The Effect of Trypan Blue on Posterior Capsule Opacification in an *Ex Vivo* Canine Model

Master’s Thesis

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By

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Abstract

**Purpose.** To determine if trypan blue (TB) reduces lens epithelial (LEC) or corneal endothelial cell (CEC) viability *in vitro* or *ex vivo*. We hypothesize that: 1) TB will reduce posterior capsule opacification (PCO) formation *ex vivo* and *in vitro* by causing LEC death through apoptosis; 2) *In vitro* treatment with TB will not damage CEC.

**Methods.** Globes were harvested from canine cadavers within 1 hour of death. Cultured LECs were treated with TB at 0, 0.05, 0.1, 0.2, or 0.3% for 30, 60, or 120 seconds. Cell morphology was evaluated and a lactate dehydrogenase (LDH) assay was performed to determine cell viability. Cultured LECs were treated with 0 and 0.3% TB for 120 seconds and an apoptosis assay was performed to assess caspase-3 activity. To evaluate the effects of TB on *ex vivo* posterior capsule opacification (PCO), following mock cataract surgery, lens capsules were treated with 0 and 0.3% TB at the above times and maintained in culture for two weeks. Capsules were monitored for changes in cell density and morphology; histology was performed at experimental completion. Finally, CECs were treated with 0 and 0.3% TB for 120 seconds and an LDH viability assay was performed.

**Results.** Following acute exposure, TB did not significantly reduce LEC density at any of the concentrations tested. While TB-treated LECs demonstrate higher rates of cell death compared to vehicle control, the difference was not significant. Induction of apoptotic signaling was found in LEC cultures treated with TB for an extended period of
time. *Ex vivo* PCO formation was not significantly different in any treatment group. CECs treated with TB or vehicle showed no significant differences in cell death.

**Conclusions.** Trypan blue induced low levels of LEC death via apoptotic signaling cascades, but was not effective at reducing *ex vivo* PCO formation. Trypan blue did not induce CEC death at the concentrations and treatment times evaluated. Therefore, treatment with intraocular TB at the time of cataract surgery is safe to the CECs but will not prevent PCO formation. Funded by ACVO Vision for Animals Foundation grant (VAF2014-01). Trypan blue provided by Acrivet.
Dedicated to the people who have inspired, supported and loved me most: my mother, Barbara Brash; my father, Mark Brash; my fiancé, Matthew Hurm and my loving dog, Ruby Sue.

To my mother and father, who taught me from a young age what hard work and dedication are and encouraged me to find a career that made me smile everyday. With their support, I have been able to successfully achieve all my dreams.

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CHAPTER 1

INTRODUCTION

*The Lens: Normal Anatomy and Physiology*

The main function of the normal crystalline lens is to refract incoming light rays to a point source on the retina.\(^1\) In order to achieve this function the lens must be transparent, be in a stable and appropriate position within the eye and have a biconvex structure that changes during accommodation.\(^1\)\(^-\)\(^3\) The lens is located within the posterior chamber of the anterior segment of the eye and is suspended by numerous zonules that extend from the ciliary body to the equator of the lens.\(^2\) Contraction and relaxation of the ciliary body musculature alters tension on the zonules, changing the shape of lens, resulting in accommodation. Accommodation is a rapid change in the refractive power of the eye, which allows objects at different distances to be focused on the retina.\(^4\) Accommodation happens as a response to a blurred retinal image and occurs due to one or more mechanisms including changing the corneal curvature, changing the distance between the cornea and retina or changing the curvature or position of the lens within the eye.\(^4\) The lens is held in place by the tension of the zonular ligaments that suspend the lens circumferentially and by the gentle pressure applied by the anterior vitreous face,
where it rests in the patella fossa. The normal canine lens measures approximately 7 mm in axial thickness and 10 mm in diameter. It is responsible for approximately 15-19 diopters of refractive power inside the canine eye.

During embryogenesis, the lens is derived from surface ectoderm and is composed of two types of cells in the adult lens: the lens epithelial cells and the lens fiber cells. A capsule surrounds the lens, which is comprised of the basement membrane of the lens epithelium. Histologically, this membrane is periodic acid-Schiff (PAS) positive and consists primarily of type IV collagen. The thickness of the lens capsule varies by region and with age. The thinnest region of the adult lens capsule is the posterior capsule with an average thickness of 2-4 µm. The average thickness of the capsule at the equator is 8-12 µm, and the thickest region of the capsule is anterior with an average thickness of 50-70 µm. The lens capsule is secreted continuously throughout life by a monolayer of cuboidal lens epithelial cells (LECs) that line the anterior and equatorial surfaces of the lens capsule. The posterior lens epithelium forms the embryonic primary lens fibers; therefore, the posterior lens capsule is not lined by LECs. These features are the primary reason why the anterior lens capsule is thicker than the posterior lens capsule and why the anterior lens capsule continues to thicken with age.

In addition to synthesis of the lens capsule the LECs are responsible for creating all lens fibers through a process called terminal differentiation. This process involves fiber cell elongation, expression of large amounts of protein (crystallin), acquisition of specialized plasma membrane interdigitations, the loss of all intracellular organelles, and internalization. The majority of the lens consists of lens fibers formed continuously.
throughout life; the lens fibers are divided into two main sections: the inner nucleus and the outer cortex. The oldest portion of the lens is called the embryonic nucleus and it is found in the innermost region of the lens nucleus. The other regions of lens fibers encountered extending outwards from the embryonic nucleus include the fetal nucleus, adult nucleus, and cortex.\textsuperscript{2,3} Lens epithelial cells continuously undergo terminal differentiation throughout life, with the outermost cortical lens fibers being the newest fibers produced. As the lens continues to grow, these cortical fibers become internalized and will become incorporated into the adult nucleus.\textsuperscript{2,3}

Lens transparency is a result of the avascular nature of the lens, its state of relative dehydration, the lamellar arrangement of lens fibers, and the solubility of the lens proteins.\textsuperscript{2} Lens transparency is essential for visual acuity and lens clarity. Due to the avascular nature of the lens it must derive its metabolic needs primarily from the aqueous humor and to a lesser extent from the vitreous humor. The exchange of nutrients and wastes occurs across the semi-permeable lens capsule that surrounds the lens. The transparency of the cells and lens capsule is maintained as long as they are being properly nourished.\textsuperscript{2,3,6} The oxygen concentration within and around the lens is much lower than in other parts of the body. A small quantity of oxygen is required by the lens and is obtained from the aqueous humor. The lens, therefore, relies primarily on anaerobic glycolysis for most of its metabolic activity. The glucose required for the glycolytic metabolism is also obtained from the aqueous humor.\textsuperscript{3,6} The lens maintains a relatively dehydrated state consisting of approximately 60-75\% water and with age, the total water content of the lens decreases.\textsuperscript{2,3} Na-K-ATPase pumps, which are located in the anterior
epithelium, act to maintain this state of relative dehydration within the lens. These pumps maintain the Na+ and K+ gradient across the lens capsule by actively transporting K+ ions and amino acids into the lens and transporting Na+, Cl- ions out of the lens thus keeping water concentration low and aiding in maintaining the transparency of the lens.6

Transparency of the lens also depends on the highly ordered lens fiber arrangement. This regular and well-organized arrangement of the lens fibers develops during lens fiber maturation. During this process the lens fiber cells develop regions for unified cell attachment and become devoid of intracellular organelles.2,3 The lens contains approximately 35% protein, which is a higher proportion than any other tissue in the body.6 This high protein concentration within the lens fiber cells is essential for maintenance of the refractive index, which requires the lens to be denser than that of the surrounding aqueous media.6 There are two primary forms of lens proteins: soluble (crystalline) and insoluble (albuminoid). The soluble proteins comprise about 85% of the total lens proteins.2,3,6 This percentage decreases with age and varies with species and lens pathology. In the normal lens, insoluble protein levels are highest in the nuclear region and soluble protein levels are highest in the cortical region. When the lens becomes cataractous the concentration of insoluble lens proteins increases leading to decreased transparency.3,6

Cataract Formation and Treatment

A cataract is defined as any opacification of the lens or its capsule.7 Cataracts become clinically significant when there is sufficient loss of lens transparency to result in
impaired vision.\textsuperscript{1} Cataract formation is the most common intraocular lesion and is the leading cause of reversible vision loss in dogs and humans.\textsuperscript{1,7,8} Cataracts are classified according to etiology, age of onset, location of cataract within the lens, appearance of cataract, and stage of progression of cataract.\textsuperscript{1} There are many different etiologies for cataract formation including inherited, developmental, diabetes mellitus, hypocalcemia, hypercupremia, metabolic disorders, toxin exposure, nutritional deficiencies, lens injury, lens instability, age-related degenerative processes, uveitis, exposure to ultraviolet light, radiation, electric shock, and retinal degeneration.\textsuperscript{1} Fortunately, cataracts are surgically treatable, most commonly achieved by performing lens extraction via phacoemulsification followed by artificial intraocular lens (IOL) implantation to restore emmetropia. This procedure is the most common intraocular surgery performed in both canine and human patients.\textsuperscript{8-10} In general, cataract surgery carries a favorable outcome with short-term success rates exceeding 85-90\%.\textsuperscript{11,12} The rate of surgical success declines over time with an overall surgical success rate of 79\%.\textsuperscript{12} The most common complications of dogs undergoing phacoemulsification are uveitis, postoperative ocular hypertension, intraoperative hemorrhage, endophthalmitis, corneal disease, glaucoma, retinal detachment, and posterior capsule opacification.\textsuperscript{1,11-13}

\textit{Posterior Capsule Opacification and Clinical Significance}

Posterior capsule opacification (PCO) is the most common long-term postoperative complication in both human and canine patients.\textsuperscript{1-3,11,14} Posterior capsule opacification is also commonly referred to as secondary cataract, after cataract, capsular
It is a universal complication of modern cataract surgery that occurs in up to 50% of human surgical patients within 5 years postoperatively.\textsuperscript{2,15,17} Cataract surgery is currently the most common operative procedure in the world and is expected to reach 30 million surgeries per year by 2020.\textsuperscript{8,19} Cataract surgery initially provides excellent restoration of vision but is undermined by secondary loss of vision due to PCO which requires further surgical intervention to restore vision.\textsuperscript{8,19} This places a large burden on both the patient and the health care providers finances.\textsuperscript{19} The incidence of PCO increases in younger patients in the human literature and has a reported incidence of 100% in pediatric patients.\textsuperscript{2,9,18,20} In the veterinary literature, PCO has been reported to occur in 62-100\% of all patients, \textsuperscript{2,9,11} and age is not reported as a significant factor in canine patients.\textsuperscript{2,3,9} This discrepancy was explained by the fact that most dogs are physically mature by 2 years of age.\textsuperscript{2,3,9} Surgical technique and patient host factors have been found to influence PCO formation in humans and continue to be areas of research in both the human and veterinary ophthalmology field.\textsuperscript{2,3,9,21}

Posterior capsule opacification is a fibrotic condition that is a consequence of proliferation, migration, and transformation of residual LECs, normally present as a monolayer on the inside of the anterior lens capsule, onto the posterior lens capsule.\textsuperscript{9,22} The initial response of these residual LECs is to differentiate into fibroblast like cells via epithelial-to-mesenchymal transformation (EMT), resulting in fibrous plaque formation on the posterior lens capsule.\textsuperscript{2,3,8,9,14,15,23} PCO is divided into two distinct types: Fibrous type and pearl type.\textsuperscript{2,3,6,8,9,15} The fibrous type of PCO results from the anterior and some equatorial LECs that proliferate and form fibrous tissue by undergoing fibrous
metaplasia.\textsuperscript{3,6,15} The pearl type of PCO results from the formation of large, round, swollen epithelial cells that form clusters of differentiated equatorial cells are called “Bladder” or “Wedl” cells and clinically are observed as Elschnig’s pearls.\textsuperscript{7,15} It is likely that both types of LECs have the capability to contribute to both forms of PCO and most clinical PCO is composed of a combination of both the fibrous and pearl types.\textsuperscript{1,15}

Posterior capsule opacification formation is most commonly initiated at the time of cataract surgery and continues to progress during the postoperative period; however, a large majority of cataractous lenses have been shown to already display LEC migration onto the posterior lens capsule at the time of surgery as evidenced by pre-existing posterior and anterior capsular plaques.\textsuperscript{6,8,9,15,24} When PCO occurs postoperatively, it is initiated by a wound healing response secondary to tissue trauma induced by cataract surgery and combined with a foreign body reaction to the artificial lens implant.\textsuperscript{2,3,22,25,26} Surgeons remove most of the LECs during cataract surgery, however, due to surgical constraints, some of these cells remain in the capsular bag post operatively.\textsuperscript{2,3,15,16,22,26} The remaining lens epithelial cells, normally cuboidal in shape, first transform into spindle shaped myofibroblast-like cells.\textsuperscript{2,3,16,23} This process is called epithelial-to-mesenchymal transformation (EMT) and results in transdifferentiation of LECs into mesenchymal cells.\textsuperscript{15,19,22} Mesenchymal cells have migratory and invasive properties that result in migration of these cells onto the posterior lens capsule where they proliferate and form plaques.\textsuperscript{2,3,15} Residual LECs also undergo other cellular morphological alterations such as cell elongation, organelle loss, nucleolar chromatin condensation and aberrant basement membrane synthesis.\textsuperscript{2,3,8,15,27} Epithelial-to-mesenchymal
transformation can be triggered by any inflammatory response, such as lens induced uveitis or cellular trauma from cataract surgery. As mentioned previously, PCO formation occurs both pre and post operatively. Pre-operative PCO formation has most frequently been attributed to pre-existing lens induced uveitis, however PCO formation has been associated with cataract formation with or without lens-induced uveitis. Studies have been performed showing a causative relationship between lens-induced uveitis and PCO formation; PCO formation increases when uveitis is present and PCO may form in association with uveitis in both the pre- and postoperative time periods. The exact mechanism of PCO formation remains unknown, but most likely involves a multifactorial pathogenesis that involves both pre- and post surgical factors.

Clinically, PCO results in progressive opacification of the central visual axis due to the presence of fibrotic cells, which lead to a significant reduction in visual acuity by resulting in capsule plaques, image distortion, capsular haze, and light scattering. Visual acuity in adult humans is often decreased 20-40% of normal within 2-5 years post cataract surgery due to PCO formation. While there are no veterinary studies evaluating the effect of PCO on visual acuity, extrapolation from these human data would suggest a substantial decrease in visual acuity in the canine population. When transformed LECs continue to proliferate, they also result in secondary complications due to the mechanical forces exerted by the fibrotic cells. This can result in striae or folds of the posterior capsule and potentially, traction induced decentration of the IOL. Other consequences of PCO include difficulty in visualizing the peripheral fundus during
examination and localized endophthalmitis also termed lens induced uveitis due to
retained lens cortex and cortical proliferation.\textsuperscript{2,3,15}

\textit{Treatment and Prevention of Posterior Capsule Opacification}

Many factors during the cataract extraction process have been found to influence
PCO severity including capsulorhexis technique, use of hydrodissection, composition of
the irrigating solution, composition of viscoelastic material, use of capsule polishing, and
type of IOL implant.\textsuperscript{1,7-10,15,27,32-35} Numerous pharmacologic agents have also been tested
and have been shown to inhibit \textit{in vitro} PCO formation by causing LEC death. These
drugs were studied using a variety of techniques including delivery directly into the
anterior chamber at the time of cataract surgery, placement in the intraocular irrigating
fluids during surgery, or drug impregnation of the IOL or capsular tension ring
device.\textsuperscript{1,21,24,27,36-40} However, when many of these drugs are used \textit{in vivo} the
concentrations needed to inhibit LEC proliferation and migration had significant toxic
effects on the corneal endothelial cells, iris, ciliary body epithelial cells, and retina.\textsuperscript{1,21,39}

In human ophthalmology, Nd:YAG laser posterior capsulotomy is the most
commonly used technique for treatment of PCO.\textsuperscript{8-10,15,18,21} Unfortunately, this procedure
is expensive and not without risk. Potential Nd:YAG complications include damage to
the IOL, intraocular pressure elevation, cystoid macular edema, retinal detachment, IOL
luxation, and endophthalmitis.\textsuperscript{11,12,15,18} Nd:YAG laser capsulotomy was investigated in
normal canine cadaver eyes and a therapeutic margin between energy that reliably
achieved posterior capsulotomies with minimal damage to the IOL was found.\textsuperscript{12,41}
Clinical canine patients with PCO were not studied. It was suggested that patients with thickened and wrinkled capsules may pose a problem since these changes would likely reduce the therapeutic effect of the laser procedure as the energy needed to disrupt the posterior capsule would likely damage the IOL.\textsuperscript{14}

In canine patients, there is no current proven therapy for prevention of PCO. Therefore, understanding the factors leading to PCO formation and developing methods for delay and prevention of PCO are of great clinical importance.

\textit{Trypan Blue}

Trypan blue is a vital dye that has been used extensively in both research and clinical ophthalmology settings. Dyes are chemical compounds that bind to various substances to induce a color change. When dyes are used to color living tissue or cells they are referred to as a vital dye.\textsuperscript{42,43} Trypan blue belongs to the azo group of dyes, which is a large class of synthetic organic dyes that are formed when a diazonium ion reacts with either a phenol or an amine. The azo bond allows visible light to be absorbed by the dyes and results in high intensity brilliant staining.\textsuperscript{42} Trypan blue is a large, anionic, hydrophilic dye with the formula $C_{34}H_{24}N_6Na_4O_{14}S_4$ and a molecular weight of 960.79 Daltons.\textsuperscript{42,43} This dye has traditionally been used in research settings to identify dead or damaged cells. Trypan blue will stain the nuclei of damaged or dead cells, while live cells and tissues with intact cell membranes usually are not colored because their selective control of cellular membrane transport does not allow trypan blue to bind.\textsuperscript{43} In ophthalmology, trypan blue was initially used by eye donor banks to help identify dead or
damaged endothelial cells in donor corneas. One study found that the use of 0.3% trypan blue for 1 minute followed by 0.2% alizarin red S for 1 minute was most effective in assessing cell viability of donor cornea endothelial cells. Trypan blue has also been widely used in cataract surgery, vitrectomy surgery, and more recently in epiretinal membrane removal surgery. Trypan blue is commercially available in a 0.15% concentration solution for vitreoretinal surgery and in a 0.06% concentration for cataract surgery. These solutions typically contain small amounts of sodium salts, 8.2 mg of NaCl and water.

**Trypan Blue’s Use in Cataract Surgery**

Trypan blue has been the most frequently used agent to stain intraocular structures in cataract and anterior segment surgeries. The use of trypan blue dates back to the 1970’s when it was used to stain the corneal endothelium preoperatively. In the late 1990’s it became popular for its use intraoperatively to aid in visualization of the anterior lens capsule. Phacoemulsification has become the standard surgical procedure for cataract extraction in both human and veterinary medicine. This surgical technique requires the creation of a continuous curvilinear capsulorhexis (CCC). The visibility needed to create an ideal capsulorhexis is partially dependent on a red reflex in human cataract surgery or tapetal reflex in veterinary cataract surgery. The view of the anterior lens capsule can become significantly compromised in eyes with a white or dense cataract or a corneal opacity. If there is poor visualization of the capsulorhexis the rate of surgical complications increases. Melles et al. first described the use of trypan
blue, in cataract surgery, to stain the anterior lens capsule to allow better visualization of the capsulorhexis when the red reflex is absent.\textsuperscript{47} In that study, 0.1% trypan blue was used successfully in all cases to achieve homogenous staining of the anterior lens capsule and enhanced visibility of the tearing edge to allow a safe capsulorhexis to be performed.\textsuperscript{47} In a study by Chang et al., trypan blue at 0.1% was found to have the most satisfactory staining effect when compared to other vital dyes and other concentrations of trypan blue.\textsuperscript{49} Trypan blue was found to be superior compared to other dyes because it selectively stains the basement membrane of the anterior lens capsule, is easily washed from the anterior chamber, and does not cause permanent discoloration of ocular tissues.\textsuperscript{50}

Due to the popularity of trypan blue use in cataract surgery, studies were performed to evaluate its effect on the ultrastructure of the anterior lens capsule as well as the effect on the biomechanical properties of the capsule. Rangaraj et al. evaluated whether 0.1% trypan blue alters the surface integrity of the anterior lens capsule.\textsuperscript{51} In this study, electron microscopy was used to compare the surface ultrastructure of anterior lens capsules treated with or without 0.1% trypan blue. Anterior lens capsules harvested from human eyes after routine capsulorhexis and treated with 0.1% trypan blue exhibited identical features to untreated capsules. A normal capsule lined with epithelial cells with normal nuclei and cytoplasmic organelles was visualized. The only difference detected in the trypan blue treated capsules was the appearance of the external surface of the capsule, which looked focally ragged and irregular.\textsuperscript{51}
Histologic studies have also been performed to evaluate trypan blue treated anterior lens capsules. A study by Singh et al. evaluated the layers of the lens capsule that stained with the trypan blue and the extent of dye accumulation in these layers. This report found dye retention mostly in the portion of the lens capsule adjacent to the LEC layer with minimal laminar staining in the surrounding superficial lens capsule. This feature allows the surgeon to easily distinguish the lens capsule from the surrounding lens cortex to allow for safe and easy completion of the capsulorhexis.

The biomechanical changes in the anterior lens capsule after trypan blue staining were first evaluated using porcine cadaver eyes. In general, application of trypan blue to the anterior lens capsule followed by at least 1 minute of light irradiation increased the elastic stiffness and reduced the extensibility of the capsule. This effect was hypothesized to be due to the role of trypan blue as a photosensitizer which resulted in capsule collagen crosslinking. Trypan blue, as previously stated, was found to accumulate mainly in the area adjacent to the epithelial cell layer at the level of the anterior lens capsule basement membrane. The basement membrane of the lens capsule is primarily composed of type IV collagen, where the photo-oxidative effects on anterior capsule collagen crosslinking would primarily take place. Other studies confirmed these same findings that trypan blue significantly decreased the elasticity and increased the stiffness of the capsule. This effect was found to be more pronounced in diabetic patients. The influence of decreased elasticity on the CCC resistance to tearing was evaluated in a study by Jaber et al. In this study, no significant difference was found in CCC strength between the trypan blue stained capsules and control capsules. Thus,
although trypan blue may reduce the elasticity of the anterior capsule, there was no associated decreased resistance to tearing or difficulty with CCC performance.\textsuperscript{55}

The ability of trypan blue to reduce the density and viability of the LEC has also been studied. Nanavaty et al. evaluated the \textit{in vivo} effect of anterior capsule staining with 0.0125\% trypan blue on the density and viability of LECs.\textsuperscript{56} This study found a significant decrease in LEC density covering the anterior lens capsule in eyes treated with trypan blue; overall LEC viability was considerably reduced.\textsuperscript{56} Further studies using transmission electron microscopy compared trypan blue treated LECs to untreated controls. Two distinct epithelial cell populations were observed in both groups, one population contained a more electron dense cytoplasm with more mitochondrial profiles present. In the trypan blue treated cells, a noticeable subcellular change was present in both LEC populations, this change consisted of increased electron density in the cytoplasm of the electron-dense cell type and disrupted endoplasmic reticulum in both cell types.\textsuperscript{57} This study indicated that LEC death may occur subsequent to TB treatment.\textsuperscript{57} This was further investigated in a subsequent study by Portes et al. evaluating LEC morphology and cell death signaling pathways in trypan blue treated capsules.\textsuperscript{58} Trypan blue-induced cell toxicity was present in all treated LECs, which was observed as a noticeable change in cell morphology and size.\textsuperscript{58} Two pathways of LEC death were noted: apoptosis and autophagy.\textsuperscript{58} This observed decrease in LEC viability and density caused by trypan blue led to the investigation of trypan blue as an agent capable of reducing the incidence of PCO.\textsuperscript{56,59} In a clinical study by Sharma et al. 0.1 \% trypan blue was injected into the capsular bag just after cortical cleaving hydrolissection
and was allowed maximal contact with equatorial cells.\textsuperscript{59} The PCO scores were significantly lower in trypan blue treated eyes at 6 and 12 months post operatively.\textsuperscript{59} Collectively, these studies highlight the potential use of trypan blue to reduce LEC viability and the incidence of PCO, warranting further investigation into the concentration and contact time of trypan blue necessary to achieve maximal PCO reduction and to explore the mechanism by which cell death occurs.

\textit{Trypan Blue’s Safety}

Since the original description of using trypan blue to assist with capsulorhexis in cataract surgery, the dye has been used extensively with few reports of complications.\textsuperscript{45} However, the toxicity of trypan blue is well documented throughout the literature. Trypan blue is a known carcinogen and teratogen.\textsuperscript{60} In early experiments with trypan blue in 1977, unpurified dye was injected intraperitoneally in rats. Doses around 100 mg/kg were found to be teratogenic and doses around 300 mg/kg were found to be oncogenic.\textsuperscript{61} Complications such as these are rarely encountered in intraocular surgery because trypan blue is injected locally into the anterior chamber at doses around 0.005 mg/kg and the dye is only present for a short period of time before it is rinsed out of the eye.\textsuperscript{62} Trypan blue has also been reported to be the inciting cause of toxic anterior segment syndrome in two patients that received 0.1 mL of 0.1\% generic trypan blue during routine cataract surgery.\textsuperscript{63} Both patients received dye from the same lot number and eventually the cause of the sterile inflammation was traced back to an unidentified impurity in the trypan blue solution.\textsuperscript{63} In cataract surgery, it is known that even after rinsing, some dye will be left
behind especially if it enters the posterior segment. Thus, studies have been performed to
evaluate any potential toxicity associated with trypan blue to the corneal endothelium, the
LEC, and the retina.\textsuperscript{1,43,45,56,58,62,64-70}

In general, the corneal endothelium is the ocular tissue of greatest concern when
injecting any substance intracameral.\textsuperscript{45,64} The corneal endothelium is a monolayer of
cells that have limited proliferative ability and any potential insult to this cell layer poses
a risk for permanent harmful effects such as bullous keratopathy.\textsuperscript{1,3,64} An early
uncontrolled case series where 0.1% trypan blue was injected into the anterior chamber
found no effect on endothelial cell counts during 8 years of follow up.\textsuperscript{46} Additional
studies have found that intraocular use of trypan blue at concentrations as high as 1% for
two minutes is non-toxic to human corneal endothelial cells.\textsuperscript{47,65} In a study by Chang et
al., concentrations of trypan blue between 0.01 and 0.4% caused no significant toxicity to
rabbit corneal endothelial cells.\textsuperscript{64} These studies indicate a wide therapeutic range for
trypan blue with little concern for endothelial cell toxicity.

As previously discussed, the effect of trypan blue on LECs has been evaluated
using different techniques in multiple studies. Although, one \textit{in vitro} study found no
effect on the viability of LECs at concentrations of trypan blue ranging from 0.0025\% to
0.5\%, other \textit{in vivo} studies and \textit{ex vivo} studies have shown a cytotoxic effect of trypan
blue on LECs.\textsuperscript{56-59,67}

Trypan blue has most recently been studied for its use as an aid in vitreoretinal
surgery for staining epiretinal membranes in proliferative vitreoretinopathy.\textsuperscript{71} As such,
the toxicity of trypan blue to the neural retina and retinal pigment epithelium has been
studied. Trypan blue, at a concentration of 0.06% with one minute of retinal contact time, has been found to be sufficient at staining the epiretinal membrane without staining the underlying neural retina. Concentrations of trypan blue between 0.06 and 0.2% have been used for intravitreal application and animal studies have revealed a low potential for retinal toxicity. In vitro studies have been performed evaluating the effect of trypan blue on cultured human retinal pigment epithelial cells and neurosensory retinal cells. These studies demonstrated that cultured retinal pigment epithelial cells as well as neurosensory retinal cells had no direct toxicity changes due to trypan blue application at concentrations as high as 0.3% for up to five minutes. In addition, no toxicity was found when the cells were exposed to trypan blue and light for 10 minutes. When the retinal pigment epithelial cells were incubated with 0.25% trypan blue for 30 minutes, toxicity was observed; however, when shorter incubation times were used these toxic effects were not observed. In an ex vivo study of porcine retinas it was found that exposure to 0.15% trypan blue for one minute did not cause any histological damage to retinal cells. In an in vivo study by Veekeneer et al., gas compression vitrectomy was performed in rabbits and 0.1 mL of balanced salt solution or trypan blue (either 0.06% or 0.2%) solution was injected into the posterior segment. The dye was left in the posterior segment and four weeks later eyes were enucleated. Light and electron microscopic examinations were performed and it was discovered that the inferior retina in all animals treated with 0.2% trypan blue had damage to the photoreceptor layer and marked disorganization of the retina. This effect was likely secondary to gravity and the effect of gas compression, which resulted in sequestration of TB in the inferior retinal cavity.
No histologic changes were observed in the dorsal retina of the 0.2% trypan blue group, in the control eyes, or in eyes treated with 0.06% trypan blue. These studies suggest that trypan blue is safe for vitreoretinal surgery and displays little risk to the retina when used at concentrations of 0.15% or less for short periods of time.\textsuperscript{43,45}

\textit{Purpose of the Study}

The purpose of the present study was to test the hypothesis that trypan blue will reduce canine PCO \textit{ex vivo} and \textit{in vitro} by causing LEC death, through apoptosis. The other specific aim of the current study was to evaluate if trypan blue causes damage to canine CECs.
CHAPTER 2

MATERIALS & METHODS

Tissue Sample Collection

All protocols were performed in accordance with the Association for Research in Vision and Ophthalmology 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 globes were harvested from grossly healthy dogs with normal eyes that were humanely euthanized at a local animal shelter for population control purposes. The age of the dogs included in this study was estimated using dentition and the degree of nuclear sclerosis. Globes were collected within one hour of death, were rinsed in a 1:50 dilute betadine solution, stored in sterile phosphate-buffered saline (1X PBS), and refrigerated prior to lens capsule dissection.

Trypan Blue Stock Solution

The trypan blue and control solutions used throughout this study were donated by Acrivet (Acrivet Blue, S&V Technologies AG /Acrivet® Hennigsdorf, Germany). Sterile trypan blue was used at concentrations of 0.0%, 0.05%, 0.1%, 0.2%, and 0.3%. The
trypan blue was mixed in physiological sodium chloride solution, buffered with phosphate buffer, and sterilized. All solutions were stored at room temperature and protected from freezing and from light.

Mock Cataract Surgery
Sham cataract surgery was performed on harvested globes, as previously described.\textsuperscript{27,32,72} Excess conjunctiva and Tenon’s capsule was excised from the perilimbal region, and a #10 scalpel blade was used to create a partial thickness scleral groove approximately 1 mm posterior to the limbus around the entire circumference of the eye. Using a #10 scalpel blade, a stab incision was made into the anterior chamber, and Stevens tenotomy scissors were used to excise the cornea from the remainder of the globe along the circumferential groove. Using Vannas scissors, the iris was incised at its base and removed exposing the anterior lens. An anterior axial capsulotomy was performed using Vannas scissors and Utrata forceps were used to complete a continuous curvilinear capsulorhexis. Cortical cleaving hydrodissection was performed, injecting 0.2 mL of sterile PBS through a 27-gauge cannula placed between the anterior lens capsule and the cortical fibers. The lens nucleus and cortex were gently removed using a lens loop. The capsular bag was filled with either 0.2 mL of vehicle control solution or 0.3\% trypan blue solution which was allowed to remain in the capsule for 0.5, 1, or 2 minutes (\textit{n}=36). The lens capsule was then rinsed with 10 mL of PBS. Finally, residual LECs and cortical material was removed via gentle manual irrigation and aspiration using 10 mL of PBS and a coaxial cannula; no effort was made to polish the posterior lens capsule. Capsules
were then removed from their zonular and vitreal attachments using Vannas scissors. Curved dressing forceps were used to carefully extract the lens capsule from the globe prior to placement in a sterile cell culture dish with 5 mL of serum-free Dulbecco’s minimum essential medium (DMEM, Gibco, Carlsbad, NY) supplemented with 1% antibiotic/antimycotic (Invitrogen Corporation, Grand Island, NY). The capsules were then placed in a 37°C / 5% carbon dioxide incubator. The serum-free DMEM was removed and replaced every 48 hours and the capsules were incubated for a total of 14 days.

Phase Contrast Microscopy and Capsule Scoring

Phase contrast microscopy and photomicrographs were used to monitor and document LEC morphology and migration within the capsule for 14 days. Capsules were scored every 48 to 72 hours using phase contrast microscopy and graded based on a set grading scheme (Table 1). The score for each capsule was based on three categories: percent area of entire capsule populated by LECs, morphologic indicators of epithelial-to-mesenchymal transformation (EMT) such as LEC elongation and pseudopodia, and migration of LECs onto the axial posterior capsule. Capsules were given scores using a scale from 0-6 as detailed in Table 1.
<table>
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<tr>
<th>Capsule Score</th>
<th>% Total area of capsule populated by LEC</th>
<th>Presence of morphologic indicators of EMT</th>
<th>Migration of LEC onto axial posterior capsule</th>
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</thead>
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<td>None</td>
<td>None</td>
</tr>
<tr>
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<td>None</td>
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</tr>
<tr>
<td>4</td>
<td>&lt;60%</td>
<td>Present</td>
<td>31-60% confluent</td>
</tr>
<tr>
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<td>&lt;95%</td>
<td>Present</td>
<td>61-95% confluent</td>
</tr>
<tr>
<td>6</td>
<td>&lt;100%</td>
<td>Present</td>
<td>&gt;95% confluent</td>
</tr>
</tbody>
</table>

**Table 1:** Description of Capsule Score Grading Scheme

**Histopathology**

After capsules were monitored and scored for 14 days, the lens capsules were fixed in 10% neutral buffered formalin for 24 hours, immersed in PBS, then embedded in paraffin. All capsules were submitted for histological processing, prepared on a slide in 5 µm sections, and routinely stained with hematoxylin and eosin. Light microscopy was used to manually count LECs on the anterior and posterior capsule from four different sections. The sections were made in the sagittal plane through the center of the capsule.
Canine Lens Epithelial Cell Culture

Anterior lens capsules were obtained from freshly harvested canine globes, as described above. The anterior lens capsules with adherent LECs were incubated in 0.5 mL of 0.25% trypsin and 1X EDTA (Gibco) at 37°C for 7-10 minutes. After incubation was complete, 1.0 mL of complete DMEM (cDMEM; 10% fetal bovine serum and 1% antibiotic/antimycotic solution [Gibco]) was added to the sample to stop the enzymatic activity of trypsin. The solution, including the lens capsule, was centrifuged at 1800 rpm for 5 minutes. The supernatant was aspirated and discarded with care as to not dislodge the capsules in the tube. The cells were then reconstituted in 1 mL of cDMEM and transferred, along with the capsules, into a 25 cm² laminin-coated culture flask (Beckton-Dickinson, Franklin Lakes, NJ). The total volume of cDMEM was brought to 5 mL by adding 4 mL of cDMEM. The cells were incubated in a humidified incubator at 37°C and 5% CO₂. Complete media was replaced every 2-3 days until the LEC reached 90% confluence as evaluated by phase contrast microscopy.

Lactate Dehydrogenase Cytotoxicity Assay

Cultured canine LECs were treated in a 96 well plate with 0.0, 0.05, 0.1, 0.2, or 0.3% trypan blue for 0.5, 1, or 2 minutes (n=20). Prior to treatment each cell culture well was washed with 100 µL of PBS. Following treatment with trypan blue each cell culture well was washed with 100 µL of PBS and fresh culture media (cDMEM) was placed on the cells. Treated cells were then incubated for 24 hours prior to evaluation of cell viability using a lactase dehydrogenase (LDH) cytotoxicity detection assay (Roche,
Indianapolis, Indiana). This is a colorimetric assay used for the quantification of cell death and cell lysis, based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant. The media that the treated cells were incubated in was removed from the sample wells, centrifuged at 2400 rpm for 10 minutes at 4°C, then transferred to a new tube and stored at 4°C. To perform the LDH cytotoxicity assay 100 μL of each test media was added to each well of an untreated 96-well plate. In addition to the trypan blue test samples, the following controls were used: 2% Triton X-100 solution was used as the positive LDH release control and untreated cell supernatant was used as a spontaneous LDH release control. Once all sample wells were filled, 100 μL of reaction mixture was added to each well with a multichannel micropipette. The plate was then incubated for 25 minutes at room temperature protected from light. The microplate was then read at 490 nm with a reference wavelength of 650 nm. Those test groups with the highest absorbance exhibited the greatest amount of cell death and cell lysis.

*Lens Epithelial Cell Morphology*

 Cultured canine LECs were treated with 0.0, 0.1, 0.2, and 0.3% trypan blue for 1 minute in a 12-well plate. Following treatment, LECs were evaluated using phase contrast microscopy and photomicrographs for morphologic changes within treated LECs.
Apoptosis Assay

Cultured canine LECs were treated in a 96-well plate with 100 uL of 0.0, 0.3% trypan blue or 3% H$_2$O$_2$ for 30 minutes (n=5). Prior to treatment each cell culture well was washed with 100 uL of PBS. Following treatment each cell culture well was again washed with 100 uL of PBS and fresh culture media (cDMEM) was placed on the cells. The cells were incubated in a humidified incubator at 37$^\circ$ C and 5% CO$_2$ for 30 minutes prior to evaluation of caspase 3/7 release using the Apo-ONE Homogenous Caspase 3/7 Assay (Promega, Madison, WI). This is a fluorescence assay used for the quantification of apoptosis activity based on the measurement of caspase 3/7 release from the cytosol of damaged cells into the supernatant. After the cells incubated for 30 minutes, 100 uL of each test media was added to each well of an untreated, black 96-well plate. Once all sample wells were filled, 100 uL of Apo-ONE Caspase 3/7 reagent was added to each well with a multichannel micropipette. The contents of the plate were gently mixed using a plate shake for 30 seconds. The microplate was then read at 499 nm with emission maximum at a wavelength of 521 nm for 18 hours. Those test groups with the highest fluorescence activity exhibited the greatest amount of apoptosis activity.

Canine Corneal Endothelial Cell Lactate Dehydrogenase Cytotoxicity Assay

Fresh canine corneas were harvested, as described above from canine cadavers within 1 hour of death. Freshly enucleated globes were stored in sterile 1x PBS for 24 hours or less. Extraocular tissues were removed from the globes and the cornea was sharply excised 2 mm posterior to the limbus. The cornea was placed epithelial-side
down in a sterile culture well. The endothelial cells were gently washed with 1 mL of 1x PBS. The PBS was then removed via gentle aspiration and replaced with 200 µL of 0.0, 0.3% trypan blue or 3% H2O2 for 120 seconds (n=9). The treatment was removed after 120 seconds and the corneal cup was submerged in 5 mL of keratinocyte Basal Medium 2 (Clonetics, Allendale, NJ) and incubated for 24 hours at 4°C. The corneal cup was discarded and the medium was centrifuged at 2400 rpm for 10 minutes at 4°C. The supernatant was removed and the media was used to assess LDH Activity.

To perform the LDH cytotoxicity assay 100 µL of each test media was added to three wells of an untreated 96-well plate. Once all sample wells were filled, 100 µL of reaction mixture was added to each well with a multichannel micropipette. The plate was then incubated for 25 minutes at room temperature protected from light. The microplate was then read at 490 nm with a reference wavelength of 650 nm. Those test groups with the highest absorbance exhibited the greatest amount of cell death and cell lysis.
CHAPTER 3

RESULTS

Ex Vivo Capsule PCO Model

Capsules were evaluated and scored using inverted microscopy every 48 to 72 hours post treatment for a total of 14 days. Data reported are the average of six capsules for each treatment group recorded as percent confluence, capsule score, and total LEC count per histology section. Lens capsules treated with 0.0% trypan blue for 0.5, 1, and 2 minutes reached 69.2 +/- 36.7, 69.2 +/- 34.1, and 83.3 +/- 28.8% confluence in 14 days, respectively (Figure 1). Those treated with 0.3% trypan blue for 0.5, 1, and 2 minutes reached 84 +/- 32.6, 50 +/- 39.9, and 65 +/- 38.34% confluence in 14 days, respectively (Figure 1). This data demonstrates no significant difference between any treatment groups. The capsules were also scored at each observation over the two-week study period. The capsule score (as outlined in the materials and methods) evaluated the total area of capsule populated by LECs, the presence of morphologic changes associated with EMT, and migration of LECs onto axial posterior capsule (Table 1). The lens capsules treated with 0.0% trypan blue for 0.5, 1, and 2 minutes had average capsule scores of 4.8 +/- 1.2, 4.8 +/- 1.3 and 5.17 +/- 1.2 respectively (Figure 2). The lens capsules treated with 0.3% trypan
blue for 0.5, 1, and 2 minutes had average capsule scores of 5.5 +/- 1.2, 3.67 +/- 1.6 and 4.5 +/- 1.38 respectively (Figure 2). No significant differences were detected between any treatment groups. At the end of the study period, capsules were formalin fixed and histologic cell counts were performed. Data reported are the average total cell count of six capsules for each group. As observed in Figure 3, capsules from the 0.0% trypan blue group treated for 0.5, 1, and 2 minutes had average cells counts of 90.3 +/- 78.3, 185.7 +/- 68.6, and 183.1 +/- 90.9, respectively. Capsules treated with 0.3% trypan blue for 0.5, 1, and 2 minutes had average cell counts of 122.6 +/- 46.5, 111.6 +/- 53.3, and 128.4 +/- 40.1, respectively (Figure 3). Again, no significant differences were found when comparing treatment groups. All three data sets were in agreement that trypan blue did not result in statistically significant differences compared to controls.
Figure 1: Percent Confluence on Posterior Capsule.

The 0.3% trypan blue treatment groups showed similar capsule coverage compared to control groups at each observation point over the course of the 14-day study period. No significant differences were noted between treatment groups.
Figure 2: Lens Capsule Score Over 14 Days.

The 0.3% trypan blue treatment groups showed similar capsule scores compared to control treatment groups at each observation point over the 14-day study period. No significant differences were noted between treatment groups.
Figure 3: Histologic Cell Counts

Although trypan blue treated lens capsules had a small decrease in the number of LECs present on the lens capsule compared to the vehicle, no significant differences were noted.
**Lactate Dehydrogenase Cytotoxicity Assay: Canine Lens Epithelial Cells**

Cultured canine LECs were treated in with 0.0, 0.05, 0.1, 0.2, or 0.3% trypan blue for 0.5, 1, or 2 minutes (n= 20). Following treatment, a LDH cytotoxicity detection assay was performed to quantify cell death and cell lysis based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant. This is a colorimetric assay and results are reported as an average +/- the standard deviation of absorbance from 15 test wells for each treatment group read at 490 nm with a reference wavelength of 650 nm. In addition to the trypan blue and control test groups, 2% Triton-X-100 solution was used as the positive LDH release control. The average absorbance for the 0.0% trypan blue test group treated for 0.5, 1, and 2 minutes was 0.14 +/- 0.07, 0.16 +/- 0.08, and 0.15 +/- 0.7, respectively. In the 0.05% trypan blue test group cells treated for 0.5, 1, and 2 minutes had an average absorbance of 0.18 +/- 0.06, 0.19 +/- 0.07, and 0.18 +/- 0.05, respectively. The average absorbance for the 0.1% trypan blue test group treated for 0.5, 1, and 2 minutes was 0.19 +/- 0.5, 0.20 +/- 0.05, and 0.17 +/- 0.06, respectively. Cells treated with 0.2% trypan blue for 0.5, 1, and 2 minutes had an average absorbance of 0.20 +/- 0.05, 0.19 +/- 0.05, and 0.17 +/- 0.08, respectively. The average absorbance for the 0.3% trypan blue test group treated for 0.5, 1, and 2 minutes was 0.17 +/- 0.07, 0.17 +/- 0.05, and 0.16 +/- 0.04, respectively. Finally, the positive control (2% Triton-X-100) resulted in an average absorbance of 1.4 +/- 0.19. This data is depicted in Figure 4; no significant differences were found in LDH release from trypan blue treatment groups and control groups when compared to each other.
Figure 4: Lactase Dehydrogenase Activity Released From Treated Lens Epithelial Cells

No significant differences were noted when comparing control and trypan blue treatment groups; the positive control (2% Triton-X-100) significantly increased LDH release. This demonstrated that there is no increased cell lysis and cell death between trypan blue groups and the vehicle control.
**Lens Epithelial Cell Morphology**

Cultured canine LECs were treated in a 12 well plate with 0.0, 0.1, 0.2, and 0.3% trypan blue for 1 minute. Following treatment, cell morphology was evaluated using phase contrast microscopy and photomicrographs. Morphologic changes were noted in the trypan blue treated LECs (Figures 5-9), and included decreased LEC size, condensed nuclei and chromatin clumping. These changes are more prominent in the LECs treated with 0.2 and 0.3% trypan blue indicating that higher concentrations of trypan blue induce greater amounts of LEC death and lysis.
Figure 5: Lens Epithelial Cell Morphology- Control

Normal LECs with no apparent morphologic changes.
Figure 6: Lens Epithelial Cell Morphology - 0.1% Trypan Blue

Subtle morphologic changes present, such as anisocytosis present in LECs.
Figure 7: Lens Epithelial Cell Morphology- 0.2% Trypan Blue

Lens epithelial cell morphologic changes present such as anisocytosis (red arrow), anisokaryosis (white arrow) and chromatin clumping (green arrow).
Figure 8: Lens Epithelial Cell Morphology- 0.3% Trypan Blue

Prominent morphologic changes present in 0.3% treated LECs including anisocytosis (red arrow), anisokaryosis (white arrow) and chromatin clumping (green arrow).
Figure 9: Lens Epithelial Cell Morphology- side-by-side comparison

Lens epithelial cell morphologic changes are noted in the LECs treated with trypan blue. Cellular changes include decreased LEC size, condensed nuclei and chromatin clumping, these changes are more prominent in the 0.2 and 0.3% trypan blue treated cells.
Apoptosis Assay: Canine Lens Epithelial Cells

Cultured canine LECs were treated in a 96 well plate with 0.0 and 0.3% trypan blue for 30 minutes prior to evaluation of caspase 3/7 release using the Apo-ONE Homogenous Caspase 3/7 Assay. This fluorescence assay is used to quantify apoptosis activity based on the measurement of caspase 3/7 release from the cytosol of damaged cells into the supernatant. Results reported are the average relative fluorescence units +/- the standard deviation of study groups at the 12-hour post incubation time point. The 0.0% trypan blue test group had an average of 344.4 +/- 210.3 fluorescence units and the 0.3% trypan blue test group had an average of 1067.4 +/- 533.1 fluorescence units (Figure 10). These results demonstrate a significant increase in caspase 3/7 activity in the 0.3% treatment group, which indicates apoptosis is the mechanism by which trypan blue causes cell death. In this assay 0.3% trypan blue was used to treat the cells for an extended period of time (30 minutes) compared to the maximum time of 120 seconds in the LDH assay. This allowed for increased cell death/ lysis so that a mechanism for cell death could be determined.
Figure 10: Lens Epithelial Cell Apoptosis Assay

A significant difference was noted between the LECs treated with 0.0 and 0.3% trypan blue. This indicates that cells treated with 0.3% trypan blue for 30 minutes had increased caspase 3/7 activity, which signified increased apoptosis activity.
**Lactate Dehydrogenase Cytotoxicity Assay: Canine Corneal Endothelial Cells**

Fresh canine corneal cups were harvested and treated with 0.0, 0.3% trypan blue, or 3% H$_2$O$_2$ for 120 seconds (n=9) and incubated for 24 hours. The supernatant was used to assess for LDH activity release. In this colorimetric assay results are reported as an average +/- the standard deviation of absorbance from 9 corneal cups for each treatment group read at 490 nm with a reference wavelength of 650 nm (Figure 11). The 3% H$_2$O$_2$ test group was used as a positive control for maximal LDH release. The 0.0% trypan blue treated corneal cups had an average absorbance of 0.78 +/- 0.1 which was similar to the average absorbance of the 0.3% trypan blue treated corneal cups which has an average absorbance of 0.62 +/- 0.01. However, the positive control group, the 3% H$_2$O$_2$ treated corneal cups had an average absorbance of 3.12 +/- 0.70, which is significantly different than the trypan blue and control treatment groups. This data is depicted in Figure 6 and indicates that there is no increased cell death to canine CECs when 0.3% trypan blue is used for 120 seconds.
No significant difference was found between 0.0 and 0.3% trypan blue treated canine CECs indicating no increased cell death or cell lysis when using high concentrations of trypan blue.
CHAPTER 4

DISCUSSION

Cataract formation is the most common intraocular lesion and is the leading cause of visual impairment in canine and humans.\textsuperscript{1} Cataract surgery is the most common intraocular surgery performed worldwide in both humans and canines and allows restoration of vision to affected patients. Posterior capsule opacification remains the most common long-term complication following cataract surgery in both species.\textsuperscript{11,14} It is a universal complication that occurs between 62-100\% of all canine patients and up to 50\% in adult human patients and 100\% of all pediatric patients.\textsuperscript{9,20} Post operatively, residual LECs migrate, proliferate, and differentiate due to a variety of factors including surgical technique, patient age, and patient host factors.\textsuperscript{8,9,21} The end result of PCO is progressive opacification of the central visual axis that results in significant reduction in vision that can lead to secondary blindness.\textsuperscript{30} Despite recent studies performed to reduce and prevent PCO formation using different surgical modalities and pharmaceuticals, there remains no current treatment to completely inhibit PCO formation \textit{in vivo}. This study investigated a novel approach to reduce PCO formation by utilizing trypan blue in an \textit{in vitro} and \textit{ex vivo} canine model.
Trypan blue is a vital dye that is frequently used in ophthalmology to stain intraocular structures in cataract and anterior segment surgeries. The dye was first used to identify dead cells in donor corneas prior to keratoplasty and thus has been used to assess tissue viability. Trypan blue has primarily been used in intraocular surgeries to stain the anterior lens capsule in cataract surgery which aids in visualization for creation of a capsulorhexis. It has since been used not only in cataract surgery, but also in vitrectomy surgery and in epiretinal membrane removal. Trypan blue has been shown to reduce the overall density and viability of LEC through apoptosis and autophagy. This was further evaluated by Sharma et al., whose study determined that 0.1% trypan blue injected into the capsular bag of humans at the time of cataract surgery decreased PCO formation. Further investigation is necessary, however, to determine the concentration and contact time of trypan blue necessary to achieve maximal PCO reduction, to explore the mechanism by which cell death occurs, and to determine if this model is translational from human to canine LECs.

In the present study, an ex vivo canine model of PCO was used to determine if trypan blue treatment had an effect on canine LEC morphology and cell viability. First, we determined the cytotoxic effect of trypan blue on LECs by culturing canine LECs and performing a LDH cytotoxicity assay. LECs were treated with concentrations of trypan blue ranging from 0.05-0.3% or vehicle control for 0.5, 1, or 2 minutes. Although the trypan blue treated LECs demonstrated higher amounts of LDH release, indicating increased cell death compared to the vehicle, this difference was not significantly different between test groups. In addition to the LDH assay, cell morphology was also
evaluated for signs of cell death and lysis. Cell morphology was evaluated after cultured LECs were treated with vehicle control, 0.1, 0.2, or 0.3% trypan blue for 1 minute (Figure 5-9). Morphologic changes were noted in the trypan blue treated LECs including decreased LEC size, condensed nuclei, and chromatin clumping. These changes were more prominent in the LECs treated with higher concentrations of trypan blue indicating that trypan blue can induce morphologic changes consistent with LEC lysis and death. These observations indicate that higher concentrations and potentially longer treatment times of trypan blue may induce greater levels of LEC death.

An ex vivo canine lens capsule model was used to determine the effect of trypan blue on PCO formation. This is a well-established model that was first described in dogs by Davidson et al. and effectively recapitulates in vivo PCO formation. This model is ideal because a natural capsule and LEC matrix environment is created with the same spatial cell and tissue arrangements that are observed clinically. In this study, lens capsules were treated with 0.3% trypan blue or vehicle control for 0.5, 1, or 2 minutes. At the termination of the study, the 0.3% trypan blue treated capsules showed similar capsule scores and percent posterior lens capsule confluence compared to control treatment groups. Histologic cell counts corroborated the tissue culture observations. Cumulatively, this indicates that treatment with trypan blue has the potential to induce cell death; however, longer treatment times and higher concentrations are likely necessary.

It is also possible that the decrease in histologic cell counts in trypan blue treated capsules compared to control capsules could be explained by the surgical technique. The
capsules treated with trypan blue after cataract extraction highlighted areas on the anterior and equatorial lens capsule where increased sheets of LECs remained. Therefore, during manual irrigation and aspiration the remaining LECs were easier to detect and remove. This could explain why the histologic cells counts were decreased in the trypan blue treated cells compared to control capsules.

The mechanism of action by which trypan blue causes LEC death has been minimally explored *in vitro*. Authors have suggested two pathways by which trypan blue may reduce LEC viability, specifically via apoptosis and autophagy.\(^{58,59}\) Portes et al. performed a prospective study to evaluate the effect of 0.1% trypan blue staining used during cataract surgery on LECs and capsules. Immunohistochemical tests were used to evaluate anterior lens capsules for the expression of beclin-1, a marker of cell death by autophagy.\(^{58}\) A TUNEL assay was also performed on the anterior lens capsules to identify cell death via apoptosis. LECs exposed to trypan blue were positive for beclin-1 expression and were TUNEL positive, suggesting that trypan blue causes LEC death via autophagy and apoptosis.\(^{58}\) Unfortunately, in this study, the figure demonstrating beclin-1 expression was of poor quality and unconvincing and untreated controls were not shown for comparison. In addition, beclin-1 is a marker that has been shown to have crossover between regulation of apoptosis and autophagy.\(^{73}\) As such, it is possible that beclin-1 expression was not related to autophagic signaling but rather, involved only in apoptotic signaling.

In the current study, because acute exposure to trypan blue resulted in subtle changes indicative of cell stress, it was hypothesized that extended exposure to trypan
blue would increase LEC death. To that effect, cultured canine LECs were treated with vehicle control or 0.3% trypan blue for 30 minutes and used to evaluate caspase 3/7 release from the cytosol of damaged cells using an apoptosis assay. Caspase -3 and -7 are members of the cysteine aspartic acid-specific protease (caspase) family who play a key effector role in apoptosis of mammalian cells.\textsuperscript{74-77} Induction of apoptosis and activation of caspases can result from a variety of stimuli including growth factor withdrawal, exposure to radiation or pharmacologic agents, or initiation of the Fas/ Apo-1 receptor mediated cell death process.\textsuperscript{78,79} Active caspases participate in a cascade of cleavage events that disable homeostatic and reparative enzymes resulting in structural disassembly and death of cells.\textsuperscript{78,79} We demonstrated that prolonged treatment with 0.3% trypan blue resulted in a significant difference of caspase 3/7 activity indicating increased apoptotic signaling when compared to vehicle controls. Although this finding indicates that apoptosis is the mechanism by which trypan blue causes cell death and supports our theory that prolonged treatment times and higher concentrations of trypan blue would result in more cell death. Further confirmation with additional tests such as western blot, TUNEL Assay or electron microscopy are necessary to further support apoptosis as the mechanism by which cell death occurs.

Trypan blue’s toxicity has been evaluated extensively in the literature and these studies have shown that low doses of trypan blue is non-toxic to intraocular structures, including the corneal endothelial cells, iris, ciliary body epithelium, and retina.\textsuperscript{44,46,62,64,69,71} These studies have validated its safety for intraocular use. Toxicity to the CECs is of great concern because these cells have limited proliferative ability and any
potential insult to this cell layer poses a risk for permanent harmful effects to the corneal endothelium.\textsuperscript{1,3,44,64} Intraocular use of trypan blue at concentrations as high as 1\% for two minutes has been found to be non-toxic to human CECs.\textsuperscript{1,47,65} To date, there are no published studies that document the effect of trypan blue on canine CEC.

In order to assess CEC toxicity \textit{in vitro} canine CECs were treated with 0.0 or 0.3\% trypan blue for 2 minutes. Using a LDH assay no significant differences were found when comparing the vehicle control and 0.3\% trypan blue treated canine CECs. This indicates no increased endothelial cell death or cell lysis when using 0.3\% trypan blue for 2 minutes. However, it is unknown if higher concentrations of TB or if TB remained in contact with CECs for longer treatment times if toxicity would be observed, as shown in LECs treated with 0.3\% TB for 30 minutes.

The main limitation to our study was that all experiments were performed \textit{in vitro}. Laboratory conditions have the ability to be carefully controlled; however, they can never completely mimic what happens at the cellular level \textit{in vivo}. The \textit{ex vivo} capsule model has been widely used in many species to evaluate PCO formation and prevention strategies, however, it lacks the influence from other ocular tissues that likely play a role in LEC behavior after cataract surgery.\textsuperscript{16,19,25,72} Our study also lacked placement of an IOL within the capsule bag, which has been shown to limit PCO formation \textit{in vivo} by creating a barrier for LEC migration along the posterior lens capsule.\textsuperscript{21,80-82}

A potential future experimental aim would be to use higher concentrations of trypan blue for longer treatment times. Our results indicate that trypan blue induces LEC death by apoptosis and that higher concentrations and longer treatment times may be
necessary to have an effect at reducing PCO formation. However, there is concern that much longer treatment times than already evaluated in this study would limit the clinical practicality of this treatment, as it would extend surgical times tremendously. In addition, further studies would need to be performed to evaluate chronic corneal endothelial cell exposure to higher concentrations of trypan blue.

In conclusion, the purpose of this study was to determine if trypan blue could induce LEC death and decrease PCO formation in vitro and ex vivo. We also sought to determine if trypan blue treatment could reduce corneal endothelial cell viability. Overall, our study found that trypan blue did not significantly reduce LEC density in vitro and ex vivo. Trypan blue treated LECs did demonstrate higher rates of cell death compared to vehicle control; this difference was not significant. Induction of apoptotic signaling cascades was found to be the mechanism by which prolonged trypan blue treatment led to LEC lysis and death. Finally, endothelial cells treated with trypan blue or vehicle control showed no significant differences in cell death. Therefore, Trypan blue was not effective at reducing ex vivo PCO formation and trypan blue did not induce endothelial cell death.
References


