areas of bright fluorescence when autophagosomes are present (Avagliano, 2013). Figure 7 represents two photomicrographs of LC3 immunofluorescence in LEC. Nuclei (blue) are stained with DAPI. Autophagosomes (green) indicate positive LC3 staining. The photomicrograph in Figure 7A depicts immunofluorescence for LC3 in the vehicle-treated group. Only a small amount of diffuse cytoplasmic LC3 background can be detected. By comparison, the photomicrograph in Figure 7B depicts positive punctate immunofluorescence for LC3 in the LEC treated with 10 µg/mL CsA. It is clear that LEC treated with CsA have increased lipidation of LC3. The classic punctate green fluorescence pattern characteristic of cells undergoing autophagy is evident in figure 7B.
Figure 7. LC3 immunofluorescence in LEC. (A) negligible LC3 expression is detected in the vehicle-treated LEC. By comparison, (B) treatment with 10 µg/mL CsA increases LC3 expression; the punctate localization observed is indicative of LC3-II associated with autophagosomes. Scale bar: 50 µm.

Acridine Orange Immunofluorescence

Acridine orange, or N,N,N′,N′-tetramethylacridine-3,6-diamine, is an organic compound that is used to assist in identification of cells undergoing autophagy (Bectin, Dickinson and Company, 2004). During autophagy, the autophagosomes fuse with lysosomes, and the digestive enzymes within the lysosome cause a decrease in the pH of the autophagolysosome. When acridine orange is exposed to acidic pH levels, it becomes protonated and sequestered in the compartment (Bectin, Dickinson and Company, 2004). On immunofluorescence, the dye will emit orange light when excited by blue light wavelengths (Bectin, Dickinson and Company, 2004). Therefore, autophagic vacuoles will emit orange light wavelengths if they are present in the presence of acridine orange.

Figure 8 depicts representative photomicrographs of acridine orange immunofluorescence in LEC. In Figure 8A, the vehicle-treated LEC has only trace
background of acridine orange fluorescence. When LEC are treated with 5 µg/mL CsA (Figure 8B), distinct areas of bright acridine orange fluorescence can be seen in these LEC, indicating that these CsA treated LEC contain acidic vacuoles. Treatment with 10 µg/mL CsA (Figure 8C) results in even more robust acridine orange fluorescence compared to LEC treated with 5 µg/mL CsA and controls. Such a large accumulation of acidic vacuoles supports the hypothesis that CsA-treated LEC are undergoing autophagy. Finally, in Figure 8D, when LEC are treated with 10 µg/mL CsA plus 3-MA, an autophagy inhibitor, there is a clear decrease in the fluorescence of the acridine orange in these cells. Again, this cumulative data is indicative that in the presence of 3-MA, CsA is not able to induce cellular changes associated with autophagy.
Figure 8. Acridine orange immunofluorescence in LEC. Nuclei (blue) are stained with DAPI and acidic vacuoles (orange) indicate positive acridine orange staining. LEC that are treated with (A) vehicle, (B) 5 µg/mL CsA, or (C) 10 µg/mL CsA demonstrate progressively increasing amounts of acridine orange staining. (D) treatment with 10 µg/mL CsA + 3-MA resulted in decreased acridine orange immunofluorescence. Scale bar: 50 µm.

*F-actin Immunofluorescence*

In the early stages of autophagy, the cytoskeleton of the cell is remodeled to facilitate formation of autophagosomes (Aguilera, 2012). It has been found that the cytoskeletal protein, actin, plays an important role in the initial formation of the autophagosome (Aguilera, 2012). The purpose of this experiment was to utilize
fluorescence microscopy to determine if differences in actin expression could be detected in LEC treated with CsA compared to those not exposed to CsA.

Figure 9 depicts F-actin immunofluorescence in LEC where the F-actin cytoskeleton appears bright green with immunofluorescence and the nuclei are stained blue with DAPI. When comparing vehicle-treated LEC (Figure 9A) to LEC treated with 10 µg/mL CsA for 24 hours (Figure 9B), no differences in F-actin expression or arrangement can be observed. Additionally, when LEC are treated with either 3-MA alone (Figure 9C) or 10 µg/mL CsA plus 3-MA for 24 hours (Figure 9D), no differences in F-actin can be found.

**Figure 9.** F-actin immunofluorescence in LEC. Nuclei (blue) are stained with DAPI and F-actin appears green. No differences in the immunofluorescence for F-actin in (A) the vehicle-treated, (B) 10 µg/mL CsA-treated, (C) 3-MA-treated, or (D) 10 µg/mL CsA + 3-MA-treated LEC can be found. Scale bar: 25 µm.
Chapter 4: Discussion and Conclusions

PCO is a persistent complication following cataract surgery. With cataract surgery being the most common surgery performed in our world today, costs and vision loss due to any complications are staggering (McElroy, 2014). With this in mind, finding methods to decrease and one day eliminate PCO is not only worthwhile, but imperative in continuing to provide patients with the care they need to live at their highest visual potential. Therapeutic means of reducing PCO formation have shown promise in recent research. Our lab has found success in utilizing CsA to prevent the formation of PCO following \textit{ex vivo} cataract surgery in cadaveric canine lens capsules. The aim of this research was to continue to unfold the mechanism whereby the LEC are dying following CsA exposure.

The first aim of this research was to determine the amount of time of CsA exposure needed to prevent PCO formation in cadaveric canine lenses following \textit{ex vivo} cataract surgery. It was previously determined in our lab that a concentration of 10 $\mu$g/mL CsA is needed to prevent PCO formation following \textit{ex vivo} cataract surgery (Lutz, 2013). Thus, while this research established the minimum concentration of drug needed to prevent LEC growth on the posterior capsule of the lens, it was still necessary to elucidate the minimum time needed to expose the cells to the drug. For clinical relevance, it is vital that the cells be exposed to the CsA for the least amount of time possible while still achieving PCO prevention. While CsA has been found to be non-toxic to corneal
endothelial cells in *ex vivo* experiments done in our lab, it is still unknown how CsA will affect the viability of intraocular cells *in vivo*. Therefore, the least amount of time needed to expose LEC to CsA to achieve PCO prevention is imperative information in moving forward to incorporate therapeutic prevention of PCO into routine cataract surgeries.

The experiments outlined above reveal that growth of LEC onto the posterior capsule of the lens is increasingly inhibited by longer exposure times to CsA. When cells were exposed to 10 µg/mL CsA for 4, 5, 6, or 7 days and then switched into unsupplemented DMEM for a total of 28 days, the only group that had a complete prevention of PCO formation was the 7 day treatment group. Cell growth was reduced in the 5 and 6 day treatment groups but not inhibited. Clearly, a minimum of 7 days of treatment with 10 µg/mL CsA is needed in order to achieve the goal of PCO prevention on day 28 of cell culture. While decreasing the growth of LEC may be helpful in delaying the course of PCO formation, any LEC that remain on the posterior capsule of the lens following cataract surgery have the potential to grow, replicate, and transform into vision-robbing PCO. Therefore, the goal of therapeutic treatment of PCO needs to be PCO prevention, not reduction.

With the minimum concentration of CsA and amount of exposure time needed to prevent PCO established, it is only natural to consider means of an intraocular drug delivery system. Our lab has evaluated soaking IOLs in 10 µg/mL CsA to determine how long the IOL retains the CsA in culture medium. Experiments have shown that the IOL only retains CsA for 24 hours, clearly not enough time to prevent PCO formation. Another method would be to inject CsA around the IOL during cataract surgery. Unfortunately, due to the dynamics of the aqueous humor circulation, it is unlikely that
the drug would remain at the posterior capsule of the lens for a period of 7 days at a concentration high enough to prevent PCO.

Our lab is also considering the use of thermosensitive biodegradable gels to deliver CsA to the posterior capsule of the lens. Recent biomedical research shows potential of utilizing poly(ethylene glycol) (PEG) based copolymers as biomedical materials for drug delivery, because of its biocompatibility, biodegradability, and thermosensitivity (Alexander, 2014). These gels are composed of an aqueous polymer that exists in a liquid state at room temperature (Gong, 2013). The gel solidifies through hydrogen bonding into a hydrogel material at body temperatures (Gong, 2013). This allows the gel to remain within the lens capsule. This gel is biologically inert and has the ability to degrade over time (Alexander, 2014). Additionally, the thermogel has been found to work well with hydrophobic drug delivery (Alexander, 2014), a characteristic that may make release of CsA, a hydrophobic drug, far more feasible. Preliminary experiments have shown that after 7 days, there is sufficient CsA remaining in the capsule to prevent PCO formation.

From a mechanistic standpoint, it is necessary to understand what is happening to the LEC when exposed to CsA. Based on previously performed viability data, it is known that the CsA induces LEC death; however, the mechanism by which this occurs was previously unknown. As such, another important focus of this research was to determine the mechanism whereby CsA induces cell death, thereby preventing PCO formation. A better understanding of how CsA affects the physiology of the LEC allows for a better understanding of the inherent behavior of these cells, and increases our knowledge of the pathological processes within the lens. Because cataract formation is the number one
cause of blindness throughout the world, studying the behavior of the cells that make up the lens may lead to increased knowledge of how to prevent pathological changes within the lens, including cataract formation.

In order to determine the mechanism responsible for LEC death, we evaluated the main pathways of cell death: apoptosis, necrosis, and autophagy. While autophagy is not a type of “cell death”, the process has been found to be upregulated in several disease states (Choi, 2013). Therefore, autophagy may have a dual role, one as a pro-death component, and another as a protective mechanism to preserve and recycle cellular components in times of stress (Pallet, 2009). Apoptosis, or programmed and organized cell death, is a normal process of death in a cell. It is not always pathological and is often seen in normal growth (Kanduc, 2002). Previous experiments in our lab ruled out apoptosis as the main initiating mechanism of death in LEC exposed to CsA. Necrosis is when a cell dies due to pathological circumstances. The cell dies in an unordered fashion and causes damage to surrounding tissues (Kanduc, 2002). Again, previous experiments in our lab have also ruled out necrosis as the form of cell death displayed by the LEC exposed to CsA.

Results from several experiments in this research point to autophagy as the main pathway facilitating cell death in LEC exposed to CsA. The first supporting evidence of autophagy-mediated cell death comes from TEM analysis. TEM analysis is the most sensitive method of identifying cells undergoing autophagy because it allows visualization of the morphologic changes associated with autophagy (Eskelinen, 2005). During times of stress, a cell may undergo autophagy by utilizing their endoplasmic reticulum or mitochondria to engulf and recycle organelles in a double membrane bound
compartment (Eskelinen, 2008). In the present data, these double membrane compartments were visible in CsA-treated LEC via TEM. As observed in Figure 3, a LEC undergoing apoptosis (Figure 3B) looks very different morphologically from a control LEC (Figure 3A). Apoptotic cells are characterized by many small blebs of cytoplasmic material that break off of the main body of the cell, along with a small, condensed nucleus (Kanduc, 2002). The cells in Figures 4 and 5 have very different morphologic characteristics compared to an apoptotic LEC (Figure 3B). Clearly, CsA-treated LEC are not undergoing apoptosis. Rather, the internal morphological characteristics of these LEC are comprised of double membrane compartments that are filled with organelles and cytoplasmic contents. This data is highly supportive of cells undergoing autophagy. This autophagic response appears to be dose dependent as many more double membrane bound, vacuolated compartments can be found in the cells exposed to 10 \( \mu \)g/mL CsA as compared to the cells exposed to 5 \( \mu \)g/mL CsA. Again, it seems that a higher concentration of CsA is needed to induce enough of a cell death response to lead to PCO prevention, and this dose response is evident using TEM.

The double membrane structure of the autophagosomes can be derived from different organelles. They can arise from the mitochondria, which is a double membrane bound organelle, or from the ER (Eskelinen, 2008). When we search the TEM photomicrographs, it is difficult to find evidence of intact ER, while many intact mitochondria are present in the pictures (data not shown). This may provide a clue that the ER is the source of the double membrane bound autophagosomes. The ER is a main contributor to protein synthesis in the cell. When a cell is undergoing stress, it stops producing proteins. The ER has an “unfolded protein response” pathway that is activated
during times of stress (Yoon, 2009). Both Pallet et al. (2009) and Ceichomska et al. (2013) have shown that CsA induces autophagy through the ER unfolded protein response in renal tubular cells and malignant glioma cells, respectively. It is possible that, due to the absence of ER in our TEM photomicrographs, CsA may be inducing the LEC to undergo autophagy through the unfolded protein response in the ER. Current studies in our lab are being conducted to fully elucidate the pathway whereby autophagy is occurring in the LEC.

Several other experiments were done to determine if the LEC were undergoing autophagy, the first being western blot analysis of LC3-I and LC3-II in CsA-treated LEC. LC3-II was upregulated in LEC exposed to 10 µg/mL CsA, compared to both the vehicle control and LEC treated with 5 µg/mL CsA. LC3 expression was reduced in LEC exposed to the autophagy inhibitor, 3-MA. These results indicate that both LC3-I and LC3-II levels are upregulated in LEC exposed to CsA as compared to the vehicle, where only a small amount of basal LC3 expression is seen. Again, the amount of LC3 expression appears to depend on the dosage of CsA to which the LEC are exposed. A darker band of LC3 expression is seen in cells exposed to 10 µg/mL CsA versus 5 µg/mL CsA. The dosage of CsA continues to be important in the level of cell death seen in the LEC exposed to CsA.

During autophagy initiation, LC3-I is lipidated to become LC3-II (Cherra, 2010). LC3-II expression represents the presence of mature autophagosomes (Avagliano, 2013). Therefore, it appears that more cells are in the mature, autophagosome state than the initiation phase. This leads to the conclusion that the LEC are completing the process of autophagosome formation before they enter into the cell death cycle. The western blot
analysis provides further evidence that the cells are not only initiating the process of autophagy, but that they are also completing the process of autophagosome formation.

Immunofluorescence of several autophagy markers was evaluated in this paper. The first was LC3 immunofluorescence. LC3 is a known molecular marker for autophagy, and appears as bright, punctate areas of green fluorescence in the presence of autophagosomes (Avagliano, 2013). Figure 7 represents LC3 immunofluorescence in LEC. CsA-treated LEC display robust expression of LC3 as compared to the vehicle treated LEC. The punctate areas of fluorescence appear to coalesce near the cell membrane and are also dispersed throughout the cytoplasm. This association of the autophagosomes with the cell membrane is a known characteristic of autophagy (Aguilera, 2012). These photomicrographs provide further evidence that the LEC are indeed undergoing autophagy when exposed to CsA. The expression of LC3 in these CsA-treated cells is robust and undeniably present.

F-actin immunofluorescence was also performed in the LEC exposed to CsA. It has been found that the cytoskeletal protein, actin, plays an important role in initial autophagosome formation (Aguilera, 2012). This experiment looked at F-actin expression in LEC exposed to a vehicle, 10 µg/mL CsA, 10 µg/mL CsA plus 3-MA, or solely 3-MA for a total of 24 hours. The expression of F-actin did not appear to differ in any of the four treatment groups. This suggests that F-actin expression was not upregulated and F-actin did not undergo remodeling compared to baseline observed in the vehicle-treated LEC. It is important to note that F-actin upregulation or rearrangement is most commonly seen in the initial stages of autophagy. We presume that most of the LEC in our experiments (after 24 hours of treatment) seem to contain autophagosomes in the middle
to later stages of autophagy. It may be worthwhile to look at F-actin expression after a shorter treatment period (less than 24 hours). Also, it may be useful to look at changes in other cytoskeleton components, such as microtubules.

It seems that CsA causes environmental stress to the LEC. These cells may attempt to conserve themselves and enter into the autophagy pathway. The LEC do not simply “surrender” to the CsA and undergo necrosis. This suggests that the CsA is not directly toxic to the cells, but rather, acts through a more sophisticated pathway, maintaining some form of homeostasis before the LEC die. This programmed form of cell death provides a more conservative means of ridding the posterior capsule of LEC. The autophagy pathway is tightly linked with the apoptotic pathway, and the two can facilitate programmed cell death (Pallet, 2009). I hypothesize that the ultimate pathway to cell death in the LEC is through apoptosis due to the close link between the autophagy and apoptosis pathways. Possibly, the LEC undergo apoptosis sometime after 24 hours of treatment with CsA. The disorganized nature of necrosis is undesirable as it causes damage to nearby tissues and can illicit an inflammatory response. If CsA induced a necrotic response within the eye, it may produce far too many complications for use of CsA for therapeutic means to prevent PCO. On the contrary, LEC exposed to CsA appear to undergo a more organized and conservative pathway to cell death, a more beneficial outcome when it comes to therapeutic prevention of PCO. Further understanding of this pathway, and also of the effects of CsA on surrounding ocular tissues, could lead to a viable intraocular treatment plan for the prevention of PCO.

The third and final immunofluorescence assessed in LEC was acridine orange staining. Acridine orange becomes protonated and fluoresces when exposed to low pH
environments (Bectin, Dickinson and Company, 2004). Although acridine orange in not autophagy specific, positive staining is supportive of our autophagy hypothesis. During late stage autophagy, the autophagosome fuses with the lysosome to form the autophagolysosome (Heymann, 2006). The lysosome releases its digestive enzymes into the autophagosome, which naturally causes a decrease in the pH inside of the structure (Heymann, 2009). Figure 9 clearly shows that acridine orange is increased in LEC exposed to both 5 µg/mL and 10 µg/mL CsA as compared to the vehicle; this appears to be dose dependent. This suggests that more cells are undergoing autophagy and many of these cells are in the later stages of autophagy, where the autophagosome fuses with the lysosome. The expression of acridine orange in the 10 µg/mL CsA plus 3-MA group appears similar to the levels expressed in the vehicle group suggesting that when autophagy is inhibited, the LEC express near baseline levels of acridine orange. This supports the conclusion that autophagy is indeed inhibited in these cells as there is possibly limited fusion between autophagosomes and lysosomes. Again, this evidence supports the conclusion that more cells are in the late stages of autophagy than the initial stages.

Cumulatively, the data suggests that LEC are undergoing autophagy when exposed to CsA. This is evident through the robust expression of LC3-II seen on western blot analysis and immunofluorescence, and through accumulation of acridine orange positive vesicles. Specifically, most of the LEC treated with 10 µg/mL CsA seem to be in a later stage of autophagy as opposed to the initiation stage. The LEC exposed to CsA do not upregulate or reorganize F-actin, which may be important in the initiation phase of autophagy. Through indirect evidence obtained during TEM analysis, the autophagosome
double membrane appears to be derived from the endoplasmic reticulum as opposed to the mitochondria. Also, the morphology of the LEC seen through TEM have a classic appearance of cells undergoing autophagy.

This data begins to establish the basic cellular mechanism whereby CsA induces lenticular cell death, supporting continued development of novel approaches to utilize CsA for the prevention of PCO. The autophagy pathway is highly organized and regulated, suggesting that induction of cell death via these means could lead to a safe and effective means of preventing PCO in vivo. We now know the minimum concentration of CsA and the exposure time needed to prevent the formation of PCO ex vivo. The next main step in this process is to find an effective means of intraocular drug delivery to the lens capsule. Thermosensitive biodegradable gels provide a possible option to deliver CsA to the lens capsule over an extended time period at the necessary concentration to halt the progression of LEC growth. It is encouraging that we have come this far in discovering the mechanism whereby CsA can prevent PCO formation. There is promise that an intraocular drug delivery system may one day eliminate the most common and costly complication of cataract surgery. This could provide a necessary and a cost-effective means of eliminating PCO throughout the world and decrease the incidence of PCO related-blindness.
Appendix A: Statistical Analysis of LEC Growth

Figure 10. Descriptives for growth of LEC following the various exposure times to control, vehicle, or 10µg/mL CsA. The outcome measure (Outcome28) is the grade (0-6) of the cell growth on day 28 of cell culture. The two independent comparison groups are the treatment groups (control, vehicle, or 10µg/mL CsA) and exposure times to the specific treatment (4, 5, 6, or 7 days).

<table>
<thead>
<tr>
<th>Descriptives</th>
<th>Statistic</th>
<th>Std. Error</th>
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<tbody>
<tr>
<td>Outcome28 Mean</td>
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<td>.21181</td>
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<tr>
<td>95% Confidence Interval for Mean</td>
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<td></td>
</tr>
<tr>
<td>Lower Bound</td>
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<td></td>
</tr>
<tr>
<td>Upper Bound</td>
<td>4.4354</td>
<td></td>
</tr>
<tr>
<td>5% Trimmed Mean</td>
<td>4.1259</td>
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</tr>
<tr>
<td>Median</td>
<td>4.0000</td>
<td></td>
</tr>
<tr>
<td>Variance</td>
<td>3.365</td>
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</tr>
<tr>
<td>Std. Deviation</td>
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</tr>
<tr>
<td>Minimum</td>
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<tr>
<td>Maximum</td>
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<td></td>
</tr>
<tr>
<td>Range</td>
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<td></td>
</tr>
<tr>
<td>Interquartile Range</td>
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<td></td>
</tr>
<tr>
<td>Skewness</td>
<td>-.870</td>
<td>.277</td>
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<tr>
<td>Kurtosis</td>
<td>-.121</td>
<td>.548</td>
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Figure 11. Data was normalized using the Shapiro-Wilk and Kolmogorov-Smirnov tests of normality.
Figure 12. Descriptives for growth of LEC following the various exposure times to control, vehicle, or 10µg/mL CsA for a two-way ANOVA. The outcome measure (Outcome28) is the grade (0-6) of the cell growth on day 28 of cell culture. The two independent comparison groups are the treatment groups and exposure times. Treatment 1 is the control. Treatment 2 is the vehicle. Treatment 3 is 10µg/mL CsA. Exposure 1 is 7 days. Exposure 2 is 6 days. Exposure 3 is 5 days. Exposure 4 is 4 days. Based on Levene’s Test of Equality of Error Variances, we can assume variances are equal (p=0.069).
Tests of Between-Subjects Effects

<table>
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<tr>
<th>Source</th>
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<th>F</th>
<th>Sig.</th>
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<td>Intercept</td>
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<td>1285.714</td>
<td>2094.828</td>
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<tr>
<td>Treatment</td>
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<tr>
<td>Exposure</td>
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<td>Treatment * Exposure</td>
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<td>Error</td>
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<td>.614</td>
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<tr>
<td>Total</td>
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<tr>
<td>Corrected Total</td>
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</table>

a. R Squared = .845 (Adjusted R Squared = .818)

**Figure 13.** Two-way ANOVA. There is a significant interaction between treatment group and exposure time (p<0.0001).

**ANOVA**

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>df</th>
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<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>Within Groups</td>
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<td>.508</td>
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<tr>
<td>Total</td>
<td>14.625</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 14.** One-way ANOVA for control group. Fail to reject the null hypothesis. There is no difference in cell growth based on exposure time for the control group (p=0.059).

**ANOVA**

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
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<td>3</td>
<td>1.444</td>
<td>1.635</td>
<td>.213</td>
</tr>
<tr>
<td>Within Groups</td>
<td>17.667</td>
<td>20</td>
<td>.883</td>
<td></td>
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</tr>
<tr>
<td>Total</td>
<td>22.000</td>
<td>23</td>
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</tr>
</tbody>
</table>

**Figure 15.** One-way ANOVA for vehicle group. Fail to reject the null hypothesis. There is no difference in cell growth based on exposure time for the vehicle group (p=0.213).
Figure 16. One-way ANOVA for CsA group. Reject the null hypothesis. There is a difference in cell growth based on exposure time for the CsA group. At least one group mean for the exposure times is different from the others (p<0.0001).

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
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<td>3</td>
<td>18.191</td>
<td>38.622</td>
<td>.000</td>
</tr>
<tr>
<td>Within Groups</td>
<td>10.833</td>
<td>23</td>
<td>.471</td>
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<tr>
<td>Total</td>
<td>65.407</td>
<td>26</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Figure 17. Post-Hoc: Tukey HSD for CsA group. There is a significant difference between exposure 1 and 2, 1 and 3, 1 and 4. (p<0.0001). There is a significant difference between exposure 2 and 4. (p=0.001). (Exposure 1 is 7 days, exposure 2 is 6 days, exposure 3 is 5 days, exposure 4 is 4 days).


Sigma 3-Methyladenine. Package Insert. Sigma-Aldrich, Inc.


